# Human skeletal muscle possesses both reversible proteomic signatures and a retained proteomic memory after repeated resistance training

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## Abstract

Investigating repeated resistance training separated by a training break enables exploration of the potential for a proteomic memory of skeletal muscle growth. Our aim was to examine skeletal muscle proteome response to 10-week resistance training (RT1) followed by 10-week training cessation (i.e. detraining, DT), and finally 10-week retraining (RT2). Thirty healthy, untrained participants conducted either periodic RT (RT1-DT-RT2, n=17) or a 10-week no-training control period (n=13) followed by 20 weeks of RT (n=11). RT included twice-weekly supervised whole-body RT sessions, and resting vastus lateralis biopsies were obtained for proteomics analysis using high-end DIA-PASEF's mass spectrometry. First RT period altered 150 proteins (93% increased) involved in e.g. energy metabolism, cytokine signalling, and protein processing compared with minor changes during the no-training control period. The proteome adaptations were similar after the second RT compared to baseline demonstrating reproducibility in proteome adaptations to RT. Many of the proteins were reversed towards baseline after detraining and increased again after retraining. These reversible proteins were especially involved in aerobic energy metabolism. Interestingly, several proteins remain elevated after detraining, including carbonyl reductase 1, histone H1.5, and many calcium-binding proteins. Specifically, Calpain 2 (CAPN2), a calcium-activated protease was recently identified as an epigenetic muscle memory gene. We show for the first time that human skeletal muscle experiences retained levels of several proteins even into detraining. This shows that resistance training evokes long-term protein changes in skeletal muscle and is the first study to demonstrate a proteomic memory of muscle growth following resistance training in human skeletal muscle.

Key Words: Muscle memory, proteomics, detraining, hypertrophy, calpain-2

## Key points

• Repeated resistance training in humans separated by a training break (i.e. detraining) enables the identification of temporal protein signatures over the training, detraining, and retraining periods as well as studying reproducibility of protein changes to resistance training.

• Muscle proteome adaptations were similar after a second period of resistance training when compared to baseline demonstrating reproducibility in proteome adaptations to earlier resistance training.

• Many of the proteins were reversed towards baseline after detraining and increased again after retraining. These reversible proteins were especially involved in aerobic energy metabolism.

• Several proteins remain elevated after detraining, including carbonyl reductase 1, histone H1.5, and Calpain 2 (CAPN2).

• Human skeletal muscle experiences retained levels of several proteins even into detraining demonstrating that resistance training can evoke long-term protein changes in skeletal muscle and a potential proteomic memory of muscle growth following resistance training.

Running title: Resistance training, detraining and retraining proteomics

## Introduction

Resistance training (RT)-induced muscle hypertrophy and strength may be regained to an enhanced level after training breaks (i.e. detraining), a phenomenon termed muscle memory (Sharples & Turner, 2023). In a broader sense, it can also refer to a variety of very long-term tissue-level effects of exercise. Thus, it can also potentially affect muscle maintenance and, therefore, an individual's health and wellbeing, even in situations when there are breaks from exercise.

Previous (Egner *et al.*, 2013) and more recent (Murach *et al.*, 2020; Eftestøl *et al.*, 2022) cellular and molecular muscle memory studies have concentrated on 'cellular' memory, i.e. retention of myonuclei during detraining in rodents. To date, human muscle memory and long-term adaptation studies to RT have primarily focused on either myonuclear retention (Psilander *et al.*, 2019; Snijders *et al.*, 2019; Cumming *et al.*, 2024) or retained epigenomic and transcriptomic signatures in skeletal muscle of young adults (Seaborne *et al.*, 2018) and older individuals (Blocquiaux *et al.*, 2022). In addition, other types of exercise, such as high-intensity interval training (HIIT) have been investigated in humans (Pilotto et al., 2024) and progressive weighted wheel running in rodents (Wen et al., 2021) using a training, detraining and retraining design. Collectively, these studies suggest that there may be retention of myonuclei and epigenetic modifications, in particular retained DNA methylation signatures from earlier training into detraining leading to continued elevation of gene expression even during detraining. However, it is currently unknown whether there are long-term or retained signatures following exercise training at the protein level.

Proteomics analysis, involving the quantification of thousands of proteins from a single muscle sample can reveal complex adaptations to RT in skeletal muscle. Unfortunately, due to the high cost of proteomics analysis, many studies have had very small n-sizes, and/or the samples have been pooled decreasing statistical power. Nevertheless, a few recent proteomic studies have revealed that RT (Robinson *et al.*, 2017; Roberts *et al.*, 2024; Jessen *et al.*, 2024) and other types of high-intensity exercise (Robinson *et al.*, 2017; Deshmukh *et al.*, 2021) can induce the remodelling of skeletal muscle at the individual protein level. Moreover, some studies have investigated the effects of muscle disuse on muscle proteome (Murgia *et al.*, 2022). However, there is no evidence of long-term proteome adaptations following detraining after an earlier period of RT and how this affects protein changes to a later repeated period of RT. This poses the question of whether there is a memory of resistance exercise induced muscle growth evident at the protein level.

We hypothesized that RT would significantly and systematically affect the muscle proteome and that we would discover proteins with reversible (i.e. return to baseline levels during detraining) and some

with retained signatures after detraining. However, due to the novelty of the study design and unbiased proteomic approach, we did not adopt an a priori hypothesis regarding which proteins or biological processes would be affected. Using this approach, we aimed to identify temporal protein signatures over the training, detraining, and retraining period of which the retained proteins identified following earlier training may be implicated in skeletal muscle memory.

## Methods

### Study design and setting

This is a secondary analysis of a randomized and controlled study, of which the basic phenotype data and detailed description of study design, study participants, recruitment, and phenotyping methods have been recently published (Halonen *et al.*, 2024). The study design is illustrated in Fig. 1. Briefly, participants were randomly assigned to periodic RT (PRT) or continuous RT (CRT) that included a control period of no-RT (Fig. 1). The PRT group completed a 10-week RT period (RT1), a 10-week detraining period (DT), and a repeated 10-week RT period (RT2) identical to RT1. The CRT group began with a 10-week non-RT period, followed by a 20-week continuous RT period.

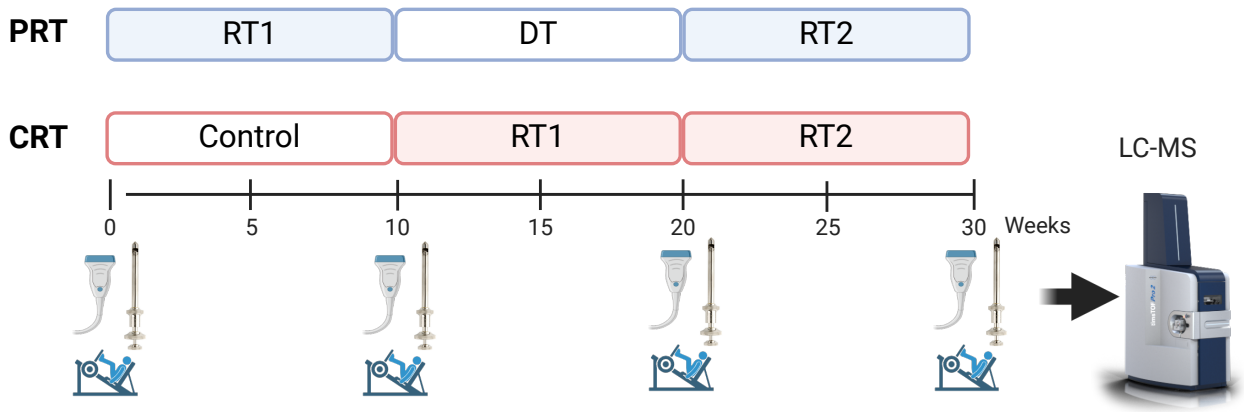


**Figure 1.** Study design. RT = resistance training. DT = detraining (no-RT). The periodic resistance training (PRT) group completed a 10-week initial RT period (RT1) followed by 10 weeks of no-RT/ detraining (DT) and then a second period of 10 weeks RT (RT2) with proteome analysed from all the participants n=17. The continuous resistance training (CRT) group completed a period of 10 weeks of no-RT (control) followed by continuous RT for 20 weeks (two periods of 10 weeks denoted RT1 & RT2 on figure) with n=13, of which n = 11 completed the training with available biopsies for proteomics.

## Participants

Fifty-five eligible males and females volunteered to participate in the original study of which forty-two participants completed the intervention (Halonen *et al.*, 2024). Homogeneity between groups at baseline was achieved by separately dividing male and female participants into matched pairs by the combined Z score for BMI and age. For the present study, we included participants where we had at least 20 mg biopsy samples available for proteomics analysis resulting in a total of 30 participants. In the PRT group, which is the main focus of the present study there were n = 17 (33.8 ± 5.2 years, 6 females/ 11 males), and in the CRT group n = 13 (31.8 ± 4.0 years, 6 females / 7 males) of which 2 (1 female/ 1 male) had samples only from the first time-points, i.e. before and after the control period). Inclusion criteria were: Age within the range 18-40 years, no regular RT history (> 10 RT sessions a year), not participating in systematic endurance-type training (> 2 endurance exercise sessions lasting > 30 minutes per week for the last six months), body mass index (BMI) within the range 18.5-30 kg/m$^2$, and not currently consuming any anti-inflammatory drug(s). Exclusion criteria were: A history of medication that could affect exercise responses, use of creatine, any acute or chronic illness affecting cardiovascular, respiratory, musculoskeletal, and/or endocrine function, any other condition that may limit the ability to perform RT and testing (e.g., uncontrolled hypertension, diabetes, arthritic conditions, neuromuscular complications), or blood-borne diseases, diseases and medication affecting blood clotting, allergies to anaesthetic drugs, and severe psychological disorders.

The study received ethical approval from the ethics committee at the University of Jyväskylä (857/13.00.04.00/2021). The study was conducted according to the declaration of Helsinki. All the applicants were informed of all potential risks of the study and had the possibility of withdrawing from the research project at any time, after which they signed an informed consent document. Personal data was stored and handled according to the ethical and GDPR guidelines of the University of Jyväskylä. The study has been registered into ClinicalTrials.gov (ID: NCT05553769).

## Vastus lateralis muscle size and strength

The measurements and analysis have been explained in detail in (Halonen *et al.*, 2024). Briefly, Vastus lateralis (VL) muscle cross-sectional area (CSA, cm$^2$) was measured using a B-mode axial plane ultrasound (model SSD-α10, Aloka, Tokyo, Japan), which has a high correlation with MRI in detecting changes in muscle CSA (Ahtiainen *et al.*, 2010). The ultrasound images were analysed using ImageJ software (version 1.54). The coefficient of variation percentage (CV%) for VL CSA from test to retest was 1.6%. One participant from the PRT group was excluded from the VL CSA analysis due to poor image quality, however this participant was included in all the other analyses.

## RT intervention

Both groups underwent the two identical 10-week RT periods, but the difference between the groups was that the CRT group performed the first and second 10-week RT periods continuously, and the PRT group had a 10-week detraining period between the two RT periods (Fig. 1). The twice-weekly exercises were leg press (4 x 8-10 reps), knee extension (4 x 8-10 reps), Smith machine bench press (3 x 8-10 reps), barbell biceps curl (4 x 8-10 reps), and chest supported seated row (4 x 8-10 reps) with two-minute rest periods between the sets. The participants were instructed to perform 8-10 reps in each set with approximately 1-2 repetitions in reserve, except in the final set of each exercise in the second training session of every week, when the last set was performed until volitional failure. To enable progression, the number of repetitions was then used to adjust the training loads for the following week; if the number of performed repetitions was more than 10, the load was increased, and if the repetitions were less than eight, the load was decreased. A more detailed description of the training is explained in (Halonen *et al.*, 2024). All training sessions were performed at the laboratory of the University of Jyväskylä's Faculty of Sport and Health Sciences, and the sessions were instructed and supervised to ensure correct exercise techniques.

## Control period and detraining

During the control period in the CRT group and after the first 10-week RT period in the PRT group (start of DT), the participants were instructed to continue their habitual lifestyle but to avoid any form of resistance or endurance-type training or any other unaccustomed exercise for the next 10-week period. Physical activity and other lifestyle changes during the detraining period were assessed with a survey (Halonen *et al.*, 2024) and the participants were also contacted via email in the middle of the detraining period to ensure that they did not participate in any form of RT. These analyses suggested there was no RT conducted during the detraining period (Halonen *et al.*, 2024).

## Muscle biopsy obtainment

The muscle biopsies were obtained at 0, 10, 20, and 30 weeks, at rest ~6 days after the last exercise bout except in the CRT group after 10-weeks of RT1 where the biopsy was taken ~3 days after the last exercise bout to minimize training break in the middle of the continuous 20-week RT intervention. Biopsies were taken from vastus lateralis 1/3 of the length from the patella towards the greater trochanter with local anaesthetic and a 5-mm Bergström biopsy needle together with suction as previously described (Hulmi *et al.*, 2009). The biopsy was always from the right leg. The second biopsy was taken approximately 1 cm medially to the first biopsy, the third biopsy 1 cm laterally to the first biopsy, and the fourth biopsy 1 cm medially to the second biopsy. The muscle

sample was cleaned of any visible connective tissue, adipose tissue, and blood and frozen immediately in liquid nitrogen (-180 °C) and stored at -80 °C.

**Sample preparation for proteomics analysis**

Twenty mg of muscle tissue was disrupted and lysed using “high” setting with Beatbox (PreOmics, Germany) using Tissue kit 24x (P.O.00128, PreOmics). The total protein concentration of the homogenates was measured with Bio-Rad Protein Assay Dye (#5000006, Bio-Rad Laboratories). The samples were stored frozen at -80°C overnight followed by digestion. 50 µg of total protein from each sample was taken for proteomics analysis. Sample digestion and purification were done on all samples at the same time with an iST HT kit (P.O.00067, PreOmics) according to the manufacturer’s instructions. After desalting and purification, the samples were dried in a centrifuge concentrator (Concentrator Plus, Eppendorf). The dried peptides were reconstituted in 30 µl buffer A (0.1% (vol/vol) TFA, 1% (vol/vol) acetonitrile (#83640.320, VWR) in HPLC grade water (#10505904, Fisher Scientific)). For the DIA analysis, the resuspended peptides were further diluted 1:50 in buffer A1 (1% formic acid in HPLC water). Twenty µl was loaded into an Evotip (Evosep, Denmark) according to the manufacturer's instructions and run at the same time.

**Mass spectrometry and data analysis**

The samples were analysed using the Evosep One liquid chromatography (LC) system coupled to a hybrid trapped ion mobility quadrupole TOF mass spectrometer (Bruker timsTOF Pro2, Bruker Daltonics) (Meier *et al.*, 2018) via a CaptiveSpray nano-electrospray ion source (Bruker Daltonics). An 8 cm × 150 µm column with 1.5 µm C18 beads (EV1109, Evosep) was used for peptide separation with the 60 samples per day method (21 min gradient time). Mobile phases A and B were 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile, respectively. The MS analysis was conducted in the positive-ion mode with dia-PASEF method (Meier *et al.*, 2020) with sample optimized data independent analysis (dia) scan parameters. We performed DDA in PASEF mode from a pooled sample to be able to adjust dia-PASEF parameters optimally. To perform sample-specific dia-PASEF parameter adjustment, the default dia-short-gradient acquisition methods were adjusted based on the sample-specific DDA-PASEF run with the software “tims Control” (Bruker Daltonics). The following parameters were modified for each sample type: m/z range: 415.0 – 1215.0 Da; mass steps per cycle: 32; mean cycle time: 1.27 s. The ion mobility windows were set to best match the ion cloud density from the sample type-specific DDA-runs.

To analyse diaPASEF data, the raw data (.d) were processed with DIA-NN v1.8.1 (Demichev *et al.*, 2020, 2022) utilizing spectral library generated from the UniProt human proteome (USP000000589,

downloaded 21.08.2023 as a FASTA file, 20159 proteins). During library generation, the following settings were used with fixed modifications: carbamidomethyl (C); variable modifications: acetyl (protein N-term), oxidation (M); enzyme:Trypsin/P; maximun missed cleavages: 1; mass accuracy fixed to 1.5e-05 (MS2) and 1.5e-05 (MS1); Fragment m/z set to 200-1800; peptide length set to 7-50; precursor m/z set to 300-1800; Precursor changes set to 2-4; protein inference not performed. All other settings were left to default.

**Statistical and bioinformatics analysis of the proteomics data**

The input file to further DIA data analysis was the DIA-NN Report.pg_matrix. For data pre-processing, an in-house R-script was utilized. Raw intensity values were log2 transformed and median-normalized. Afterward, missing values were imputed using QRILC imputation (https://CRAN.R-project.org/package=imputeLCMD). For time comparison, p-values were calculated with paired sample t-test and groups with Student t-test using python package SciPy (Virtanen *et al.*, 2020), and adjusted using Benjamini-Hochberg (false discovery rate, FDR) method via statsmodels package (Seabold & Perktold, 2010). For the analysis, stringent $FDR < 0.05$, Fold change (FC) >|1.2| filtering was used if not otherwise mentioned. ShinyGO (http://bioinformatics.sdstate.edu/go/) (Ge *et al.*, 2020) v0.81 was used to identify enriched Gene Ontology (GO) terms from the GO database (biological processes (BP), cellular components (CC) and molecular function (MF). We used an FDR cutoff of 0.05 and the whole proteome as background. Categorical annotations for each protein in the form of GO terms of BP, CC and MF were extracted from the UniProt database. Overrepresentation analysis was carried out on lists of proteins from different comparisons using the Reactome database (Jassal *et al.*, 2019). Statistically overrepresented were identified comparing the input set to the background distribution of all pathways, helping to reveal key biological processes associated with the data. The figures showing significant pathways versus number of identified proteins and FDR values were created in the R programming language (version 4.3.3) and the ggplot2 package (Wickham, 2016). To show the connections between the significant pathways and the individual proteins, Sankey diagrams were also created in R as were the Volcano plots. The pathway entitled Metabolism was omitted as a too generic term from these figures.

All other figures were made with GraphPad Prism software (version 10.0, GraphPad Software Inc), Microsoft Excel (version 2410), or using Proteomaps approach (Liebermeister *et al.*, 2014). Part of the Venn diagrams were generated using: https://bioinformatics.psb.ugent.be/webtools/Venn/. In Proteomaps each protein is represented by a polygon of which size corresponds to the magnitude and consistency of the change after RT (i.e. fold change * -Log10(FDR)). It also situates functionally related pathways in adjacent regions based on KEGG Orthology identifiers. To plot temporal changes

in proteins across the intervention, we undertook Self Organising Map (SOM) profiling of the change in standardised mean protein content within each time-point using Partek Genomics Suite as previously described earlier (Turner *et al.*, 2020). SOM analysis plots a standarized mean (shifts mean to a value of 0 and scales to a standard deviation of 1) for the group of samples across each time-point to identify temporal signature changes over the time-course of the intervention.

## Results

### Changes in muscle size and strength

In this subcohort of our study (Halonen *et al.*, 2024), the first 10 weeks of RT increased muscle CSA in the VL and 1 RM strength in the leg press ($P < 0.001$, Table 1). Moreover, when comparing periodic RT (PRT) and continuous RT (CRT) during the whole intervention (weeks 0 to 30), no significant differences were observed in muscle size or strength (Table 1). This occurred because the muscle size and strength lost during detraining ($P < 0.001$) were quickly regained during retraining in the PRT group. In contrast, the CRT group experienced a somewhat slower rate of gains in muscle size and strength during the second 10-week RT period (Halonen *et al.*, 2024).

**Table 1.** Characteristics of the participants analysed for proteomics.

| **Group** | **Δ% VL CSA** | | | **Δ% 1 RM** | | |
|---|---|---|---|---|---|---|
| | **(0-10 wk)** | **(0-20 wk)** | **(0-30 wk)** | **(0-10 wk)** | **(0-20 wk)** | **(0-30 wk)** |
| **PRT** | 16.0 ± 8.5*** | 3.9 ± 7.7*** | 19.4 ± 9.5 | 20.8 ± 13.3*** | 15.4 ± 10.6 | 29.7 ± 16.2 |
| **CRT** | 1.3 ± 4.4 | 23.2 ± 10.1 | 27.4 ± 11.9 | 1.6 ± 4.4 | 22.2 ± 11.1 | 28.6 ± 12.3 |

1 RM strength measured in leg press. N = 16 in PRT except in the 1 RM (n=17) and n=11 analysed for proteomics in the CRT-group. *, ** or *** $p < 0.05$ – $p < 0.001$ difference between PRT and CRT. #The data with full n-size has been published earlier (Halonen et al., 2024).

### RT affects on the muscle proteome

Using an FDR cutoff of 0.01 on both peptide and protein levels, we identified 3610 proteins of which 3391 were unique. This led to the quantification of 3127 and 3130 unique proteins in PRT and CRT groups, respectively. The first RT period (RT1) in PRT significantly ($FDR < 0.05$, FC >|1.2|) altered 150 proteins compared to only three proteins in the no-RT control period (Fig. 2A). See all the time-

point comparisons in Supporting information, Supplement 1. The large majority 93% (140) of the total 150 proteins identified had increased levels. Within cellular processes and pathways, the altered proteins belonged to e.g., aerobic respiration, TCA cycle, amino acid metabolism, cytokine signalling, chaperones and protein processing (Figure 2B, Supporting information, Supplement 2).

Next, we examined the changes following RT2 and whether these were similar to changes after the earlier RT1 period. When RT2 was compared to baseline, 217 proteins were significantly altered (186 / 86% increased, Fig. 2A). Most of the proteins (66.6% or 100/150) altered after RT1 were also significantly altered after RT2 (Fig. 2C) and abundance changes correlated between RT1 and RT2 (Fig. 2D). Also, the top 50 upregulated proteins (Fig. 2E) as well as the cellular pathways and processes (Figure 2B, Supporting information, Supplement 2) were similar after RT1 and after RT2 (vs. baseline). This was confirmed further, as no proteins were significantly altered when post RT2 and RT1 timepoints were compared. However, there were 117 unique proteins increased after RT2 (but not after RT1), with these proteins enriched in aerobic metabolism pathways and processes (Supporting information, Supplement 2). When post-RT2 was compared to pre-RT2 (post-DT), only 17 proteins were significantly altered (Fig. 2C). However, half of these were shared in both RT1 and RT2, and the direction and overall magnitude of the responses were very similar (Fig. 2C and 2F).

**Temporal protein signatures identifies both reversible and retained proteins after RT**

Proteome signatures in the PRT group were further analysed by self-organizing map (SOM) profiling. For the SOM analysis, we identified 285 proteins that were significant in at least one of the possible six comparisons between the time-points (Fig. 3A). It highlighted four predominant temporal signatures (Fig. 3B). Signature 1 included the smallest proportion of proteins (14.0%) that were decreased after RT. Signature 2 included proteins (20.3%) that were increased after RT1, fully returned to baseline, and then increased again after RT2. Signature 3 included proteins (37.2%) that were increased after RT1 and then returned towards, but not fully to baseline levels after DT, and then once again increased after RT2. Finally, signature 4 included proteins (28.4%) increasing after RT1 and then retained as elevated after DT and RT2. In addition, so that researchers can check the temporal profile of their protein of interest across RT1, DT, and RT2, we conducted SOM analysis also using all detected proteins (with no statistical or fold change cut-offs) (Supporting information, Supplement 2) and included them as a resource in Supporting information, Supplement 1.

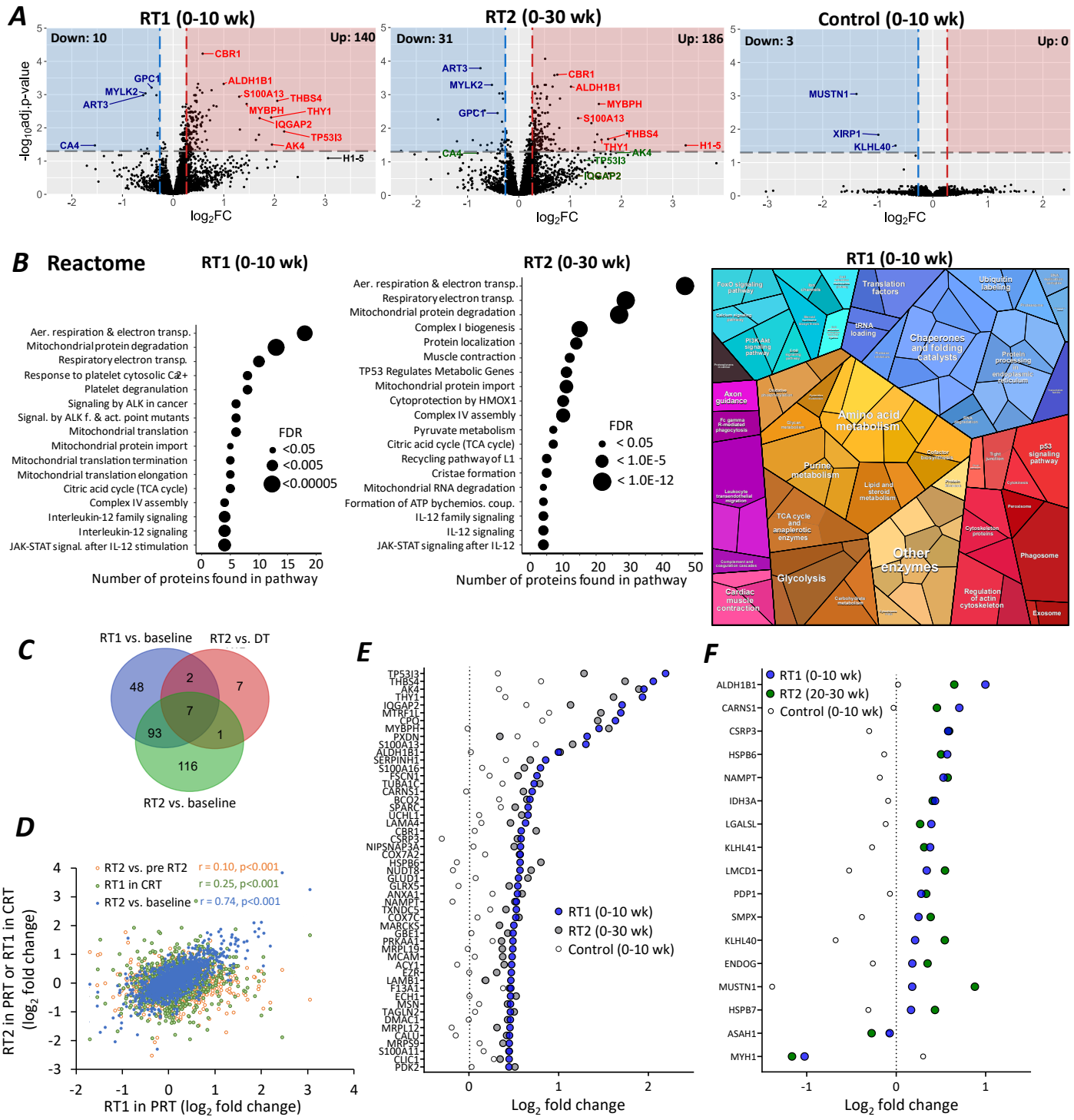


**Figure 2.** *A*, Volcano plot of the significantly altered proteins after the first 10-week RT (RT1) and RT2 in the PRT condition (n =17) and control (no RT) in the CRT condition (n=13). In the plots highly altered (Log2FC * -Log10(FDR)) proteins are labelled. Note that $-\log_{10}$ of 0.05 is ~1.30 and $\log_2$ of 1.2 is ~0.26. *B*, Reactome pathway analysis of RT1 and RT2 vs. baseline and Proteomaps of the RT1 period in the PRT group. See also Supporting information, Supplement 2. *C*, Venn Diagram analysis of the statistically differentially (*FDR* < 0.05, FC >|1.2|) regulated proteins following RT1 and RT2 compared to baseline (RT1+RT2) and RT2 vs. pre RT2 (RT2), illustrating the number of common overlapping and unique significant proteins across comparisons in the PRT group. *D*, Pearson´s correlation of protein abundance changes in different comparisons (in PRT between RT1 and RT2 vs. baseline, between RT1 in PRT and RT1 in CRT, and between RT1 and RT2 vs. DT). *E*, Top 50 upregulated proteins (*FDR* < 0.05) in PRT group after RT2 (vs. baseline) and changes in the same proteins during RT1 (vs. baseline) and control period (n=11). *F*, Altered proteins (*FDR* < 0.05 and FC> |1.2|) in RT2 vs. DT comparison (n=17) and respective protein changes in RT1 in the PRT group and control period in the CRT group (n=11).

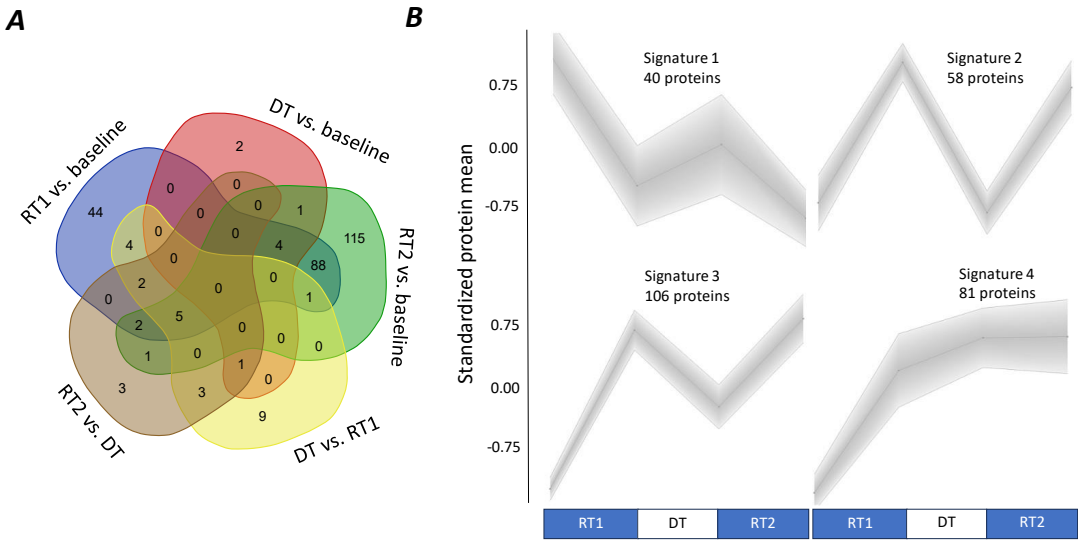


**Figure 3.** *A*, Venn diagram analysis of the statistically differentially regulated proteins: 1) RT1 vs. baseline, 2) DT vs. baseline, 3) RT2 vs. baseline, 4) DT vs. RT1 (pre-DT) and 5) RT2 vs. DT (pre-RT2) (*FDR* < 0.05, FC >|1.2|). The 6th comparison: RT2 vs. RT1 is not shown as it did not produce any statistically significantly different proteins. The figure illustrates the number of common overlapping and unique significant proteins across each comparison. There were 285 proteins that were significantly different in at least one of the pairwise comparisons highlighted in the Venn diagram. *B*, SOM profiling of 285 proteins identified in the Venn diagram above in the PRT group over the 30 weeks time-course of baseline, RT1, DT and RT2.

## Proteins with reversible signature after RT1

Many proteins changed in response to RT1 and then returned towards baseline during detraining (signature 2 defined above). To further investigate this protein signature with more stringent filtering, we used the following criteria: 1) *FDR* > 0.5 at post DT vs. baseline (i.e. high probability of no change) and 2) DT vs. RT1 decreased > 70 %. With this criteria, we found 42 proteins of which 36 confirmed to also belong to signature 2 (containing 58 proteins) and illustrated in Fig. 4A. These 36 reversible proteins are involved in energy metabolism, especially in mitochondria (Fig. 4B, Supporting information, Supplement 2) and in molecular function within GO terms especially enriched in oxidoreductase and dehydrogenase functions (Fig. 4C).

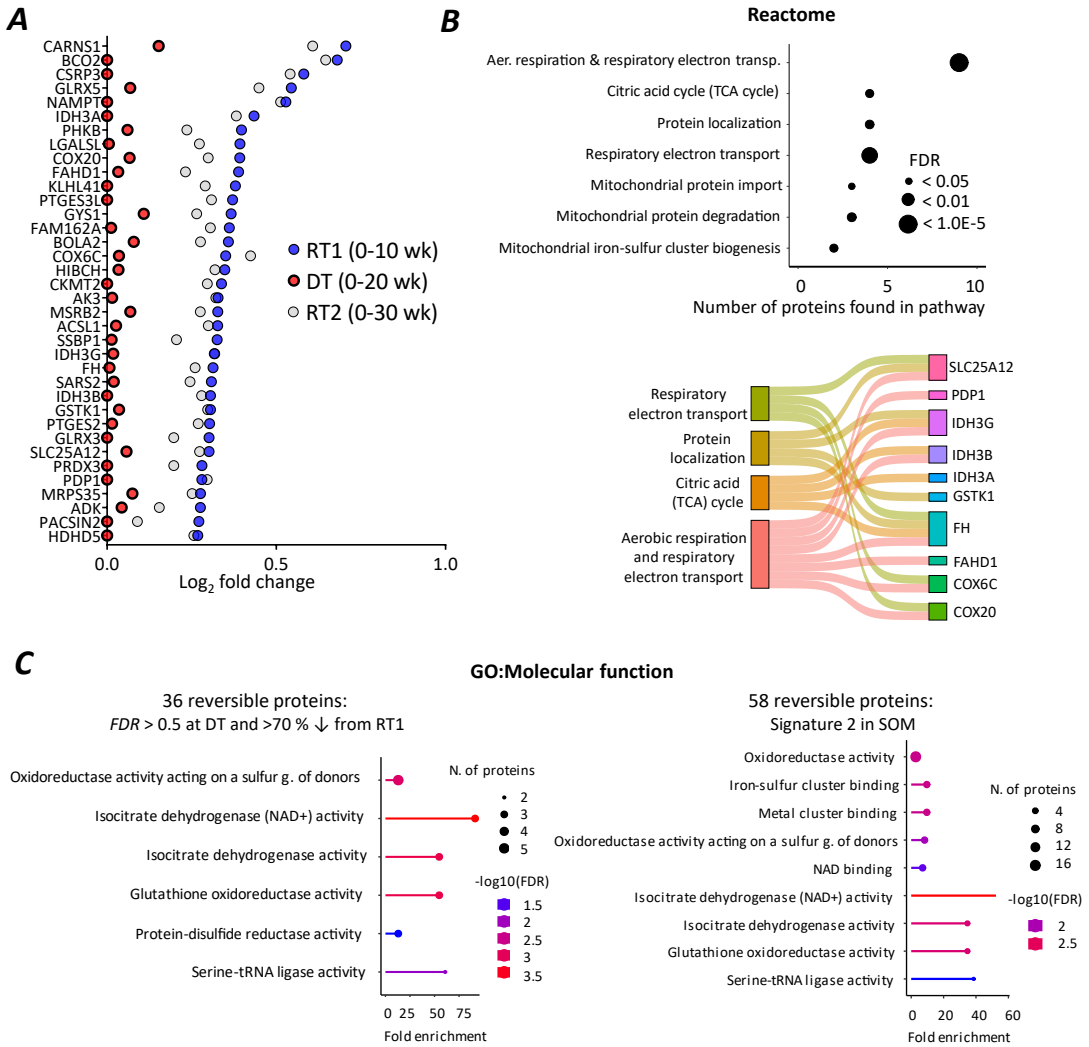


**Figure 4.** *A*, 36 proteins, which increased after RT1 in the PRT group restored to the baseline after DT with the following criteria of: 1) *FDR* > 0.5 at post RT+DT vs. baseline and 2) relatively decreased > 70 % from the post-RT1, i.e. DT vs. RT1 as well as belonging to signature 2 (see Figure 3). *B*, Reactome pathway analysis together with Sankey-diagram show that reversible proteins to RT after DT belong to e.g., aerobic energy metabolism processes and have molecular function roles of especially oxidoreductase and dehydrogenase ©.

## Proteins with retained or 'memory' signatures after RT1, DT, and RT2

After DT, we found eight proteins altered compared to baseline (*FDR* < 0.05, FC > 1.2, Fig. 5A). Of those proteins, four were also increased after RT1 the majority contained within SOM profiling signature 4 as retained as increased after training into detraining, including NAD-dependent malic enzyme (ME2), protein S100-A13 (S100A13), and carbonyl reductase (CBR1) as well as fascin (FSCN1) that was contained within SOM signature 3 (Fig. 5B). In addition, the protein with the largest fold change increases after DT vs. baseline, also in retained protein signature 4, was histone H1.5 (FC=14.2, *FDR* = 0.05), which was also the most altered protein (9.5-fold, *FDR* < 0.05) after RT2 vs. baseline (Fig. 5B). Indeed, H1-5 was already increased at a large magnitude after RT1 (8.3-fold), but due to large variation, it was not statistically significant (*FDR* = 0.08). Whereas, other H1 proteins (H1-0 and H1-10) showed no changes or even small decreases after RT1 (H1-0, 16 %, *FDR*

= 0.05). Looking at the time-course within CRT and PRT groups, reveals that out of these five proteins (ME2, S100A13, FSCN1, CBR1 and H1-5), especially CBR1 and H1-5 were RT-induced "memory" proteins belonging to signature 4 of retained proteins and being consistently increased by RT and staying elevated following DT and RT2 (Fig. 5B).

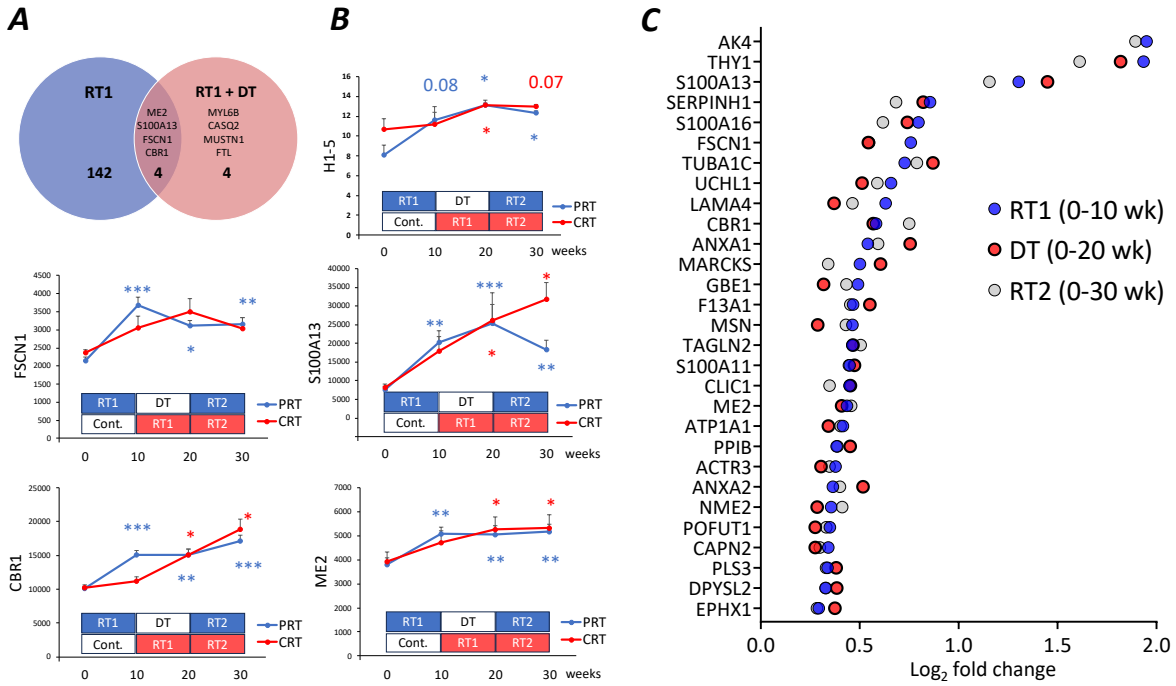


**Figure 5.** *A*, Venn Diagram analysis of the statistically differentially regulated proteins following RT1 and RT1+DT compared to baseline illustrating the number of common overlapping and unique significant proteins across comparisons. *B*, Proteins increased after RT1 and retained as increased after DT and RT2. *C*, 29 proteins, which significantly increased after RT1 in the PRT group and remained elevated after DT and post RT2 (*FDR* < 0.1 and FC > 1.2). N=17 in PRT and n=11 in CRT. Notice that H1-5 is plotted as $\log_2$ values due to robust changes and large variation, while others are plotted by applying the inverse $\log_2$ transformation to estimate their original values. The statistics were, however, always conducted from $\log_2$ values. * <0.05, ** <0.01 and *** <0.001 or exact FDR-value (if *FDR* < 0.10) vs. baseline. Data is presented as mean ± Standard Error of the Mean (SEM).

As the above approach used a very stringent statistical approach *FDR* < 0.05, however, for discovery *FDR* < 0.10 or more robust approaches have been widely used in human exercise proteomics data. Therefore, focusing again on the 150 proteins altered by RT1 that maybe retained, we next used slightly more inclusive approach for the post-DT vs. baseline comparison (*FDR* < 0.10 in the whole proteome) while maintaining that the fold change still had to be greater than 1.2 (more than 20% increase). We identified 37 retained proteins (that included the four retained proteins identified above) to still be elevated after detraining following earlier training, of which 29 were also confirmed to remain as elevated also after RT2 (vs. baseline, *FDR* < 0.10 and FC > 1.2) (Fig. 5C). To gain further insight into cellular and biological processes of these retained proteins, we conducted bioinformatics analysis of these 29 proteins as well as 81 retained proteins based on SOM signature 4. The retained

proteins were especially overrepresented in cytokine signalling, RHO-signalling and muscle contraction (Fig. 6A-B, Supporting information, Supplement 2) and in molecular function roles of calcium-binding, actin filament binding and phospholipase inhibitor activity (Fig. 6C).

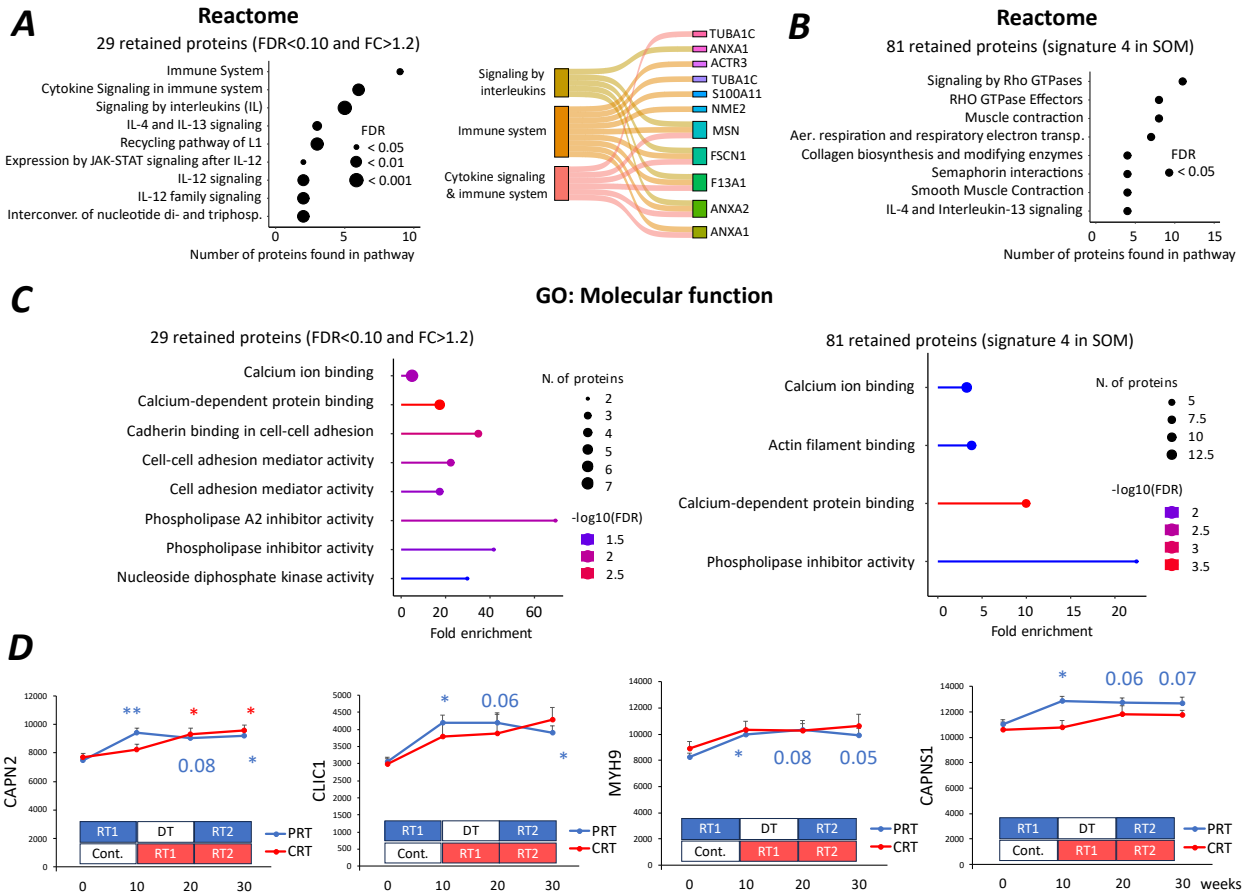


**Figure 6.** *A* and *B*, Reactome pathway analysis together with Sankey-diagram illustrates that the retained proteins are enriched in cytokine signalling, biosynthesis, and folding, sorting and degradation pathways and functions. *C*, The 29 and 81 retained proteins (SOM) in GO have as a molecular function especially in calcium or calcium-dependent protein binding, actin filament binding and phospholipase inhibitor activity. *D*, CAPN2, CLIC1, MYH9 and CAPNS1 proteins increasing after RT1 and maintaining their levels after DT still above the baseline. N=17 in PRT and n=11 in CRT. * <0.05, ** <0.01 and *** <0.001 or exact FDR-value (*FDR* < 0.10) vs. baseline. Data is presented as mean ± SEM.

Next we compared these 29 retained 'memory' proteins to previously published epigenetic memory genes, i.e. retained hypomethylation of the genes DNA after both RT and DT (Seaborne *et al.*, 2018) and after HIIT training and detraining (Pilotto *et al.*, 2024). We found that previously described epigenetic memory genes CAPN2, CLIC1 and MYH9, which are retained as hypomethylated, also demonstrate retained protein levels after DT following the earlier training in the present study. Of those proteins, CAPN2 and MYH9 were also identified to be hypomethylated genes following RT that also increase in 181 integrated skeletal muscle transcriptomes after RT (Turner et al., 2019). Of those two, only CAPN2 had a similar time trend also in the CRT group, i.e. increased as a result of

RT with no change during the control period (Fig. 6D). CAPN2 is a catalytic subunit of calpain-2 forming a heterodimer complex with the small regulatory subunit (CAPNS1) to be a functional protease (Hyatt & Powers, 2020), where in the present study CAPN2's regulatory subunit CAPNS1 also demonstrates a retained protein profile after detraining that was similar to CAPN2 (Fig. 6D).

**Majority of the proteins decreased after RT1 have a reversible signature with one protein that is retained as down**

Ten proteins decreased significantly after RT1 in the PRT group (Fig. 7A), of which almost all returned towards the baseline after 10 weeks of DT and were then decreased again after RT2 when compared to baseline (8 of 10 at *FDR* < 0.10 and 5 of 10 at *FDR* < 0.05). Interestingly, however, we identify one slightly, but consistently (FDR<0.01) decreased protein at RT1 (*FDR* < 0.01), DT (*FDR* = 0.06) and RT2 (*FDR* < 0.001), Low density lipoprotein receptor-related protein-associated protein 1 (LRPAP1) with a retained down profile also after DT (*FDR* = 0.06) and RT2 (*FDR* < 0.001) (Fig. 7B). Finally, after RT2 when compared to pre-RT2, two proteins decreased, MYH1 (IIX isoform) and ASAH1 of which there was also similar magnitude (~51 %, *FDR* = 0.066) decrease in MYH1 following RT1 (Fig. 2E), as well in the CRT group (~52 %, *FDR* = 0.138).

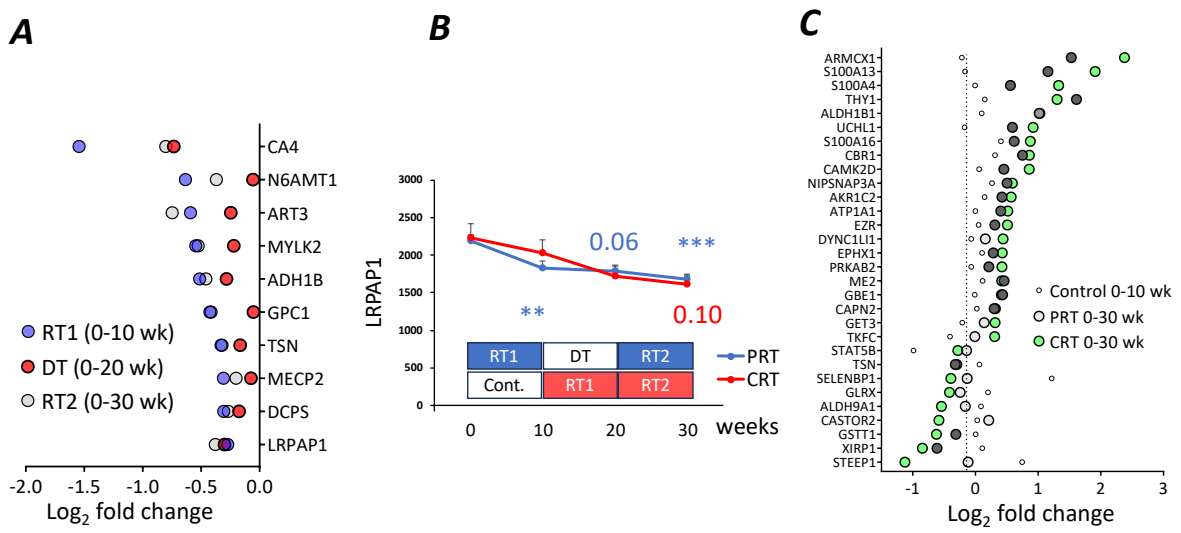


**Figure 7.** *A*, Ten proteins significantly decreased in response to RT1 in CRT group. *B*, LRPAP was a protein with decreased retained signature. * <0.05, ** <0.01 and *** <0.001 or exact FDR-value (*FDR* < 0.10) vs. baseline. *C*, Significantly altered proteins in CRT group (n=11) at 0 to 30 weeks plotted with the same proteins in the PRT (n=17) group and during the control period in the CRT group (n=13). Dark grey indicates *FDR* < 0.05 alteration also in the PRT group.

**Outcomes of PRT when compared to CRT**

As shown in Table 1 and earlier (Halonen *et al.*, 2024), the eventual outcome (i.e. change from week 0 to 30) in muscle size or strength did not differ between PRT and CRT. Similarly, when comparing concurrent proteome adaptations, there were no significant between-group differences in the change (*FDR* > 0.3, Supporting information, Supplement 2). This suggests that skeletal muscle proteomic adaptation to 20 weeks of RT is not strongly affected by the training break. Finally, to demonstrate similar responses in these two groups, the significantly altered proteins in the CRT group from 0-30 weeks behaved very similarly also in the PRT group during the same time-period (Figure 7C).

## Discussion

In this study, we report substantial and reproducible remodelling of human skeletal muscle proteome following repeated RT. Several proteins returned towards baseline after the training break while being increased again after a second period of RT. These proteins are especially involved in aerobic energy metabolism. However, many proteins remained elevated even after ten weeks of detraining. The proteins with the retained profile include those involved in calcium and actin filament binding, cytokine signalling and muscle contraction. Herein, we are the first to display that human skeletal muscle possesses long-term protein changes and a proteomic memory of muscle growth via RT.

Earlier epigenetic/transcriptomic studies in humans (Seaborne *et al.*, 2018; Pilotto *et al.*, 2024) and mice (Wen *et al.*, 2021) have reported retention of epigenetic modifications after training (RT, HIIT, and weighted wheel running, respectively), which can lead to continued elevation of gene expression during detraining. The present study now provides novel evidence to extend these results to the protein level. More specifically, of the individual proteins retained as altered after the 10-week training break, we identify calpain 2 (CAPN2), histone H1.5, carbonyl reductase 1 (CBR1), and low-density lipoprotein receptor-related protein-associated protein (LRPAP1) as potential 'muscle memory' proteins. GO term Molecular function showed that many of the retained proteins have a role in calcium-binding. One of the calcium-binding proteins was calpain-2 (CAPN2), which is also one of the few consistent hypomethylated muscle memory genes to RT (Seaborne *et al.*, 2018; Turner *et al.*, 2019) and HIIT (Pilotto *et al.*, 2024). This gene transcript was also increased in 181 integrated skeletal muscle transcriptomes after RT (Turner *et al.*, 2019) and was recently one of the most upregulated genes with a delay after a single bout of resistance exercise (Edman *et al.*, 2024). The reliability of the result is also increased by the fact that this protein was consistently increased by RT in the present study and that its small regulatory subunit (CAPNS1) (Hyatt & Powers, 2020), also

followed a similar retained protein signature. Recently, calpain-2 was also increased after 10 weeks of RT, especially post-exercise, and to decrease already in 24 hours following a single exercise bout (Scarpelli et al., 2024), suggesting that it does not have very long half-life in human skeletal muscle. Calpain-2 has an important role in the remodelling of skeletal muscle, such as essential myofibril disassembly during growth and atrophy stimuli, like exercise and inactivity, respectively (Hyatt & Powers, 2020). When analysing the retained proteins using SOM-approach, we identified many proteins to be involved in muscle contraction and thus also linked to myofibrils (CAMK2D, ANXA1, TPM4, ANXA2, CASQ2, MYL6B, VIM, CES2). On the other hand, RHO-signalling was also identified using this approach. The proteins overrepresented in that pathway (TUBA1C, TUBB2A, TPM4, ARPC2, ARPC3, MYH9, PPP2R5D, VIM, IQGAP2, MYH10, and CKAP4) are predominantly associated with cytoskeletal organization and cell structure, and thus serving important functions in skeletal muscle. Thus, it appears that several proteins essential for muscle function (e.g. muscle contraction, cytoskeleton and calcium-binding) have persisting levels in skeletal muscle following RT which substantiates the effect on these pathways identified to be enriched in hypomethylation and gene expression after RT (Turner *et al.*, 2019).

Some of the RT-induced retained proteome remodelling includes proteins involved in biosynthesis and folding, sorting, and degradation processes, but similar processes were observed also in reversible signatures (Supporting information, Supplement 2). To expand these, also the overall RT-induced proteome involved processes such as chaperones and folding catalysis, protein processing in endoplasmic reticulum. These are well in line with a recent muscle proteomics study (Jessen *et al.*, 2024) and studies showing that RT can induce endoplasmic reticulum (ER) stress and activate the unfolded protein response (UPR) pathways (Hentila *et al.*, 2018). Intriguingly, we found that LRPAP1 was the only protein to be decreased after RT that was also retained as decreased following detraining and the second RT period. LRPAP1 primarily functions as a molecular chaperone, but we are not aware of it being investigated in the context of exercise, skeletal muscle biology.

In the epigenetic control of transcription, the gene expression is also regulated by histones and their modifications. We found that linker histone H1-5 was highly increased by RT, and it remained highly increased following detraining and retraining. Its GO terms are related to its involvement in maintaining chromatin compaction, and its depletion can decrease cell growth (Li *et al.*, 2012). We are unaware of any H1-5 studies in skeletal muscle, thus warranting further studies to investigate its potential role in RT responses. Another retained up protein was CBR1, where its transcript levels are significantly increased by RT based on MetaMEx (https://www.metamex.eu/) without an effect of inactivity (Pillon *et al.*, 2020) and its protein levels were also slightly ($FDR < 0.10$) elevated earlier

after a shorter RT-intervention (Jessen *et al.*, 2024). It is upregulated during skeletal muscle differentiation and regeneration and appears to be an essential regulator of these processes in vitro through decreasing ROS levels and lipid peroxidation (Lim et al., 2013), suggesting that it may be involved in the continuous remodelling of skeletal muscle following RT.

Our bioinformatics analysis showed that many of the retained proteins are involved in cytokine signalling pathways, especially of IL-4, IL-12, and IL-13. IL-13 and its receptor are expressed in skeletal muscle and their levels and signalling appear to be increased after exercise training (Prokopchuk *et al.*, 2007; Knudsen *et al.*, 2020), and are important for aerobic adaptations through STAT-signalling (Knudsen *et al.*, 2020). The JAK-STAT signalling pathway is also crucial in muscle regeneration by controlling satellite cell activity, myogenic differentiation and potentially enhancing muscle repair, although it can also have muscle wasting effects if chronically upregulated (Moresi *et al.*, 2019). This interplay of the immune, regeneration, and metabolic pathways may be important for continuous skeletal muscle remodelling after RT.

Many proteins followed retained or semi-retained signatures following 10 weeks of training break. For many proteins, we expect that this reflects their long-term expression, i.e. potentially identifying them as muscle memory proteins. However, due to long half-lives in skeletal muscle (10 days on average in mice) (Rolfs *et al.*, 2021) some of the protein elevations are probably retained during detraining, as speculated earlier after inactivity due to spaceflight and bed rest (Murgia *et al.*, 2022). Nevertheless, we found several proteins decreasing back to baseline after detraining. These reversible proteins were particularly enriched in energy metabolism, mitochondrial function, redox homeostasis, and especially the TCA cycle and electron transport chain. This is in line with the known short *in-vivo* half-life of enzymes and fast decrease of aerobic capacity after detraining (Mujika & Padilla, 2000). One example of a reversible RT-upregulated protein essential in skeletal muscle energy metabolism was nicotinamide phosphoribosyltransferase (NAMPT). It is a rate-limiting enzyme in $NAD^+$ production, a coenzyme essential for muscle (Beltrà *et al.*, 2023). NAMPT has been previously shown to be upregulated by RT (de Guia *et al.*, 2019; Lamb *et al.*, 2020) and we expand these findings showing that the effect is reversible and also repeatable upon later retraining (See Supporting information, Supplement 2).

This is the first study to investigate in a longitudinal (within-individual) design whether the proteome responses to exercise are systematic. Our study also included a control period to exclude results due to, for example, seasonal changes or other random effects. Our data shows a much more robust effect of RT than the control period on proteomics, but we speculate that also potential seasonal changes may affect some muscle proteins as seasonal changes in gene expression are known to exist in human

cells (Dopico *et al.*, 2015). The three proteins altered during the no-training control period in the CRT group included MUSTN1, XIRP1, and KLHL40. These proteins demonstrated a decrease during the control period, perhaps due to a seasonal effect (from late winter to early summer) (See Supporting information, Supplement 2). For example, XIRP1 also simultaneously decreased in the PRT group. On the other hand, MUSTN1 and KLHL40 increased after RT2 in the PRT group but not after RT1 when their potential decreasing seasonal effect occurs. MUSTN1 is a protein recently shown to be a secreted protein in skeletal muscle, which is decreased after hindlimb unloading and rescued after reloading (Ducommun *et al.*, 2024). We expand these findings showing that this protein is also increased by RT in humans. Another kelch-like family protein KLHL41 recently identified as RT-induced protein (Jessen *et al.*, 2024) did not change during the control period, but was consistently responsive to both RT1 and RT2 (See Supporting information, Supplement 2). Interestingly, RT-induced changes in MUSTN1 correlated strongly and consistently with changes in KLHL40 and KLHL41 (See Supporting information, Supplement 2) and when the changes in *MUSTN1* were correlated with all other gene expression changes, *KLHL40* emerged as the strongest link also in MetaMEx (https://www.metamex.eu/) (Pillon et al., 2020). This suggests MUSTN1 and KLHL40 (and potentially also KLHL41) may have common upstream regulation, which should be investigated mechanistically in the future.

We report in our periodic RT group reproducible protein changes after the first RT and the whole period, as two thirds of the RT1-induced proteins were also induced after the second RT period when compared to the baseline. Moreover, when the second RT period was compared to its pre state (i.e. to post detraining), more than half of the proteins altered behaved very similarly after RT1 and overall, significantly correlated with the responses in RT1. We report that based on fold change (with $FDR < 0.05$), the top upregulated proteins after the first RT were Quinone oxidoreductase PIG3 (TP53I3), Thrombospondin-4 THBS4, Adenylate kinase 4 (AK4), Thy-1 membrane glycoprotein (THY1) and Ras GTPase-activating-like protein (IQGAP2), and with $FDR < 0.1$ H1-5, and the most decreased protein was Carbonic anhydrase 4 (CA4), all of which were also similarly altered after the whole intervention. When compared to baseline, there were more proteins increased after the second RT period than yet the first, and many of those proteins were enriched specifically in aerobic metabolism pathways and biological processes, which did not appear to occur in the continuous training group. This suggests that some proteins have greater levels after the second period of RT, but only when there was a break in between. However, the responses to the second RT when compared to its pre-state were less consistent and of smaller magnitude (see correlation Fig. 2B). Similarly, the protein remodelling during the first RT period significantly correlated between the groups.

Finally, we also compared our data to a recently published four-week RT intervention (Jessen *et al.*, 2024). Of the 140 proteins increased in the present study, about half (71) were identified. Of those proteins, 24 were also increased in (Jessen *et al.*, 2024), meaning a ~34 % overlap. We also found many similar protein changes, such as increases in KLHL40 and KLHL41, of which the latter was mechanistically shown to regulate muscle cell size *in vitro* (Jessen *et al.*, 2024). Overall, these studies suggest a considerable reproducibility of the effect of RT on muscle proteome.

The future human RT proteomics studies investigating muscle memory at the protein level should include a longer training break as well as acute bouts of resistance exercise to investigate what happens to RT response before and after a training break. In addition, combining proteomics approaches to metabolomics, transcriptomics and epigenetics and as well as other epigenetic modifications such as chromatin accessibly via ATAC-seq in the future are crucial to advance our understanding of muscle memory. Finally, the physiological significance of long-term protein changes and proteomic memory induced by RT should be investigated in the future by mechanistic experiments.

In summary, we have presented the first in-depth data on proteomic remodelling to resistance training followed by a training break and retraining. We first demonstrate that the proteome adaptations to resistance training are reproducible. We further identify proteins that are reversible or remain elevated even after more than two months of no training. This data supports the presumption that human skeletal muscle possesses a proteomic memory of muscle growth following resistance training.

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## CONFLICTS OF INTEREST STATEMENT

The authors declare that they have no conflict of interest. The results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

## DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available from the corresponding author upon reasonable request.

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