PROTEOMIC INVESTIGATIONS OF ADAPTATIONS TO EXERCISE IN HUMANS

PROTEOMIC INVESTIGATIONS OF ADAPTATIONS IN SKELETAL MUSCLE TO AEROBIC AND RESISTANCE EXERCISE IN HUMANS

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A Thesis

Submitted in Partial Fulfillment of the requirements of Liverpool John Moores University for the Degree of

Doctor of Philosophy

This research program was carried out in collaboration with McMaster University

November 2024

DOCTOR OF PHILOSOPHY (2024) (Kinesiology)

McMaster University, Hamilton, Ontario

And

Liverpool John Moores University, Liverpool, United Kingdom

TITLE: Proteomic investigations of adaptations in skeletal muscle to aerobic and resistance exercise in humans

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NUMBER OF PAGES: xv, 237

LAY ABSTRACT

While some exercises help muscles become stronger, others improve the ability to resist fatigue. However, the reasons behind these different changes are not fully understood. Learning more about these processes is important because it could help develop treatments or exercise plans to improve health, especially for people at risk of losing muscle mass. We conducted two studies to explore the reason behind these changes: In the first study, we used advanced techniques to see which proteins in muscles are activated by either aerobic (endurance) or resistance (strength) exercise. We found certain proteins that were specifically turned on by resistance exercise, which helps build muscle and strength. This discovery is important because it could help us find ways to prevent or treat muscle loss, especially as people get older. In the second study, we looked at which proteins in muscles increased or decreased with both types of exercise. We found that some proteins, especially those involved in energy production, are regulated similarly in both types of exercise. However, many other proteins respond differently to aerobic and resistance exercises. Together these studies add to our knowledge on how exercise helps muscle to become more fit and mighty.

ABSTRACT

The mechanisms leading to a hypertrophic versus an endurance phenotype, the hallmarks of resistance exercise (RE) or aerobic exercise (AE), respectively, are still largely unknown. In humans, exposure of exercise naïve persons to either AE or RE results in their skeletal muscle exhibiting generic 'exercise stress-related' signalling, transcription, and translation responses. However, with increasing engagement in AE or RE, the responses become refined, and the phenotype typically associated with each form of exercise emerges. Phosphorylation of specific residue sites has been a dominant focus, with canonical signalling pathways (i.e. AMPK and mTOR) studied extensively in the context of AE and RE, respectively. These alone, along with protein synthesis, have only begun to elucidate key differences in AE and RE signalling. Still, key yet uncharacterized differences exist in signalling and regulation of protein synthesis that drive unique adaptation to AE and RE. Omic studies are required help in understanding the mechanisms that lead to the divergent relationship between exercise and phenotypic outcomes of training. In study 1 of this thesis, 16 young, healthy subjects (n=4 pilot study and n=12 follow-up study; 6 males [M] and 6 females [F]) performed a unilateral session of AE or RE with biopsies taken before (Pre), immediately post (0h) and 3 hours (3h) following recovery. Muscle tissue (cohort 1: n=4) was subjected to deep phosphoproteomic analyses, identifying nearly 13000 individual phosphosites and unique clusters specifically associated with AE and RE. Follow-up studies (cohort 2: n=12) were performed, and the outcomes support a thesis that prolonged activation of the MKK3/6, p38, and MK2 signalling axis is a resistance exercise-specific phenomenon and is critical in the process of muscle hypertrophy. We also demonstrated that the activation of signalling through MKK3 is robustly correlated with the increase in myofibrillar

protein synthesis that occurs after RE in humans ($r^2 = 0.60$, $p<0.01$). In study 2, we sought to determine the divergent changes in the skeletal muscle proteome induced earlier and later in a training program using both RE and AE, in parallel, in healthy young males and females. We investigated muscle adaptations to AE and RE training during the early untrained versus trained state using novel deuterium oxide labelling and proteomic techniques. A total of 14 (8F/6M) healthy individuals completed 10-wk of thrice weekly unilateral resistance and unilateral aerobic training. Our data illustrated the common and unique networks of protein regulation following AE and RE. Specifically we highlighted the influence that both AE and RE have on mitochondrial proteins, likely aimed at improving metabolic function and possibly underpinning an increase in oxidative capacity and in supporting tissue protein remodelling. In both AE and RE, we identified changes in protein abundance that did not align with individual protein synthetic rates, suggesting that targeted degradation of certain proteins may be an adaptive feature of the shared response to aerobic and resistance training. To date, this work represents the most in-depth analysis of protein-specific fractional synthesis rates in human muscle in vivo in response to differential forms of exercise training. Together, these studies generate novel insights into training mode-specific muscle adaptations by measuring widespread phosphorylation and protein-specific changes in combination with individual protein synthesis rates.

ACKNOWLEDGEMENTS

To Stu, I'm extremely grateful for the opportunities you have given me. Your mentorship and guidance over the past ~5 years have allowed me to thrive as a young scientist. I could not have asked for a better supervisor. You have an incredibly ability to keep science 'fun' while maintaining a standard of excellence. You are one of the hardest working individuals I have had the pleasure to know. Thank you.

To Jatin, Connor and the rest of the group at LJMU, thank you all for welcoming me into your lab group and making my time in Liverpool and experience I will remember forever. Jatin, thank you for your patience in fielding countless questions from me regarding proteomics and LMCS. You are without a doubt, one of the most intelligent individuals I have ever met, and it was a privilege working with and learning from you.

To Drs. Gibala and Ljubicic, thank you for your continued support and guidance over the past years. It's been a pleasure to learn from both of you throughout my masters and now Ph.D. Your perspectives and insight were invaluable and truly appreciated.

To Dr. Hornberger and Wen, thank you for your collaborative support and efforts on our project together. I'm very blessed to have had the opportunity to collaborate and exchange ideas with you both.

To Drs. Chris McGlory, Changhyun Lim, James McKendry and Josh Nederveen, your mentorship as I developed myself as a researcher has been indispensable. Each of you has been there for the highs and lows along the way and I couldn't have asked for better leaders like you to guide me through it. Thank you for all the runs, bike rides, games of golf, cups of coffee and pints along the way.

To Todd, thank you for all the logistical and personal help along the way. You are the glue that holds the lab together. Thank you for teaching me so many useful lab techniques and your endless patience for my lab mistakes.

To the EMRG group, past and present, thank you. You all hold a special place in my memory. I will cherish all our experiences together and look forward to new ones in the future.

To my family, mom, dad, grandparents, thank you for your endless patience, love and support. I will always appreciate how you have made my pursuits in academia possible and have always made sacrifices without complaint so that I could have this opportunity. I will be forever grateful.

To Shannon, thank you for always being there for me. Listening to rants about muscle, protein and science, thank you for your patience and love.

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LIST OF ABBREVIATIONS

1-RM – single repetition maximum (maximal strength)
AC – acute
AE – aerobic exercise
AMPK – adenosine monophosphate kinase
AKT – Akt serine/threonine kinase
AT – aerobic training
Bx - biopsy
CAMK – Ca^{2+}/calmodulin-dependent protein kinase
CR - chronic
D_2O – deuterium
DXA – dual energy x-ray absorptiometry
eIF – eukaryotic initiation factor
eEF – eukaryotic elongation factor
FAK – focal adhesion kinase
FDR – false discovery rate
fmol – femtomole
ERK – extracellular signal-regulated kinase
FC – fold change
FSR – fractional synthesis rate
GO – gene ontology
HIIT – high-intensity interval-type training
JNK – Jun N-terminal kinase
LCMS – liquid chromatography mass spectrometry
LKB1 – liver kinase B1
MAPK – mitogen-activated protein kinase
MPS – muscle protein synthesis
MPB – muscle protein breakdown
mTORC – mammalian target of rapamycin complex
MyoPS – myofibrillar protein synthesis
PGC-1α – peroxisome proliferator-activated receptor-gamma coactivator
PIP3 – phosphatidylinositol-3,4,5-trisphosphate
PIP3K – phosphoinositide 3-kinase
PRAS – proline-rich substrate of AKT
PTM – post-translational modification
RE – resistance exercise
Rheb – Ras homolog enriched in brain
RT – resistance training
SacroPS – sarcoplasmic protein synthesis
SH2 – Src homology region 2
SIRT1 – sirtuin-1
STRADα – Ste20-related adaptor protein-α
TAZ – transcriptional activator with PDZ-binding motif
TFA – trifluoracetic acid
TMT – tandem mass tag
TSC – tuberous sclerosis complex
UBF – upstream binding factor
ULK – Unc-51-like autophagy-activating kinase
VO_2 peak – peak aerobic capacity
YAP – yes-associated protein

PREFACE:

DECLARATION OF ACADEMIC ACHEIVEMENT

FORMAT AND ORGANIZATION OF THESIS

This cotutelle thesis is prepared in the format as outlined in the School of Graduate Studies Guide (McMaster University) and Post Graduate Research (Liverpool John Moores University) for the Preparation of Theses. It includes comprehensive review of the literature, two original research papers prepared in journal article format, and a general discussion. The candidate is the first author on Chapter 1, and joint first author on Chapters 2 and 3. At the time of the thesis preparation, Chapter 1 was in submission, currently under peer review at Free Radical Biology and Medicine (FRBM).

CONTRIBUTION TO PAPERS WITH MULTIPLE AUTHORSHIPS

Chapter 1: Comprehensive literature review

Exercise-Specific Adaptations in Human Skeletal Muscle: Molecular Mechanisms of Making Muscles Fit and Mighty. Submitted to *Free Radical Biology and Medicine*. April 2024

Contribution:

A.C.Q.T. wrote the review. All authors edited and approved the final version of the manuscript.

Chapter 2:

Phosphoproteomic Investigations in Humans to Probe Critical Pathways in Resistance Exercise Adaptation.

Contribution:

A.C.Q.T., W.Z., C.C.M., T.H., and S.M.P., designed the study. A.C.Q.T., J.M., C.L., and W.Z., collected and analyzed the data. W.Z., G.M.W., J.E.H., C.G.K.F., K.W.J., N.D.S., K.L., J.J.C., T. H., performed the phosphoproteomic analysis. A.C.Q.T., and S.M.P. wrote the manuscript. All authors edited and approved the final version of the manuscript.

Chapter 3:

The dynamic proteomic response to aerobic and resistance training in human skeletal muscle.

Contribution:

A.C.Q.T., J.G.B., C.A.S., and S.M.P designed the study. A.C.Q.T., and C.A.S., collected and analyzed the data. A.C.Q.T., and C.A.S., performed the proteomic analysis. A.C.Q.T., and S.M.P wrote the manuscript. All authors edited and approved the final version of the manuscript.

CHAPTER 1:

Exercise-Specific Adaptations in Human Skeletal Muscle: Molecular Mechanisms of Making Muscles Fit and Mighty

Chapter 1: Unlocking Athletic Potential: Exploring Exercise Physiology from Mechanisms to Performance

Exercise-Specific Adaptations in Human Skeletal Muscle: Molecular Mechanisms of Making Muscles Fit and Mighty [1]

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Keywords

Protein turnover, Protein signaling, Hypertrophy, Mitochondria, Human

Article Note: This article is a contribution to the special issue entitled "Unlocking Athletic Potential: Exploring Exercise Physiology from Mechanisms to Performance." Guest Edited by Maria Carmen Gomez Cabrera (University of Valencia, Spain) & Christoph Handschin (University of Basel, Switzerland)

Abstract

The mechanisms leading to a predominantly hypertrophied phenotype versus a predominantly oxidative phenotype, the hallmarks of resistance training (RT) or aerobic training (AT), respectively, are being unraveled. In humans, exposure of naïve persons to either AT or RT results in their skeletal muscle exhibiting generic 'exercise stress-related' signaling, transcription, and translation responses. However, with increasing engagement in AT or RT, the responses become refined, and the phenotype typically associated with each form of exercise emerges. Here, we review some of the mechanisms underpinning the adaptations of how muscles become, through AT 'fit' and RT, 'mighty'. Much of our understanding of molecular exercise physiology has arisen from targeted analysis of PTM and measures of protein synthesis. Phosphorylation of specific residue sites has been a dominant focus, with canonical signaling pathways (i.e. AMPK and mTOR) studied extensively in the context of AT and RT, respectively. These alone, along with protein synthesis, have only begun to elucidate key differences in AT and RT signaling. Still, key yet uncharacterized differences exist in signaling and regulation of protein synthesis that drive unique adaptation to AT and RT. Omic studies are required to better understand the divergent relationship between exercise and phenotypic outcomes of training.

Introduction

In healthy adults, skeletal muscle comprises approximately ~40% of total body mass and is essential to functions of daily life, including locomotion, nutrient storage, and metabolic regulation (Egan and Zierath 2013, Frontera and Ochala 2015). Skeletal muscle is fundamental to athletic performance, and a certain amount of functional muscle is vital for good health. The preventative effects of exercise against non-communicable diseases such as cardiovascular disease and type 2 diabetes are, in part, attributed to skeletal muscle responses to exercise training (Saqib, Dai et al. 2020). In addition, regular exercise helps maintain muscle mass in aging, with older individuals who are in the upper tertile of mass exhibiting lower all-cause mortality and a lower incidence of cancer-related deaths (Ruiz, Sui et al. 2008). The ability of exercise to improve physical performance and health outcomes is indisputable; however, substantial molecular details are missing from our knowledge of the mechanisms that underpin the beneficial processes of muscle adaptation to exercise. Muscle adaptation is largely an intrinsic process; nevertheless, the intracellular signals that occur transiently during and in recovery following exercise cannot yet be accurately linked to the phenotypic changes in muscle content and function that are associated with chronic, repeated bouts of the exercise stimulus. Further mechanistic understanding of how exercise leads to improvements in muscle mass and metabolic function is needed to fully 'unlock' the potential of skeletal muscle and optimize health and athletic performance.

Training-induced improvements in exercise performance are a direct reflection of the specificity of the perturbations elicited by the stimulus. Therefore, research on muscle responses to exercise is largely dichotomized into either aerobic or resistance-type stimuli, which lead to distinct muscle adaptations. Aerobic exercise (AE) includes activities involving relatively low forces

over prolonged periods (minutes to hours), requiring drastic elevations in ATP usage and resynthesis via oxidative phosphorylation to sustain work output. The majority of ATP generated during aerobic exercise is derived from carbohydrate (i.e., glycogen) and fatty acid (i.e., intramuscular triglyceride and circulating free fatty acids) oxidation by elevations in mitochondrial oxidative phosphorylation (Hargreaves and Spriet 2020). The power output of the exercise session, often measured in W, estimates the rate of ATP consumption, and relative exercise intensity (as a percent of VO_2 peak) dictates the contribution of energy derived from carbohydrates and fatty acids available (Romijn, Coyle et al. 1993, Egan and Zierath 2013, Hargreaves and Spriet 2020). Regular aerobic training (AT) remodels skeletal muscle into a more oxidative, fatigue-resistant phenotype underpinned by changes in substrate utilization (i.e., altered % carbohydrate and fatty acid oxidation at relative work intensities), mitochondrial content, efficiency and capillarization (Phillips, Green et al. 1996, Hughes, Ellefsen et al. 2018). Just 14 consecutive days of AT can increase VO_2 peak by 17% in sedentary males (Egan, O'Connor et al. 2013), which suggests an early rapid phase of adaptation in response to unfamiliar exercise stimuli. The improvements in whole-body aerobic performance (i.e., VO_2 peak) are underpinned by muscle adaptions, including mitochondrial biogenesis, which enhances oxidative metabolism and alters substrate utilization (Chilibeck, Bell et al. 1998, Gerhart-Hines, Rodgers et al. 2007).

Resistance exercise (RE) is characterized by movements against higher loads for a shorter duration of time relative to those associated with AT (Hughes, Ellefsen et al. 2018). RE primarily relies on anaerobic metabolism to meet the immediate and high-energy demands of the mechanical loading of the target muscle. During very intense and brief intermittent RE, the primary source of energy is derived from the breakdown of phosphocreatine and glycogen with a

high contribution, at least initially, from non-oxidative glycolytic flux, resulting in lactate production (Hargreaves and Spriet 2020). Phosphocreatine and glycogen metabolism rapidly regenerate ATP, typically able to sustain high output for ~10-30s and 30-120s, respectively, before recovery is necessary (Parolin, Chesley et al. 1999, Chidnok, DiMenna et al. 2013). Although anaerobic energy pathways are the primary contributor to ATP production with RE if the effort is prolonged or repetitive, then oxidative metabolism of glucose and fatty acids occurs, and recovery from all forms of AE or RE is always an oxidative process (Hornberger and Esser 2004, Drummond, Fry et al. 2009, Morton, Murphy et al. 2018, Currier, McLeod et al. 2023). Regular resistance training (RT) facilitates muscle remodeling toward growth and increased force output (Goreham, Green et al. 1999). We have previously observed an 8.1% increase in *vastus lateralis* muscle cross-sectional area, assessed by MRI, following 10 weeks of RT that targeted the quadricep muscle (Stokes, Timmons et al. 2020). Although there are many variables associated with RE (i.e., load, sets, frequency, type of contraction), a recent systematic review from our lab of 192 articles emphasizes that most RT prescriptions are effective for increasing muscle strength, range of standardized mean difference vs. control (0.75-1.60) and hypertrophy, range of standardized mean difference vs. control (0.10-0.66) when compared to non-exercise (Currier, McLeod et al. 2023). The evidence is overwhelming for the efficacy of RT to consistently improve measures of muscle mass, function, and strength (McLeod, Currier et al. 2023).

Despite the divergent outcomes of the different modes of training, relatively little is known about the signaling mechanisms that are specific to the transition to an aerobic or resistance-trained muscle phenotype. Chronic responses of muscle to exercise occur over a time frame of weeks-months and cannot, as yet, be readily predicted from the transient molecular responses of muscle

to each exercise session. A greater understanding of the processes that link the acute responses of muscle during or soon after exercise to the longer-term changes in hypertrophy and metabolic phenotype are prerequisite for efficiently exploiting programmed training or lifestyle recommendations that reliably benefit performance and health. Molecular markers that are predictive of desirable changes in muscle phenotype could be used to optimize exercise prescription but there are issues to be resolved before this becomes a reality. Interindividual differences exist at baseline (untrained state), such that health and disease risk profile and responsiveness to environmental stimuli such as exercise training, acute pathological insults, or chronic responses to unhealthy environments give rise to different acute physiological responses to exercise. Muscles of individuals that are normal weight, overweight, or diagnosed with type 2 diabetes start from different baselines at the beginning of a training intervention, and the demonstrable benefits of exercise (e.g. relative change in insulin sensitivity) will differ in each case. Likewise, it is unclear which beneficial components of exercise occur irrespective of age or exhibit sexual dimorphism because the full repertoire of muscle molecular responses to exercise is unknown.

High-intensity interval-type training (HIIT) is a popular form of training that encompasses higher energy demand aerobic exercise (Gillen and Gibala 2014) interspersed with rest and requires higher force than traditional lower aerobic power activity (Callahan, Parr et al. 2021, Caparrós-Manosalva, Garrido-Muñoz et al. 2023). While there have been some studies examining the impact of intensity on post-exercise MPS (Wilkinson, Phillips et al. 2008, Di Donato, West et al. 2014, Bagheri, Robinson et al. 2022) and signalling (Callahan, Parr et al. 2021), the conclusion is less than clear as to whether HIIT resembles AE or RE in terms of the phenotypic changes it induces. Most evidence suggests HIIT definitely promotes mitochondrial biogenesis (Bishop,

Botella et al. 2019) and can lead to hypertrophy (Caparrós-Manosalva, Garrido-Muñoz et al. 2023), although this appears to be less than that achieved with RE

Recognizing that the cellular molecular events that transpire after exercise are complex, we hone in on some of the most commonly studied intracellular processes associated with the conspicuous adaptations to AE, namely mitochondrial expansion, or RE, increased myofibrillar protein accretion. Our review initially focuses on literature from targeted studies on protein phosphorylation because this is amongst the most extensively studied post-translational modification (PTM). We first review literature arising from targeted analysis of key proteins associated with signal transduction in muscle adaptations to exercise. Mechanical loading is a key differentiator between muscle responses to resistance versus aerobic training, and we, secondly, consider some leading candidate proteins in mechano-transduction and also the concept that some degree of muscle 'damage' may be necessary or involved in distinguishing muscle responses to exercise training. The third aspect of our review addresses limitations of the existing literature that currently constrain our understanding of the mechanisms underpinning muscle adaptation. We highlight issues and new avenues of research brought forth by more contemporary phospho-proteomic analyses, which have expanded the number of proteins of interest and highlighted that complex phosphorylation networks, rather than linear pathways, may underpin muscle adaptation. A lack of knowledge on the detailed time-course of molecular responses to exercise and on protein-specific dynamics in muscle are further constraints to our current understanding of muscle adaptations to exercise training. Our review concludes by highlighting the fundamental but underappreciated role of protein turnover in muscle adaption to AE and as well as RE, and we identify ideas for future experiments that clarify the processes that distinguish between muscle adaptations to AT versus RT.

Exercise-responsive phosphorylation of skeletal muscle proteins. Each session of exercise is detected in muscle as stimuli in the form of mechanical stress and metabolic perturbation. These stimuli are largely transduced through transient changes in the PTM of signaling proteins and transcription factors, which in turn alter the abundance, location, or activity of muscle proteins. The accumulated effect of repeated exercise bouts shifts the balance between the synthesis and degradation of specific proteins and elicits changes in muscle protein content and function that can lead to improvements in exercise performance. Covalent attachment by kinases or removal by phosphatases of phosphate can regulate the cellular location and activity of target proteins. Reversible phosphorylation of specific serine (S), threonine (T), and tyrosine (Y) residues is a prominent and extensively studied mechanism of intracellular signaling. Phosphorylation can be detected using antibodies specific to a phosphorylation site on a particular protein, and mechanistic studies can be conducted using phospho-mimetic or refractive proteins wherein the S/T/Y residue of interest is substituted with alanine (A) (inert/ non-phosphorylatable) or aspartic acid (D) (active/ phosphomimetic). We, however, acknowledge other PTM, including acetylation, neddylation, and ubiquitination are also transiently regulated by exercise and likely contribute to muscle adaptation. Nevertheless, phosphorylation has also been a dominant focus, and several canonical signaling pathways have been elucidated that collectively contribute to the adaptation of muscle, including mammalian target of rapamycin complex (mTORC), mitogen-activated protein kinase (MAPK) and AMP-activated protein kinase (AMPK) activation/ modification. In addition, we briefly discuss the role of reactive oxygen species (ROS), which can modulate some phosphorylation pathways.

Mammalian Target of Rapamycin (mTOR). The mTOR complex is a serine/threonine kinase that has two distinct complexes, rapamycin-sensitive mTORC1 and rapamycin-insensitive mTORC2 (Goodman, Frey et al. 2011). Each mTOR complex is defined by distinct binding partners that specify its function. Unique to mTORC1 is the subunit regulator associated protein of mTOR (raptor) and proline-rich substrate of AKT of 40kDa (PRAS40) (Jhanwar-Uniyal, Wainwright et al. 2019). mTORC2 includes similar components to mTORC1 (mLST8 and DEPTOR) but lacks raptor and instead contains the rapamycin-insensitive companion of mTOR (Sarbassov, Ali et al. 2004). Raptor has multiple points of potential interaction with mTOR, is essential for regulating mTORC1 activity, and contains the binding sites for downstream signaling targets (i.e. p70S6K and 4EBP1) (Bhaskar and Hay 2007). Exercise primarily regulates mTORC1 activity via upstream Ras homolog enriched in brain (Rheb), which in its GTP-bound state enhances the recruitment of substrates to mTORC1 (Parmar and Tamanoi 2010). The activation of mTOR kinase activity is denoted by the phosphorylation of specific serine residues (S^{2448}, S^{2481}, and S^{2446}) in addition to the phosphorylation of well-characterized downstream targets (Navé, Ouwens et al. 1999, Cheng, Fryer et al. 2004, Copp, Manning et al. 2009). The phosphorylation activation of mTOR has long been associated with muscle growth and is thought to initiate signaling for protein translation (Bodine, Stitt et al. 2001). However, mTOR S^{2448} phosphorylation also occurs following acute AE (Benziane, Burton et al. 2008), indicating mTOR S^{2448} phosphorylation is not specific to RE. It is, therefore, necessary to pair investigations on mTORC1 activation with RE- or AE- specific downstream targets to link signaling mechanisms with training outcomes. Well-defined downstream targets of mTORC1 kinase activity include proteins associated with ribosomal biogenesis and translation, including T^{389} of ribosomal protein S6 kinase beta-1(70S6K), T^{37}/T^{46} of eukaryotic translation initiation

factor 4E (eIF4E)-binding protein 1 (4E-BP1), and T^{56} of eukaryotic elongation factor-2 kinase (eEF2K; a.k.a. CaMKIII). Further, mTORC1 is known to phosphorylate master regulators of the autophagy-lysosomal pathway, such as S^{757} of Unc-51-like autophagy-activating kinase 1 (ULK1) and S^{211} of transcription factor EB (TFEB) (Kim, Kundu et al. 2011, Martina, Chen et al. 2012). Together, this implicates mTOR signaling in the regulation of both synthetic and degradative pathways with acute exercise. Although mTOR S^{2448} has been extensively used as a marker of protein synthesis/ anabolic response, it may not be a direct indicator of mTOR kinase activity, and analysis of known downstream targets is recommended to infer the presence of the active forms of mTOR complexes 1 and 2 (Figure 1) (Figueiredo, Markworth et al. 2017).

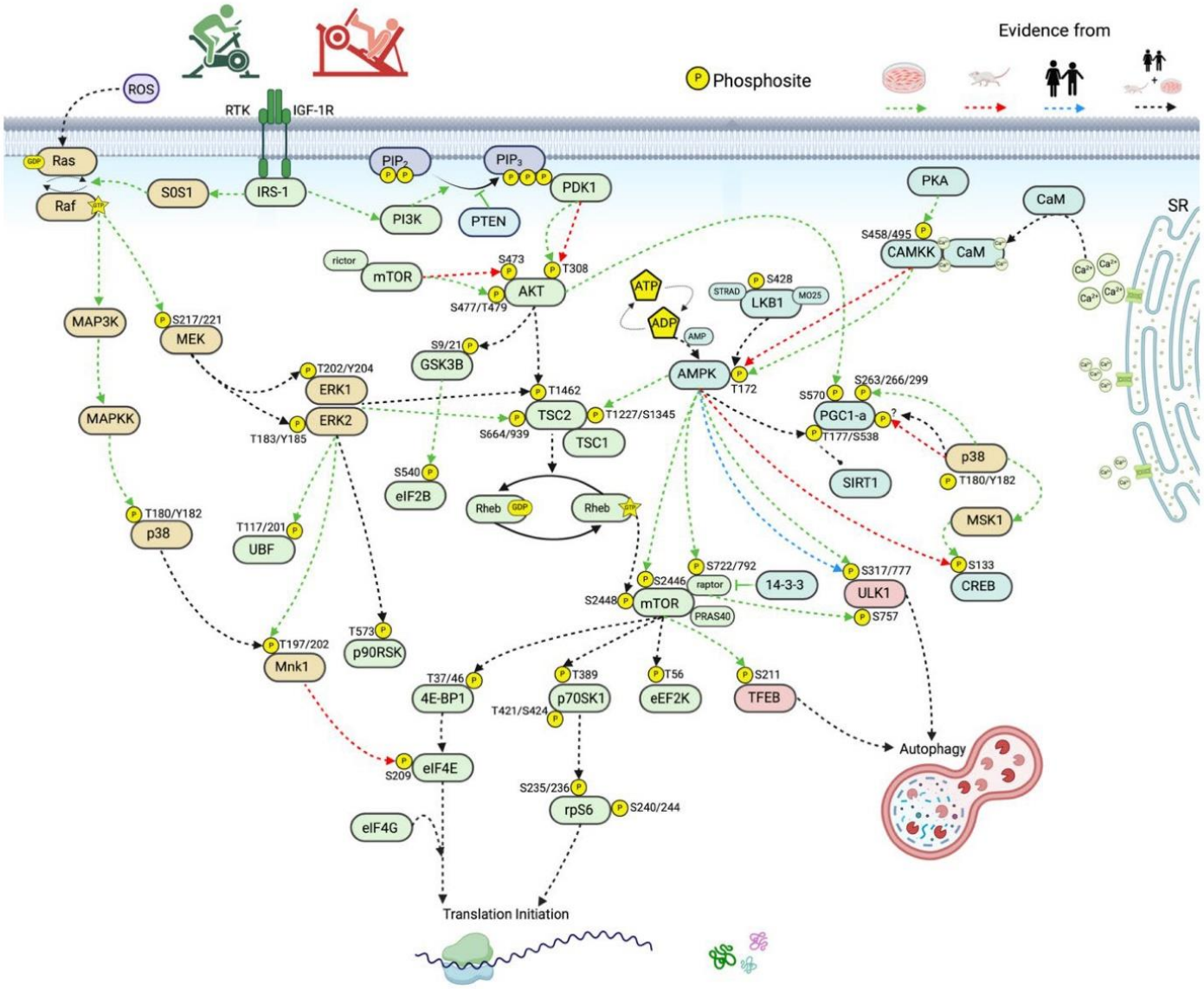


Figure 1.1. Simplified signaling network of the key proteins, kinases, and transcription factors involved in the regulation of translation initiation and autophagy following acute exercise. Targeted PTM studies following exercise have identified AKT-mTOR, MAPK, and AMPK

cascades as primary regulators of translation initiation and autophagy. The color of the arrow denotes the model of evidence used to demonstrate the link between signaling proteins.

RE stimulates mTOR activity and is the most investigated mechanism linked to skeletal muscle hypertrophy. RE-induced mTORC1 S^{2448} phosphorylation results in increases in mRNA translation initiation via downstream phosphorylation of 4E-BP1 $T^{37/46}$, resulting in disassociation of the binding protein from eIF4E allowing interaction with eIF4G and cap-dependent translation of mRNA to proceed (Gingras, Gygi et al. 1999). mTORC1 phosphorylation of p70S6K1 T^{389} induces downstream C-terminal phosphorylation of rpS6 $S^{235/236}$ and $S^{240/244}$, whose modification potentiates 40S ribosomal subunit binding the 5' mRNA cap, promoting assembly of the 43S pre-initiation complex (Hutchinson, Shanware et al. 2011). Regulation of exercise-induced signaling through mTORC1 is one mechanism that contributes to shifting the balance between anabolism and catabolism in skeletal muscle, and differences in mTOR regulation may, therefore, be a point of delineation between AE and RE signaling outcomes in skeletal muscle (Saxton and Sabatini 2017).

Upstream of mTOR, phosphoinositide 3-kinase (PI3K) phosphorylates the inositol ring of phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2), converting to phosphatidylinositol-3,4,5-trisphosphate (PIP3) at the plasma membrane (Dibble and Cantley 2015). PIP3 binds to AKT and 3-phosphoinositide-dependent kinase 1 (PDK1), increasing the phosphorylation and activation of AKT T^{308}/S^{478} by PDK1 (Pearce, Komander et al. 2010). Phosphorylation of AKT S^{478} alone is not sufficient to increase AKT activity; instead, it induces a conformational change, stabilizing the active structure and promoting phosphorylation of AKT T^{308} for full activation (Pearce, Komander et al. 2010). In human muscle, following RE, AKT S^{478} and T^{308} are often robustly

phosphorylated rapidly (immediately post-exercise) and remain elevated for ~2-3hrs post-exercise (Fry, Drummond et al. 2011, Roschel, Ugrinowistch et al. 2011). AKT activity can also be regulated by mTORC2 at AKT S^{477}/T^{479} and AKT S^{473}, hypothesized to stabilize active AKT (Liu, Begley et al. 2014). Active AKT directly phosphorylates TSC2 T^{1462}, a GTPase activating protein for Rheb, preventing signal transduction through mTOR (Long, Lin et al. 2005). At the same time, Rheb bound to GTP increases mTOR S^{2448} phosphorylation (Inoki, Li et al. 2003). In non-muscle, HEK293 cells, phosphorylation of mTOR S^{2159}/T^{2164} has also been demonstrated to alter interaction with raptor and PRAS40, found to be required for mTORC1 associated mTOR autophosphorylation at S^{2481}, promoting downstream signaling and growth (Ekim, Magnuson et al. 2011). However, phosphorylation of mTOR S^{2159}/T^{2164} has not been demonstrated in human muscle to our knowledge.

Baar and Esser (1999) (Baar and Esser 1999) first identified p70S6K phosphorylation in response to high resistance lengthening contractions and found there to be a correlation with the chronic (6-week) change in muscle mass. Work by Bodine et al. (2001) (Bodine, Stitt et al. 2001) characterized the AKT/mTORC1 pathway as an upstream regulator of p70S6K, with its ability to increase muscle mass and build upon the anabolic signaling mechanism of loading-induced hypertrophy. Bodine et al. (2001) (Bodine, Stitt et al. 2001) manipulated the AKT/mTOR signaling pathway *in vivo* to demonstrate that signaling through this pathway is a critical regulator of muscle growth, and activation is sufficient to induce hypertrophy. These data (Bodine, Stitt et al. 2001) led to the hypothesis that the prolonged significant activation (6 hours) of p70 with a transient elevation of AKT may be resistance exercise specific (high vs. low-frequency stimulation) and potentially a point of divergent signaling adaptation between AE and RE (Nader and Esser 2001). Since the work of Bodine and colleagues (Bodine, Stitt et al. 2001),

mTOR and its relationship to the regulation of muscle mass have been extensively studied (Bodine 2022). Regulation of mTORC1 is sensitive to multiple factors, including amino acids, glucose, growth factors (IGF1), and mechanical stress (Szwed, Kim et al. 2021, Bodine 2022). Mechanistic work in humans demonstrates that rapamycin, a potent inhibitor of mTORC1, treatment was sufficient to ablate the immediate (1-2 hour) post-exercise elevation of muscle protein synthesis (MPS) and blunt downstream phosphorylation of S6K1 T^{421}/S^{424} and eEF2 T^{56} (Drummond, Fry et al. 2009). Early work that established an interaction between mTORC1 with increases in protein synthesis and muscle mass has led others to focus their investigations on the canonical pathways of mTORC1 signaling in relation to myriad models of acute and chronic RT (Goodman 2019).

The ability of mechanically induced mTORC1 S^{2448} phosphorylation to regulate phosphorylation of p70 T^{389} via a completely rapamycin-sensitive mechanism has been confirmed (You, McNally et al. 2019), and mTOR signaling is detectable yet attenuated in a trained state. In trained (18 sessions) mice, acute phosphorylation of mTORC1 downstream targets p70S6K T^{389}, rpS6 $S^{235/236}$, and rpS6 $S^{240/244}$ were blunted in comparison to a single (naive) session of RE (Ogasawara, Kobayashi et al. 2013). However, both total and phosphorylated proteins of the targets p70S6K T^{389} and rpS6 $S^{235/236}$ were significantly greater than the control (non-exercising), demonstrating that mTOR signaling in trained muscle is still sensitive to mechanical stimuli (Ogasawara, Kobayashi et al. 2013). Furthermore, in human skeletal muscle, AKT S^{473} and mTOR S^{2448} are still significantly phosphorylated following RE after 10 weeks of RT (Wilkinson, Phillips et al. 2008). Additionally, in 8 chronically resistance-trained men, the phosphorylation of p70S6K T^{389} was found to be related ($r=0.34$, $p=0.03$) to myofibrillar FSR, suggesting conservation of mTORC1 signaling even in a trained state (Burd, Holwerda et al.

2010). However, transient phosphorylation of AKT S^{473} is not RE-specific and was found to be elevated (50%) immediately post-exercise in response to AE (70% VO2 peak for 60 min) in aerobic-trained individuals (Coffey, Zhong et al. 2006). Interestingly, the same study observed no change in AKT S^{473} or AKT T^{308} following resistance exercise (8 sets x 5 reps maximal intensity contraction) in previously trained individuals, further suggesting attenuation of the exercise signaling response in a trained state (Coffey, Zhong et al. 2006).

In addition to temporal differences in signaling activity following exercise, mTORC1 activation is spatially distinct and regulated by changes in subcellular localization (Tinline-Goodfellow, Lees et al. 2023). The presence of mTORC1 at the muscle periphery and mTOR translocation to the lysosome may be necessary for the elevated activity (Sancak, Bar-Peled et al. 2010); nonetheless, this has primarily been observed in addition to amino acid provision (Hodson, McGlory et al. 2017, Abou Sawan, van Vliet et al. 2018), but one study has demonstrated a rapid (<30 min) increase (20%) in mTOR translocation following resistance exercise alone (Song, Moore et al. 2017). The same group (Abou Sawan, van Vliet et al. 2018) reports mTOR translocation to the lysosomal membrane and colocalization with Rheb increased 60 and 300 minutes after AE (60 min run at 70% $VO2_{max}$), with a mixed-macronutrient meal (18g protein), observed post-exercise, concomitant phosphorylation of downstream mTORC1 targets rpS6 $S^{240/244}$ and 4E-BP1 $T^{37/46}$. In sum, RE and AE (albeit with amino acids) appear to be sufficient to induce mTORC1 spatial regulation and subsequent activation.

Mitogen-active protein kinases (MAPK). MAPK are a family of highly conserved serine/threonine kinases that can influence a variety of cellular processes in response to growth stimuli, playing a key role in skeletal muscle signaling for growth, repair, and adaptation (Brennan, Emerson et al. 2021). In humans, ERK, JNK, and p38 are the primary MAPK

involved in the regulation of muscle adaptation to contraction-type stimuli. In addition to mTOR, MAPK activation can influence protein translation via signaling through mitogen-activated protein kinase signal-integrating kinase 1 (Mnk1). The phosphorylation of Mnk1 on $T^{197/202}$ by ERK1/2 and p38MAPK has been shown to increase the phosphorylation of eIF4E on S^{209}, influencing the affinity for the 5' -mRNA cap and decreasing protein synthesis (Waskiewicz, Flynn et al. 1997, Shenberger, Zhang et al. 2007). The independent activity of MAPK signaling networks is partly dependent on exercise variables such as modality (AE vs. RE), duration, and intensity (Kramer and Goodyear 2007). For example, the extent of contraction-induced ERK1/2 T^{202}/Y^{204} phosphorylation is well correlated to the intensity of the exercise stimulus (Widegren, Wretman et al. 2000), which has been demonstrated for both ERK1/2 T^{202}/Y^{204} and ERK2 T^{183}/Y^{185} phosphorylation in an exercise intensity-dependent manner, following AE (Richter, Vistisen et al. 2004).

The MAPK are activated in an array of phosphorylation events facilitated via upstream MAPKKK and upstream Ras GTP signaling with Raf induces the phosphorylation of MEK (MAPKK1) at S^{217} and S^{221}, a known activator of ERK (Alessi, Saito et al. 1994). MAPK singling was first identified to be exercise-regulated by notable muscle-specific increases in MAPK phosphorylation and upstream MAPKK activity following exercise (Goodyear, Chang et al. 1996, Aronson, Violan et al. 1997). Subsequently, p38, JNK, and ERK signaling were hypothesized to regulate muscle growth and remodeling in response to exercise stimuli (Kimball, Horetsky et al. 1998, Zetser, Gredinger et al. 1999). ERK1/2 has since been identified as an important component of the signaling pathway for the regulation of MPS (Widegren, Ryder et al. 2001, Drummond, Dreyer et al. 2009).

In human skeletal muscle, p38a is known to be phosphorylated at T^{180}/Y^{182}, increasing its kinase activity and signaling (Hojlund, Bowen et al. 2009). However, there appear to be distinct mechanisms for the activation and subsequent signaling of p38aMAPK as compared to ERK or JNK. AE has been shown to stimulate robust phosphorylation signaling through p38aMAPK. In contrast, the effect of RE on p38aMAPK signaling is controversial, with RE-induced activation being dependent on timing, contraction type, or volume of exercise (Martineau and Gardiner 2001). It is hypothesized that the unique ROS produced from AE vs. RE stimulates p38MAPK signaling and subsequently increases the activity of PGC-1a by interacting with upstream transcription factors ATF2 and MEF2 (Akimoto, Pohnert et al. 2005). Early work in cardiac myocytes demonstrated that p38 activation is responsive to hypoxia, inducing rapid phosphorylation of Y^{182}, thought to be regulated via the Src family of tyrosine kinases and Ras (Seko, Takahashi et al. 1997). In human muscle, p38a T^{180}/Y^{182} phosphorylation is also demonstrated to occur following high-intensity (4 bouts of all-out 30s) cycling (Gibala, McGee et al. 2009). MAPK signaling is further associated with AT outcomes, as both ERK and p38MAPK activation can lead to the phosphorylation of CREB S^{133} via MSK1, initiating the transcription of genes involved in energy metabolism and mitochondrial biogenesis (Deak, Clifton et al. 1998, Bengal, Aviram et al. 2020).

In human muscle, ERK1/2 T^{202}/Y^{204} and p38MAPK T^{180}/Y^{182} are robustly (~7 and ~4.5-fold change, respectively) and rapidly (immediately post-exercise) phosphorylated in response to AE (Yu, Stepto et al. 2003). However, it should be acknowledged that ERK1/2 T^{202}/Y^{204} and p38MAPK T^{180}/Y^{182} phosphorylation is not AE-specific and has also been observed following resistance exercise (Deldicque, Atherton et al. 2008). Interestingly, AE and RE result in a similar magnitude of ERK1/2 T^{202}/Y^{204} phosphorylation (Yu, Stepto et al. 2003, Moore, Atherton et al.

2011); however, significant phosphorylation following RE has been detected at later times post-exercise (Fry, Drummond et al. 2011), which may influence the downstream outcomes. Additionally, RE in human skeletal muscle that demonstrated MAPK (ERK1/2 T^{202}/Y^{204}, p38MAPK T^{180}/Y^{182}, and JNK T^{183}/Y^{185}) phosphorylation immediately post-exercise with significant Mnk1 $T^{197/202}$ phosphorylation but no significant increase in eIF4E S^{209} phosphorylation (Williamson, Gallagher et al. 2003). The lack of increase of eIF4E S^{209} phosphorylation in this study could be attributed to biopsy timing (immediate post-exercise) or the magnitude of MAPK activation or other unknown signalling regulation (Figure 1).

A mechanism for ERK-dependent regulation of protein synthesis is thought to be mediated upstream of mTOR via TSC1-2. Specifically, ERK 1/2 phosphorylation of TSC2 S^{664} leads to TSC1-TSC2 dissociation, impairing TSC2s ability to inhibit mTORC1 signaling (Ma, Chen et al. 2005). In C2C12 myoblasts, MAPK (ERK) activation has also been demonstrated to induce TSC2 phosphorylation at multiple residues ($S^{664/939}$ and T^{1462}) and shown to regulate mTOR activity as assessed by downstream S6K1 phosphorylation at T^{389} and T^{421}/S^{424} (Miyazaki and Takemasa 2017). ERK1/2 can also modulate TSC2's GAP (GTPase-activating protein) activity towards Rheb, a negative regulator of mTORC1 activity (Huang and Manning 2008). Further, ERK1/2 T^{202}/Y^{204} is significantly phosphorylated above rest in human skeletal muscle 1 hour after RE, coinciding with increased phosphorylation of p70S6K T^{389} (Moore, Atherton et al. 2011). ERK is, therefore, responsive to both AE and RE, likely in an intensity-dependent manner, regulating protein synthesis through site-specific phosphorylation of TSC2-mTOR (Figure 1). Although some MAPK signaling does appear to converge on mTOR, it has been commonly reported as a mTORC1-independent mechanism of mechanically regulating muscle growth (Roberts, McCarthy et al. 2023). ERK2 signaling can alter protein synthesis by phosphorylation

of T^{117} and T^{201} of UBF (RNA polymerase factor 1), which is essential for transcription enhancement and regulating ribosomal gene expression (Stefanovsky, Langlois et al. 2006). ERK1/2 can also target the substrate p90RSK (p90 ribosomal S6 kinase) T^{573}, leading to the downstream activation of transcription factors, demonstrated in human skeletal muscle following RE (Williamson, Gallagher et al. 2003). In addition to contraction, ERK1/2 activation and signaling are sensitive to insulin (Goodyear, Chang et al. 1996, Wojtaszewski, Lynge et al. 1999). IGF1 knockdown has been shown to reduce ERK1/2 T^{202}/Y^{204} phosphorylation in myotubes (Saneyasu, Nakamura et al. 2022). Fluckey et al. (Fluckey, Knox et al. 2006) demonstrated that insulin-mediated elevation in muscle protein synthesis following RE is dependent on ERK1/2 T^{202}/Y^{204} and ERK T^{183}/Y^{185} phosphorylation signaling, and inhibition of ERK has been demonstrated to prevent hypertrophy induced by IGF1 (Haddad and Adams 2004). Further investigation into the exercise-induced changes in ERK1/2 MAPK signaling is necessary to determine if signaling timing and magnitude play roles in the divergent regulation of protein pool specific (i.e., myofibrillar vs. mitochondrial) synthesis associated with AT and RT.

In well-trained individuals, p38MAPK T^{180}/Y^{182} phosphorylation still occurs in response to AE (1.6-fold increase); nonetheless, the magnitude of the response may be attenuated in comparison to naïve controls (2.1-fold increase) when muscle is working at the same relative intensity (Yu, Stepto et al. 2003). This observation has also been made in rat skeletal muscle, where oxidative stress-induced activation of p38MAPK T^{180}/Y^{182} is reduced by 59% in trained muscle as compared to control (Vichaiwong, Henriksen et al. 2009). MAPK signaling, therefore, contributes to the contraction-induced mechanisms of adaptation that occur in skeletal muscle with AE and RE, possibly altering timing and magnitude with training status. Distinct modalities

(AE vs. RE), volume, and intensity of exercise may differentially regulate MAPK activation and potentially influence the divergent exercise-specific adaptations observed with training.

Adenosine Monophosphate-Activated Protein Kinase (AMPK)

AMPK is a serine/ threonine protein kinase that becomes active in response to cellular stress (i.e., low nutrient availability or prolonged exercise). AMPK acts as a central regulator of cellular metabolism and is sensitive to intracellular AMP/ADP levels (i.e., activated when the ATP:AMP ratio is lowered) and aids its function as a coordinator of growth, differentiation, and metabolism (Park, Gammon et al. 2002, Mihaylova and Shaw 2011). The most prevalent AMPKs in skeletal muscle are AMPKa1 and AMPKa2, and activity is primarily exercise-regulated via phosphorylation at sites T^{183} and T^{172}, respectively (McGee, Fairlie et al. 2009, Treebak, Pehmoller et al. 2014). AMPKa1 and a2 become activated during prolonged activity and appear to stay elevated for ~3 hours post-cessation of exercise (Wojtaszewski, Nielsen et al. 2000). However, there is some evidence to suggest exercise can differentially regulate the amplitude and timing of AMPK a1 and a2 activity (Abbott, Edelman et al. 2009). AMPKa2 T^{172} activation occurs during exercise/ contraction, functioning to both generate and conserve ATP via activation of catabolic pathways as well as suppression of protein and fatty acid synthesis (Dreyer, Fujita et al. 2006, Hardie 2007). In untrained males, cycling (60 min at 70% VO_2peak) elevated AMPK T^{172} phosphorylation 16-fold immediately post-exercise (Benziane, Burton et al. 2008). Additionally, phosphorylation of AMPK(a1 and a2) T^{172} has been observed following only 4 bouts of 30s all-out sprint cycling, demonstrating that even relatively low volume (but high intensity) aerobic-type training can cause significant cellular energy stress to stimulate altered metabolism through downstream targets of AMPK signaling (i.e. PGC-1a) (Gibala, McGee et al. 2009). A potent upstream regulator of AMPK T^{172} phosphorylation in skeletal muscle is LKB1

(Koh, Brandauer et al. 2008, Shackelford and Shaw 2009, Tanner, Madsen et al. 2013). Pseudokinase STRADα promotes the active conformation of LKB1, which is stabilized by MO25α interacting with the LKB1 activation loop (Zeqiraj, Filippi et al. 2009). LKB1 S^{428} phosphorylation alters cellular location and affinity for AMPK activation (Xie, Dong et al. 2008). When LKB1 expression in muscle is knocked down by ~90%, AMPK T^{172} demonstrates significantly reduced phosphorylation following muscle contraction (Sakamoto, McCarthy et al. 2005). Downstream, the acute increase in AMPK T^{172} activity regulates SIRT1 affinity for PGC-1a by phosphorylating PGC-1a T^{177}/ S^{538} in addition to elevating the intracellular NAD+ concentration, which in turn modulates mitochondrial protein synthesis (Jäger, Handschin et al. 2007, Canto, Gerhart-Hines et al. 2009, Canto, Jiang et al. 2010, Costford, Bajpeyi et al. 2010). Furthermore, chronic (repeated) activation of AMPK T^{172}, via exercise or pharmacological intervention results in increased mitochondrial content (Winder, Holmes et al. 2000, Zong, Ren et al. 2002, Hardie 2004, Koh, Brandauer et al. 2008) and has also been postulated to alter metabolism and improve muscle insulin sensitivity due to its ability to regulate the expression of proteins (i.e., GLUT4) involved with insulin signal transduction (Kurth-Kraczek, Hirshman et al. 1999, Jessen, Pold et al. 2003, Hawley and Lessard 2008).

During exercise, muscle protein synthesis is attenuated, presumably due to it being an energy-consuming process, and this is thought to be regulated via AMPK activity (Dreyer, Fujita et al. 2006). With RE, AMPKa2 activity is significantly increased during and immediately following (1-2 hours) exercise (Dreyer, Fujita et al. 2006). AMPK phosphorylates TSC2 T^{1227}/ S^{1345} and heightens its ability to suppress translation regulation via the mTORC1 pathway (Inoki, Zhu et al. 2003). Additionally, work performed in TSC2-depleted cells identified raptor S^{722}/ S^{792} as alternative phosphorylation targets of AMPK inducing 14-3-3 binding to raptor and subsequent

suppression of mTORC1 activity (Gwinn, Shackelford et al. 2008). AMPK can further regulate mTOR via phosphorylation of T^{2446}, which has been demonstrated to reduce activity via attenuation of phosphorylation at mTOR S^{2448} (Cheng, Fryer et al. 2004). Evidence of AMPK attenuating anabolic signaling has been demonstrated in human skeletal muscle, where individuals who performed high-intensity AE prior to RE had no significant elevation of phosphorylation at downstream targets of mTORC1, including p70SK1 T^{389} and p70SK1 T^{421}/S^{424} (Coffey, Jemiolo et al. 2009). However, although mixed MPS is repressed during RE while AMPKa2 is known to be active, there is a robust increase (75%) in MPS following RE (1-2 hours) despite AMPKa2 remaining significantly elevated from basal levels (Dreyer, Fujita et al. 2006). Further, an elevation in protein synthesis has been shown to occur concomitantly with an increase in mTOR S^{2448} phosphorylation (significantly elevated 1- and 2-hours post-resistance exercise) despite elevated AMPK (Dreyer, Fujita et al. 2006). Elevation of protein synthesis in spite of elevated AMPK activity suggests that the anabolic signaling from RE is able to overcome some level of AMPK-induced suppression. Interestingly, AMPK T^{172} phosphorylation has also been observed 24 hours following RE, suggesting alternative signaling rolls beyond immediate energy stress (Moro, Monaco et al. 2022), such as the promotion of autophagy by phosphorylating ULK1 at S^{317} and S^{777} (Kim, Kundu et al. 2011). Indeed, AMPK activation with RE can serve to more than regulate immediate energy metabolism and may also signal for alteration of mitochondrial protein synthesis, as demonstrated by increased respiration following 12 weeks of RT (Porter, Reidy et al. 2015). Furthermore, AMPK activation is also implicated in the modulation of glycolytic pathways via phosphorylation of PFK2 S^{466}, which may alter energy metabolism adaptation with chronic RT (Almeida, Moncada et al. 2004).

Although AMPK activity is regulated by both AE and RE, the differences in isoform, magnitude, and timing of AMPK T^{172} phosphorylation may be a potential mechanism for divergent functional and phenotypic adaptation with chronic AT and RT. Although much is known regarding the regulation and interplay of AMPK and mTOR with exercise, they alone do not appear to dictate/ distinguish between the divergent phenotypes observed in skeletal muscle with chronic training, which raises the question of what other signaling mechanisms occur that have not been previously considered or recognized as specific to an AT or RT phenotype.

Reactive oxygen species (ROS). Acute exercise induces disturbances to cellular homeostasis. The metabolic perturbation from exercise induces the production of ROS thought to be derived from the mitochondria and other cell compartments in response to muscle contraction and strenuous exercise (McArdle, Pattwell et al. 2005, Powers and Jackson 2008). Initially, ROS production with exercise was thought to have negative consequences on health and performance; however, ROS production is now recognized as an important mediator of signaling pathways in the adaptation of skeletal muscle (Bouviere, Fortunato et al. 2021). Skeletal muscle produces superoxide and hydrogen peroxide at rest, and exercise elicits a drastic increase in the rate of production (Vasilaki, Mansouri et al. 2006). During contraction and prolonged exercise, skeletal muscle has three main contributing sources of ROS, including the mitochondria (Sahlin, Shabalina et al. 2010), xanthine oxidase (Hellsten, Frandsen et al. 1997), and NAD(P)H oxidase enzymes (Henriquez-Olguin, Knudsen et al. 2019, Bouviere, Fortunato et al. 2021). However, there is experimental evidence that challenges mitochondria as a primary source of ROS in skeletal muscle, demonstrated by ablation of activity-induced ROS production in the presence of an NADPH oxidase inhibitor (Michaelson, Shi et al. 2010, Sakellariou, Vasilaki et al. 2013).

A likely mechanism by which ROS signals for exercise-induced adaptation in skeletal muscle is through its regulation of AMPKa indirectly and directly (redox changes to cysteine residues) (Zmijewski, Banerjee et al. 2010, Hinchy, Gruszczyk et al. 2018) and via activation of p38MAPK (thought to be responsive to hydrogen peroxide concentrations) (Torres and Forman 2003), both of which have been previously shown to increase activation in response to elevated ROS production. The attenuation of ROS (via xanthine oxidase inhibition) prevents the increase in signaling through redox-sensitive p38MAPK $T^{180/}Y^{182}$ and ERK1/2 T^{202}/Y^{204} phosphorylation, which is associated with a reduction of gene expression of mitochondrial transcription factor A (mTFA) but does not alter PGC-1a mRNA transcription or protein content with AT (Wadley, Nicolas et al. 2013). ROS production also triggers an elevation in cytosolic calcium via ROS-mediated opening of calcium-release activated channels and activation of ryanodine receptors of the T-tubules, increasing phosphorylation of AMPKa T^{172} through a CAMKKb-dependent mechanism (Hidalgo, Sánchez et al. 2006, Mungai, Waypa et al. 2011).

In addition to ROS, nitric oxide production is increased during muscle contraction (Pattwell, McArdle et al. 2004). Nitric oxide signaling can regulate AMPKa T^{172} and CAMK T^{286} phosphorylation (Lira, Soltow et al. 2007, McConell, Ng et al. 2010) and, therefore, may influence downstream GLUT4, PGC-1a, and mitochondrial gene expression. The sustained presence of nitric oxide can also stimulate mitochondrial biogenesis via guanylate cyclase and the generation of cGMP (McConell, Ng et al. 2010). However, evidence for these mechanisms is lacking in human muscle, and less is known regarding how distinct ROS production with AE and RE could elicit divergent phenotypic adaptation with chronic training.

RE and AE primarily rely on different energy systems (i.e., glycolytic vs. oxidative, respectively), causing a purported distinct generation of ROS and reactive nitrogen species,

which may contribute towards the distinct skeletal muscle adaptation with RT and AT (Spriet 1992, Bloomer and Goldfarb 2004). Although ATP generation is still significantly elevated in RE, leading to the stimulation of ROS-producing mechanisms (i.e., NOX and XO) the concentration of ROS may differ from AE and elicit unique RE-specific signaling (He, Li et al. 2016). Further evidence in human muscle is necessary to elucidate whether contraction-induced ROS plays a significant role in the determination of exercise-specific phenotype adaptation to training.

Mechanosensing and mechanotransduction

Mechanical loading of muscle regulates mass. Several proteins have been identified as potential mechanosensors that play a key role in mediating muscle mass (Mathes, Vanmunster et al. 2019, Wackerhage, Schoenfeld et al. 2019, Vanmunster, Rojo Garcia et al. 2022). Yet, the mechanistic link determining how load is sensed and then propagated as an acute biochemical signal to alter muscle protein synthesis is not fully understood (Wackerhage, Schoenfeld et al. 2019, Steinert, Potts et al. 2021). In addition to metabolic perturbance, the detection of mechanical load intrinsically in muscle may determine the downstream molecular signaling that affects a change in protein synthesis. It is important to understand which mechanosensors play prominent roles in the signaling regulation of muscle mass and how they respond under different loading/ unloading conditions. This section of the review will briefly highlight some of the prominent mechanosensors, transducers, and mediators of mechanical signaling mechanisms involved in the regulation of PTM signaling pathways and potentially initiating/ regulating protein synthesis for skeletal muscle adaptation.

Yes-associated protein (YAP). Yes-associated protein (YAP) and transcriptional activator with PDZ-binding motif (TAZ) have been identified as candidate mediators of mechanical signal transduction in skeletal muscle, regulating cell size and growth through a variety of mechanisms (Dupont, Morsut et al. 2011). Mechanically activated YAP mediates protein synthesis by regulating crosstalk between the HIPPO pathway and mTORC1 (Csibi and Blenis 2012, Tumaneng, Schlegelmilch et al. 2012). The large tumor suppressor kinases coordinate the primary mediation of YAP activity 1 and 2 (LAMP1/2) of the HIPPO pathway (Goodman, Dietz et al. 2015). A secondary upstream regulator of YAP is phosphatidic acid, which is a product of phosphatidylinositol 4,5 bisphosphate conversion by phospholipase Cγ1 in response to mechanical stimuli (Meng, Qiu et al. 2018). Also upstream of YAP is Bag cochaperone 3 (Bag3), a chaperone protein that relays matrix stiffness by redistributing YAP and TAZ in muscle progenitor cells (Gunay, Silver et al. 2021). The knockout of Bag3 reduces the nuclear localization of YAP and TAZ, effectively inhibiting mechanically induced signaling in myoblasts (Gunay, Silver et al. 2021). YAP in its active form induces miR-29, which downregulates the translation of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), an upstream inhibitor of mTOR signaling that acts via a reduction in PDK1 ability to phosphorylate AKT1 T^{308} (Tumaneng, Schlegelmilch et al. 2012). The expression of YAP has been demonstrated to increase as a response to acute muscle loading, with a strong correlation between the change in YAP expression and AKT T^{308} phosphorylation (Goodman, Dietz et al. 2015). Watt et al (2015) (Watt, Turner et al. 2015) demonstrated that inhibition of YAP by mutation of S^{79} within the C-terminus (blocking interaction with TEAD transcription factors) was sufficient to reverse hypertrophy observed from YAP overexpression. TEAD transcription factors are known to interact with miR-29; however, this result was associated with no increase in mTOR S^{2448}

activity (Watt, Turner et al. 2015). Therefore, other mechanisms for YAP to mechanically influence muscle size may exist. TEAD transcription factors can also induce the expression of high-affinity leucine transporter LAT1, which may aid muscle in amino-acid-induced mTORC1 activation and elevating protein synthesis (Hansen, Ng et al. 2015).

Additionally, YAP activity may also dictate muscle growth through its role in the proliferation and differentiation of satellite cells. Elevation of YAP stimulates the proliferation of activated Pax7+/MyoD+ progenitor cells in muscle, whereas YAP inactivation appears necessary for terminal differentiation (Nagata, Partridge et al. 2006, Watt, Judson et al. 2010). Altogether, the mechanically induced activation of YAP expression may have myriad pathways for influencing the acute signaling of MPS; however, little is known regarding the differential activity of YAP in response to AE vs. RE loading stimuli. YAP activity has primarily been studied in the context of muscle growth and it is unclear whether YAP regulation of downstream targets may also mediate adaptation towards an aerobic phenotype.

Integrin – Focal adhesion kinase (FAK). Integrins are a family of transmembrane proteins that connect the extracellular matrix to intracellular actin, a necessary component of lateral force transduction (Bell and Terentjev 2017). Despite their critical role in regulation and force transduction, integrins have no known kinase activity in human skeletal muscle (Graham, Gallagher et al. 2015) and, therefore, must interact with other proteins to propagate mechanically induced signals. Current evidence of how integrin protein signal for elevated protein synthesis *in vivo* is lacking, and most studies have utilized FAK and ILK phosphorylation as indirect markers of integrin signaling (Boppart and Mahmassani 2019). FAK is a non-receptor tyrosine kinase that is known to coordinate signaling through the costamere-associated protein complex (Graham, Gallagher et al. 2015, Wackerhage, Schoenfeld et al. 2019). Activation of FAK kinase domain

propagates mechanically induced signals and is known to regulate cytoskeletal remodeling (Parsons 2003, Bell and Terentjev 2017). There is no paucity of evidence that mechanical signals are capable of being transduced via integrin – FAK (Roberts, McCarthy et al. 2023). FAK Y^{397} phosphorylation is thought to regulate protein synthesis via the PI3K/AKT/mTORC1 signal pathway, as phosphorylation of FAK allows for binding to 85kDa subunit of PI3K through the SH2 domains of p85, leading to an elevation in its activity (Chen, Appeddu et al. 1996). Phosphorylation of FAK Y^{397} may also regulate mTORC1 activity through direct phosphorylation inhibition of TSC2 T^{1462} (Gan, Yoo et al. 2006, Crossland, Kazi et al. 2013). However, another mechanism has been identified for FAK Y^{397} as an upstream mechanosensitive regulator of p70S6K activity, coordinating protein synthesis in an AKT-independent mechanism (Klossner, Durieux et al. 2009). FAK overexpression in transfected mouse muscle demonstrated significantly greater phosphorylation of p70S6K S^{411} and p70S6K T^{421}/S^{424} at 6 and 24 hours following reloading, respectively, in comparison to control (Klossner, Durieux et al. 2009). In human muscle, RE significantly increased phosphorylated FAK $Y^{576/577}$ to a greater extent at 4 hours following exercise in an untrained state, and 10 weeks of exercise training (AT and RT) was sufficient for increasing FAK $Y^{576/577}$ phosphorylation at baseline (rest) (Wilkinson, Phillips et al. 2008). Integrin-FAK mechanosensing may, therefore, be a key player in the signal transduction pathway coordinating transient changes in PTM signaling following exercise.

Filamin C – Bag cochaperone 3 (Bag3). Filamin C is a Z-disc-associated protein with a "V" shaped structure that deforms when force is applied, opening to reveal binding sites for downstream signaling (Ulbricht, Eppler et al. 2013). Filamin C has 90+ known binding partners, highlighting its involvement and significance in skeletal muscle signaling regulation (Mao and Nakamura 2020). The growing list of binding partners for Filamin C includes ERK1/2, titin,

IGFN1, actin, and Bag3 (Mao and Nakamura 2020). Phosphoproteomic analysis has confirmed that both Filamin C and Bag3 phosphorylation status is altered in response to high-intensity exercise (Hoffman, Parker et al. 2015). Filamin C and its interaction with Bag3 has been identified as a mechanosensitive pathway with the ability to regulate downstream mTORC1 and YAP activity (Wackerhage, Schoenfeld et al. 2019). As noted previously, Filamin C - Bag3 also engages in YAP/TAZ signaling in response to acute mechanical stress (Ulbricht, Eppler et al. 2013). Additionally, Bag3 can act as a filamin signaling mediator for tension-induced increases in transcription and degradation (Ulbricht, Eppler et al. 2013). Furthermore, cochaperone Bag3 has been demonstrated to interact and sequester the mTORC1 inhibitor TSC1 (Kathage, Gehlert et al. 2017). It has also been observed that mTORC1 inhibition attenuates Bag3-mediated extracellular matrix autophagy, providing further evidence for Bag3 as a central mediator of transcription in response to mechanical stress (Kathage, Gehlert et al. 2017). Furthermore, the phosphoproteomic analysis identified AKT and PKCa as dual kinases of Filamin C S^{2234}/ S^{2237}, propagating PI3K/AKT hypertrophic signaling (Reimann, Schwable et al. 2020).

Titin. Titin is a large protein that extends from the Z-disc to the M-band with several domains that are considered to be functionally active, providing binding sites for diverse proteins, enzymes, and kinases (Kontrogianni-Konstantopoulos, Ackermann et al. 2009). Titin has also been implicated as a sensor of mechanical stimuli, with phosphorylation activity within the kinase domain of titin predicting hypertrophic changes in muscle following exercise in a load-dependent mechanism (Ibata and Terentjev 2021). The serine/threonine kinase domain of titin is associated with the M-band portion of the sarcomere, belonging to the myosin light-chain kinase (MLCK S^{143} and $S^{586/587/588}$) family of kinases, and is known to be regulated by CAMK (Gautel, Castiglione Morelli et al. 1995, Hojlund, Bowen et al. 2009, Kontrogianni-Konstantopoulos,

Ackermann et al. 2009). Specifically, it has been demonstrated that CAMKKII phosphorylates titin at several sites, including Titin S^{26}, S^{34}, S^{170}, S^{496}, T^{70}, T^{80}, T^{117} (Hidalgo, Chung et al. 2013). The kinase domain of titin is structurally and positionally optimal for sensing tension during muscle contraction, opening the protein, and exposing phosphorylation sites that may be upstream of mechanosensitive signaling pathways (Ibata and Terentjev 2021). In response to mechanical stress, titin signaling has been shown to regulate gene expression and protein turnover (Lange, Xiang et al. 2005). The work by Ibata et al. (2021) (Ibata and Terentjev 2021) and van der Pijl et al. (2018) (van der Pijl, Strom et al. 2018) demonstrate that titin may be a key mechanosensor able to propagate signaling cascades that vary in response to the proportion of mechanical stress. Titin can acutely increase protein synthesis via interaction with muscle LIM protein, which binds calcineurin and dephosphorylated nuclear factor of activated T cells (NFAT), an upstream regulator of transcription and cellular remodeling (Linke and Kruger 2010). Titin is just one example of several structural proteins that can be considered key regulators in the sensing and transduction of mechanical stimuli. Although these key sensors have been proven to be essential for the signaling regulation of protein synthesis, it is not well understood how varying factors such as the intensity, load, volume, and frequency of mechanical stimuli can be sensed and elicit divergent transient signaling responses. The application of load to skeletal muscle results in a coordinated signaling network response that produces the phenotypes commonly associated with RT and AT, yet relatively little is understood about the mechanosensory mechanisms initiating and regulating this phenomenon. The interplay of several mechanosensors may be responsible for interpreting the modality and relative intensity of load on skeletal muscle. Therefore, it is critical to continue to examine these and other mechanosensitive signaling mechanisms in the context of muscle mode-specific adaptation.

Mechanisms for adaptation

Muscle damage as a driver of adaptation. Mechanical loading (such as RE) of muscle unaccustomed to exercise can induce muscle 'damage.' Damage is especially prevalent following heavy RE in exercise naïve muscle and after eccentric contractions (Gibala, Interisano et al. 2000, Clarkson and Hubal 2002). Skeletal muscle has incredible potential for repair and remodeling, adapting to handle future stress, and impeding future damage induced by bouts of loading through altered cytoskeletal structure and contractile components (Jorgenson, Phillips et al. 2020). RE-induced damage can occur at several sites in muscle and is typically localized to the sarcomere, costamere, sarcolemma, basal lamina, contractile structures, and connective tissue (Clarkson and Hubal 2002). Muscle damage is associated with a transient decrease in force production, muscle swelling/ soreness, and metabolic perturbation due to disrupted signaling and mitochondrial damage. Unfortunately, most experimental methods for assessing contraction-induced muscle damage (Markus, Constantini et al. 2021) are based on proxies of damage and do not directly assess structural disruption to the cytoskeletal or muscle architecture. Examples of such methods are creatine kinase assays, soreness (DOMS), inflammatory markers (i.e., interleukin-6, -10, macrophages), as well as satellite cell proliferation and activation. Currently, the most direct method of assessing muscle damage is with electron microscopy, quantifying focal disruption of the sarcomere thought to occur primarily at the z-disc (z-disc smearing/streaming) or complete dissolution of the entire sarcomere (Lieber 2018). Z-disc disruption is often interpreted as structural muscle damage following heavy resistance exercise (Friden, Sjostrom et al. 1983, Roth, Martel et al. 1999). However, this notion has been challenged by Yu et al. (2004) (Yu, Carlsson et al. 2004), who observed greater Z-disc disruption on days 2-3 and 7-8 post-exercise in comparison to 1-hour post-exercise. Greater occurrence of

Z-disc streaming and smearing in the days following exercise may, therefore, be indicative of myofibrillar remodeling and sarcomerogenesis (Yu, Carlsson et al. 2004). Therefore, significant elevation of skeletal muscle proteolysis that is stimulated following exercise could present as 'damage' but instead may be remodeling and repair necessary for adaptation (Phillips, Tipton et al. 1997, Tipton, Hamilton et al. 2018). While damage is a plausible mechanism, we propose that what may appear as mechanical disruption of the sarcomere under electron microscopy could be an early stage of robust focal elevations in protein turnover in response to heavy, eccentric, or unaccustomed exercise (Moore, Phillips et al. 2005).

Contrary to the protein turnover thesis is the idea of sarcomere 'popping,' which has been proposed to occur in response to heavy or eccentric contractions (Morgan and Proske 2004). Briefly, it is hypothesized that marginal differences in contraction force along the length of the myofibril may cause disparate stretch in sarcomeres with less filament overlap. Under strenuous stimuli (eccentric contraction), contractile myofilaments no longer overlap and 'pop,' causing physical disruption to the sarcomere (Morgan and Proske 2004). Remodeling to adapt to such damage is focused on structural maintenance of sarcomere stability, which could occur rapidly without a drastic increase in muscle fiber cross-sectional area (Damas, Phillips et al. 2016). This observation may explain why some markers of 'damage' are attenuated rapidly following only a few bouts of unaccustomed exercise without measurable hypertrophy or other (known) significant adaptations (Damas, Phillips et al. 2016). Exercise-induced muscle damage is attenuated following as little as a single session of exercise and tends to decrease even more with subsequent bouts; however, significant hypertrophic adaptation is typically not reliably measurable until 6 weeks (12-15 bouts) of RT or later (Lim, Nunes et al. 2022). Notably, protein synthesis following resistance exercise correlates with hypertrophy only after muscle damage is

attenuated, as assessed by microscopy and indirect markers (i.e., reduced contraction force and CK assay) (Damas, Phillips et al. 2016). However, muscle hypertrophy occurs independent of damage, examined in naïve and trained individuals using markers of CK and soreness to assess damage (Flann, LaStayo et al. 2011). Similarly, mice that performed eccentric exercise experienced myofiber hypertrophy and an increase in Pax7+ cell content without indication of disruption (damage) in myofibers (Lueders, Zou et al. 2011). Furthermore, there is evidence that satellite cells can contribute to skeletal muscle remodeling in the absence of hypertrophy (Joanisse, Gillen et al. 2013). We propose that the initial (early training) stimulation of protein synthesis is, at least in untrained persons, more directly related to the repair and/or remodeling of skeletal muscle. At the same time, myofibrillar hypertrophy may occur with progressive attenuation of damage after subsequent bouts with chronic RT (Damas, Phillips et al. 2016). Taken altogether, although damage is commonly associated with an elevation in protein synthesis and breakdown, it does not appear to be an essential factor for anabolic signaling or hypertrophy to occur in skeletal muscle. It is, therefore, unlikely a key regulator determining the different outcomes of aerobic vs. resistance exercise training.

Protein turnover in muscle adaptation. The functional attributes of skeletal muscle are underpinned by the function of its constituent proteins (i.e., the proteome), which exist in a dynamic equilibrium of protein turnover (encompassing the balance of synthesis and degradation). MPS is the metabolic process of incorporating amino acids into new muscle proteins, and MPB is the antagonistic function whereby proteins are broken down into amino acids. Rates of MPS and MPB are dynamic and can change in response to exercise and protein ingestion. Loading and aerobic exercise induce robust, transient rises in MPS, whereas periods of disuse reduce MPS and transiently stimulate MPB (Wolfe 2006). Unlike measures of PTM,

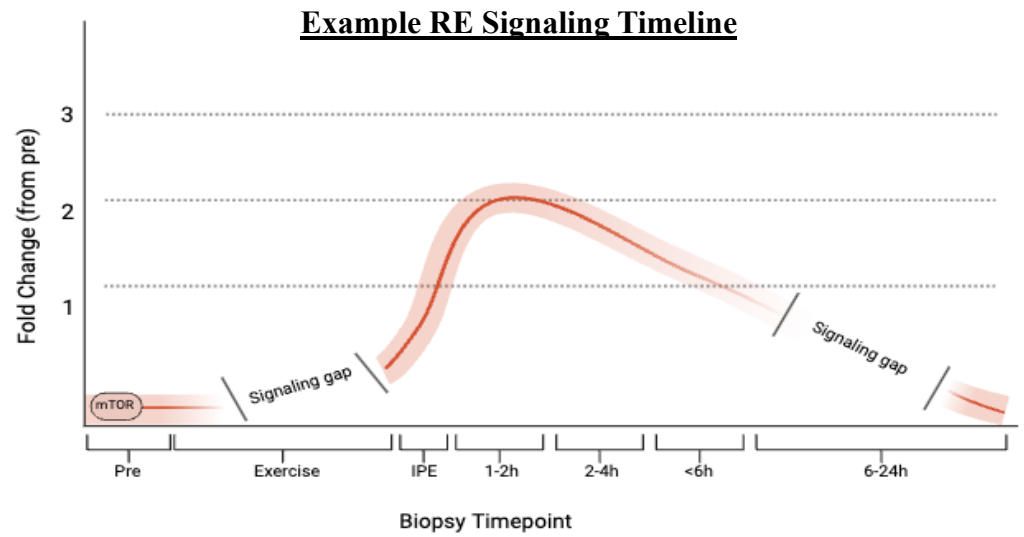
mRNA expression, or protein content, the dynamic processes of MPS and MPB cannot be assessed solely from 'snapshots' taken at isolated time points. Measurements of MPS and MPB require the use of tracer methodologies and measure the rates of incorporation of a tracer (e.g., stable isotope labeled amino acid) into muscle protein to calculate a mixed-protein fractional synthesis rate (FSR)

Typically, MPS is measured from whole muscle (bulk) or muscle sub-fractions, e.g., myofibrillar and sarcoplasmic fractions. Although bulk MPS (measured as fractional synthesis rate, typically %/h in humans) provides information on the overall efficacy of an intervention, fraction-specific data offers deeper perspectives on exercise mode-specific responses to exercise. In response to RE, Mitchell et al. (2014) (Mitchell, Churchward-Venne et al. 2014) observed an increase in myofibrillar FSR above resting values in the recovery timeframe at both 60-180 min (235 ± 38%) and 180-360 min (184 ± 28%). Whereas, after a single session of AE, bulk (mixed muscle protein) MPS is elevated by ~58% between 2-6 hours following exercise (Harber, Konopka et al. 2010). The lower MPS response to AE is not typically associated with hypertrophy, but the relative intensity of an AE stimulus may be positively associated with MPS and myofibrillar FSR (Di Donato, West et al. 2014, Bagheri, Robinson et al. 2022). For example, a higher intensity AE has been demonstrated to stimulate myofibrillar protein synthesis (~60%) over a longer time (24-28 hours) as compared to a lower intensity group (Di Donato, West et al. 2014).

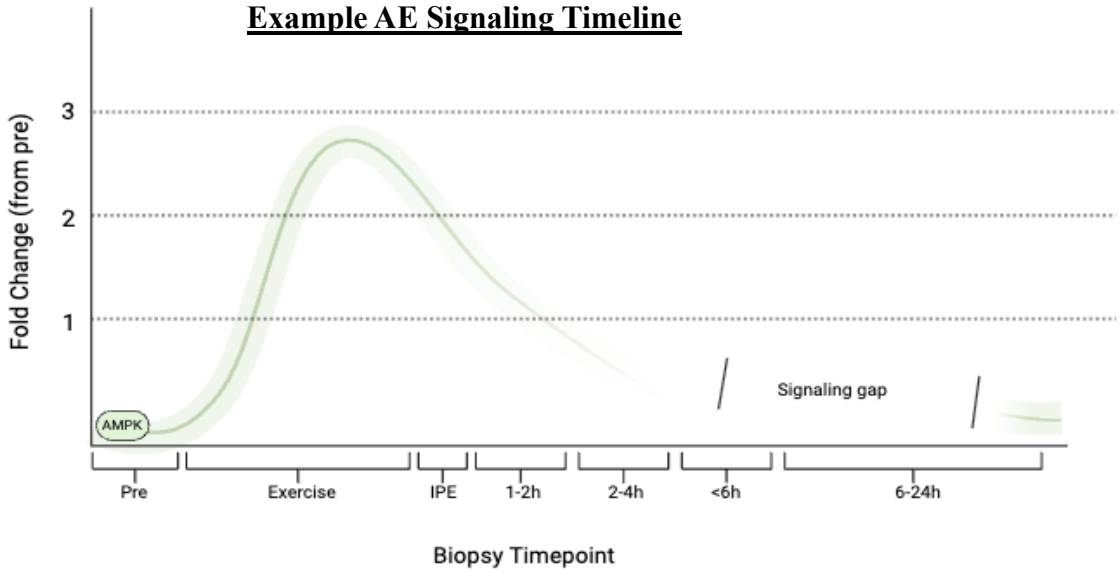
Studies such as those by Fry et al. (2011) (Fry, Drummond et al. 2011) and Moore et al. (2011) (Moore, Atherton et al. 2011) demonstrate significant regulation of kinases in the mTOR pathway (4E-BP1 and p70SK6K1) at periods of 5-6 h after resistance exercise concomitant a significant increase in protein synthesis. A few groups have examined the mTOR signaling axis (AKT/mTOR/p70S6K1) along with protein synthesis over longer periods (up to 24 hours) after

RE; however, the results are currently difficult to interpret because the data are sparsely distributed (i.e. large gaps in-between timepoints in the 24 h period) and the exercise intervention varied in loads as well as amino-acid provision (Cuthbertson, Babraj et al. 2006, Deldicque, Atherton et al. 2008, Burd, Holwerda et al. 2010, Burd, West et al. 2010, Burd, West et al. 2011, Fry, Drummond et al. 2011). Mixed results of later (6< hours) mTOR signaling denotes that mTORC1-dependent signaling may play a more significant role in the early (~1-6 hours) rise of protein synthesis following RE, but later time points (~18-36 hours) may be regulated by rapamycin-insensitive or mTORC1 independent pathways (Goodman 2019, Ogasawara, Jensen et al. 2019). Phosphorylation of mTORC1 S^{2448} is associated with a general elevation in MPS, but heightened MPS is sustained during knockout of raptor (i.e., mTORC1 inhibition), which points to an mTORC1-independent mechanism (You, McNally et al. 2019). We have attempted to summarize these time courses in Figure 1.2.

A


Example RE Signaling Timeline
Fold Change (from pre)
3
2
1
mTOR
Signaling gap
Signaling gap
Pre
Exercise
IPE
1-2h
2-4h
<6h
6-24h
Biopsy Timepoint

B


Example AE Signaling Timeline
Fold Change (from pre)
3
2
1
AMPK
Signaling gap
Pre
Exercise
IPE
1-2h
2-4h
<6h
6-24h
Biopsy Timepoint

C

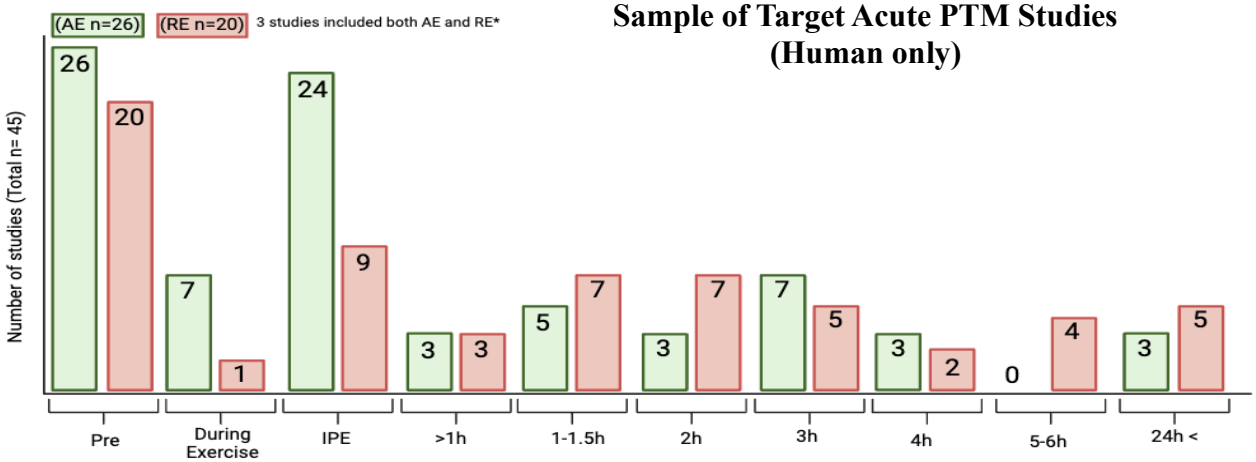

Sample of Target Acute PTM Studies (Human only)
(AE n=26)
(RE n=20)
3 studies included both AE and RE*
Number of studies (Total n= 45)
26
20
7
1
24
9
3
3
5
7
3
7
7
5
3
2
0
4
3
5
Pre
During Exercise
IPE
>1h
1-1.5h
2h
3h
4h
5-6h
24h <

Figure 1.2. Acute exercise PTM signaling timelines for representative proteins of resistance (mTOR) and aerobic (AMPK). Sixteen studies each for resistance and aerobic exercise were identified from a systemic search of the literature to have assessed (A) mTOR S2448 and (B) AMPK T172 phosphorylation following a single session of exercise. The data was used to graph the approximate average signaling response of each signaling factor following the respective exercise. (C) Forty-five studies that included data of targeted PTMs following exercise (AE and RE) were identified from a systematic search of the literature and assessed for time points of signaling (biopsies) to compare and contrast the strength of signaling data in the minutes and hours following exercise. The mean number of time points per study was 3.

The exercise-induced regulation of translation initiation has been primarily studied in the context of RE and protein accretion (Drummond, Dreyer et al. 2009). In contrast, AE has been primarily examined in relation to its regulation of transcription factors and mitochondrial genes (Long, Widegren et al. 2004). The effectors downstream of AE signaling-induced increases in MPS are relatively less well characterized in contrast to RE due to the context of previous literature.
In untrained individuals, translation initiation with AE may be in part mediated by mTORC1 S^{2448} phosphorylation and its downstream target 4EBP1 $T^{37/46}$ (Mazo, D'Lugos et al. 2021). However, the magnitude of mTORC1 S^{2448} signaling downstream targets S6K1 T^{389} and 4EBP1 $T^{37/46}$ at 1-4 hours post-exercise is less (~50% lower) following AE vs. RE, which may serve as a mechanism delineating the divergent adaptation with different exercise modalities or signaling events may follow a different time course after AE vs RE. Although AE and RE significantly

elevated mTOR S^{2448} phosphorylation 1 h after exercise, there were differences at a later time point (4 hours post-exercise) following RE (Mazo, D'Lugos et al. 2021).

Wilkinson et al. (2008) (Wilkinson, Phillips et al. 2008) hypothesized that the divergent phenotypes observed with chronic AT and RT are derived by the differential repeated stimulation of predominantly myofibrillar and mitochondrial protein synthesis elicited by RE and AE, respectively. In their seminal study, acute myofibrillar and mitochondrial protein synthesis were measured after exercise in untrained and trained muscle (following 10 weeks of training in the same participants). In the untrained state, RE stimulated similar (~70 %) increases in myofibrillar and mitochondrial protein synthesis above resting values. Whereas AE stimulated an increase in mitochondrial protein synthesis in the untrained (by 154%) and trained (by 104%) state but failed to significantly alter myofibrillar FSR (Wilkinson, Phillips et al. 2008). Additionally, after 10 weeks of training, resting myofibrillar protein synthesis was greater (~40%) in the RT condition in comparison to AT, and the same relative acute RE stimulus elevated myofibrillar protein synthesis a further 37% above resting values (Wilkinson, Phillips et al. 2008). Skeletal muscle is, therefore, able to resolve different exercise modes and elicit exercise-specific stimulation of protein synthesis and subsequent adaptation.

Developments in the application of deuterium oxide as a biosynthetic label to trace dynamic processes combined with proteomic techniques offer new possibilities to gain higher resolution information on muscle responses (i.e., changes in protein synthesis, degradation, and turnover rate) that occur in response to specific stimuli (i.e., AT compared to RT). Mixed muscle and fraction-specific measurements of protein synthesis represent an average of thousands of

proteins, which may respond differently to a particular exercise intervention. Deuterium oxide labeling with proteomic analyses of muscle samples can resolve the synthesis of individual proteins within the muscle (Burniston 2019) and has highlighted that proteins with different functions have different synthesis rates in human muscle (Stansfield, Barrett et al. 2024). The first dynamic proteomic analysis of human skeletal muscle responses to RT included parallel measurement of protein-specific synthesis and abundance data for almost 100 muscle proteins (Camera, Burniston et al. 2017), adding new detail on the selectivity of protein turnover responses to RT. The patterns of protein synthesis and abundance changes in skeletal muscle included subsets of proteins that i) increased in turnover but exhibited no change in abundance, ii) increased in abundance without a parallel rise in synthesis rate, and iii) decreased in abundance even though the synthesis rate was increased. In comparison, profiling of individual protein synthetic rates in response to aerobic-type exercise showed a significant increase in the turnover of 22 proteins (from 409 measured) associated with energy metabolism, proteasome, and cell stress in aerobic-trained muscle (Srisawat, Stead et al. 2023).

Future directions

Important considerations for acute post-exercise signalling. To date, much of our understanding of molecular exercise physiology has arisen from hypothesis-led studies targeting small numbers of proteins that have well-defined modification sites. Necessarily, the current literature includes a mix of studies at different levels (cell, animal, or human) and using different interventions (gain- loss- function, pharma, or exercise). The diversity of experimental designs is a strength but also should not be overlooked when attempting to summarise the mechanisms of exercise-induced muscle adaptation. Some mechanistic data (e.g., gain-/loss-of function in cell models) may not

apply in the context of exercise. For example, data collected in non-muscle cells or experiments that did not include exercise should be treated with caution until validated specifically in the context of exercised muscle. Changes in PTM (e.g., phosphorylation of S, T, or Y) must be interpreted against the context of cooccurring changes across the networks in which protein residue is involved. Intracellular signaling represents a computational system that reads various inputs (e.g., changes in energy status, metabolism, redox state, mechanical stress, etc.) and generates a particular output (e.g., modulation of gene expression, protein synthesis, degradation, and abundance). The processing algorithm may be different in the muscles of trained athletes versus untrained individuals, or the input may be sensed differently depending on the cellular context (e.g., redox state), leading to concepts of hormesis and bi- or multi-phasic responses to exercise.

More recently, non-targeted 'omics' studies have brought forth new hypotheses and may reveal new candidates that distinguish between AE and RE mode-specific adaptation. Højlund et al. (2009) (Højlund, Bowen et al. 2009) reported the first non-targeted data on the human muscle phosphoproteome and identified phosphorylation of muscle-specific proteins, confirming the role of phosphorylation in the regulation of skeletal muscle function. Hoffman et al. (2015) (Hoffman, Parker et al. 2015) reported phosphoproteomic profiling of muscle collected from young men before and immediately after ~10 min of high-intensity aerobic exercise and highlighted 1,004 exercise-regulated phosphosites, of which 92% were yet to be associated with upstream kinases (Hoffman, Parker et al. 2015). Surprisingly, just 5 exercise-responsive phosphorylation sites were known substrates of AMPK, which questions the narrow focus of targeted studies on 'master regulators' of exercise adaptation.

Potts et al. (2017) (Potts, McNally et al. 2017) reported phosphoproteomic profiling of mouse tibialis anterior collected 1 h after a bout of maximal intensity contractions (MIC) induced by electrical stimulation. Contractile activity resulted in significant differences in phosphorylation of 621 sites on 313 proteins, with the majority (531 sites) increasing in phosphorylation after exercise. Most exercise-responsive phospho-sites had not previously been detected and only 12 phosphosites had known upstream kinases, which were mapped to ERK1/2 and CAMKII. In a subsequent study (Steinert, Potts et al. 2021), rapamycin was used alongside the MIC protocol to investigate mTORC1-dependent and independent phosphorylation. Over 2000 unique phosphorylation sites were significantly regulated, but just 38 sites were rapamycin-sensitive, and most MIC-induced phosphorylation was rapamycin-insensitive anabolic pathways (Steinert, Potts et al. 2021).

Exercise mode-specific phosphorylation of muscle proteins was reported by Blazev et al. (2022) (Blazev, Carl et al. 2022), who identified 5,486 phosphosites (on 1,573 proteins) that were significantly regulated 1 h and 3 h after at least one of either aerobic (90 min, 60% of VO2 max), sprint (3 x 30 s all-out cycling), or resistance exercise (6 sets of 10 rep max knee extensions) in 8 healthy, untrained men (Blazev, Carl et al. 2022). Phosphorylation of 430 sites were common across the different training modes, including phosphorylation of the novel gene product C18ORF25, which was validated as an AMPK substrate (Blazev, Carl et al. 2022). In contrast, the phosphorylation of rapamycin-sensitive mTORC1 substrates was specific to different training modes (Blazev, Carl et al. 2022). Phosphoproteomic analyses can reveal unique signaling networks in response to different training modes that may accumulate to result in differences in the adaptive responses to training.

Non-targeted analyses add significant power to discover new mechanisms and, simultaneously, have exposed how little is known about signaling networks and the paucity of information on which kinases regulate which phosphorylation sites. Despite their comprehensive nature, non-targeted PTM studies so far have raised more questions than answers, and it is still unclear which key signaling events might be mechanistically linked to phenotypic muscle adaptations. As yet, few omic studies have considered more than one type of PTM. This review focuses on phosphorylation, which currently has the largest body of evidence in the context of skeletal muscle and exercise adaptation. However, crosstalk exists between different PTM (e.g., ubiquitination) on the same protein (Parker, Kiens et al. 2020). Multi-PTM omic studies are required to bring a complete understanding of intracellular signaling networks.

Currently, the full repertoire of mechanisms of exercise is unknown, and many questions remain unanswered regarding how different modes of exercise result in distinct muscle responses. Even when constrained to protein phosphorylation, higher levels of complexity exist than are currently considered. The archetypal exercise substrate, AMPKa1, has 50 known sites of post-translational modification, including 36 phospho-sites, 12 ubiquitylation sites, 1 acetylation, and 1 SUMOylation site (Phosphosite, AMPKA1 human site table). Just 20 of these sites have been investigated using targeted (low throughput; LTP) methods, and >80 % (227/280) of those studies focus on a single phosphorylation site (T^{183}). Similarly, 34 modification sites are known on the human isoform of AMPKa2, and only 12 sites have been studied by LTP targeted analyses, including the exercise responsive T^{172} mapped with 222 targeted studies from a total of 249 mapped to the entirety of AMPKa2. From a total of 109 PTM sites mapped to mTOR (Phosphosite, mTOR human site table) from discovery and targeted analyses (60 phosphorylation, 47 ubiquitylation, 2 acetylation), only 11 sites have been followed up with

targeted investigation and only 2 sites (i.e. the exercise responsive S^{2448} and S^{2481} phosphorylation sites) mapped with > 10 targeted analyses (217 and 68, respectively from a total of 304 citations providing low throughput analysis of the human isoform of mTOR).

Summary

In the future, it will be necessary to understand the interactions between different types of PTM across and within different proteins. In such studies, changes in gene expression, ribosomal activity, cellular location of proteins, and degradation rate are the immediate outcome measures that, in turn, need to be related to changes to the muscle proteome (i.e., protein abundance profile) and phenotype/ functional characteristics of the muscle. Adaptation is a time-dependent process that, so far, has been investigated by time-series studies of samples collected at discrete time points. Time-series studies attempt to describe the continuous dynamic process of adaptation using snapshots that are an incremental sequence of isolated points. Emerging new dynamic proteomic methods that combine proteomics with stable isotope labeling offer new opportunities to investigate the process that occurs between each sampling point. If a protein becomes more abundant from one time to the next, it is not clear whether more of that protein was synthesized or less degraded. Protein turnover is the mechanism of adaptation; therefore, readouts of synthesis and degradation should be the most relevant outcome or end point for studies on intracellular signaling.

Mechanistic studies cannot be conducted in humans but the analysis of molecular responses to exercise in human muscle is essential to validate data from cell and non-human animal models. Studies in humans provide the only substrate for within-subject analysis of molecular processes, which is a key advantage that is currently under-exploited. Both cell and animal experiments

necessarily use independent groups at different time points, which introduces an additional source of error because different animals or cell cultures are used at different time points. Although the number of biopsies is limited, there are examples of multi-time point studies, and in these cases, extensive omic analyses should be employed to make the most of the limited samples. Currently, the focus on limited numbers of molecular targets at too few time points limits our understanding of the crosstalk between different molecular mechanisms involved in the adaptive process with exercise.

Despite divergent outcomes of chronic training of one form of exercise or the other, relatively little is known about the exercise mode-specific acute signaling mechanisms that are proposed to result in the transition to an aerobic or resistance-trained muscle phenotype. Undoubtedly, exercise of either type (AE or RE) results in profound transcriptional responses, with some common genes being transcribed and some that are unique (Pillon, Gabriel et al. 2020). The general thesis is that with each exercise session, there are transcribed genes that are subsequently translated, and the resultant proteins accumulate to change, over time, the phenotype of the muscle (Egan and Zierath 2013). Studies on AE have often focused on linking signaling to transcription factors and changes in gene expression (Makhnovskii, Bokov et al. 2021). Studies on RE often try to link signaling to translation initiation and subsequent protein synthesis (Witard, Bannock et al. 2022). What we know far less about is what occurs when concurrent exercise is performed, combining both AE and RE, which were generally thought to oppose each other (Coffey and Hawley 2017). We propose that while the ultimate phenotype of the muscles of aerobic and resistance-trained persons are different, there may be more in common than there are distinct, at least initially, until training progresses to a point where the persistent practice of one type of exercise refines the response leading to a more distinct phenotype (Figure 1.3).

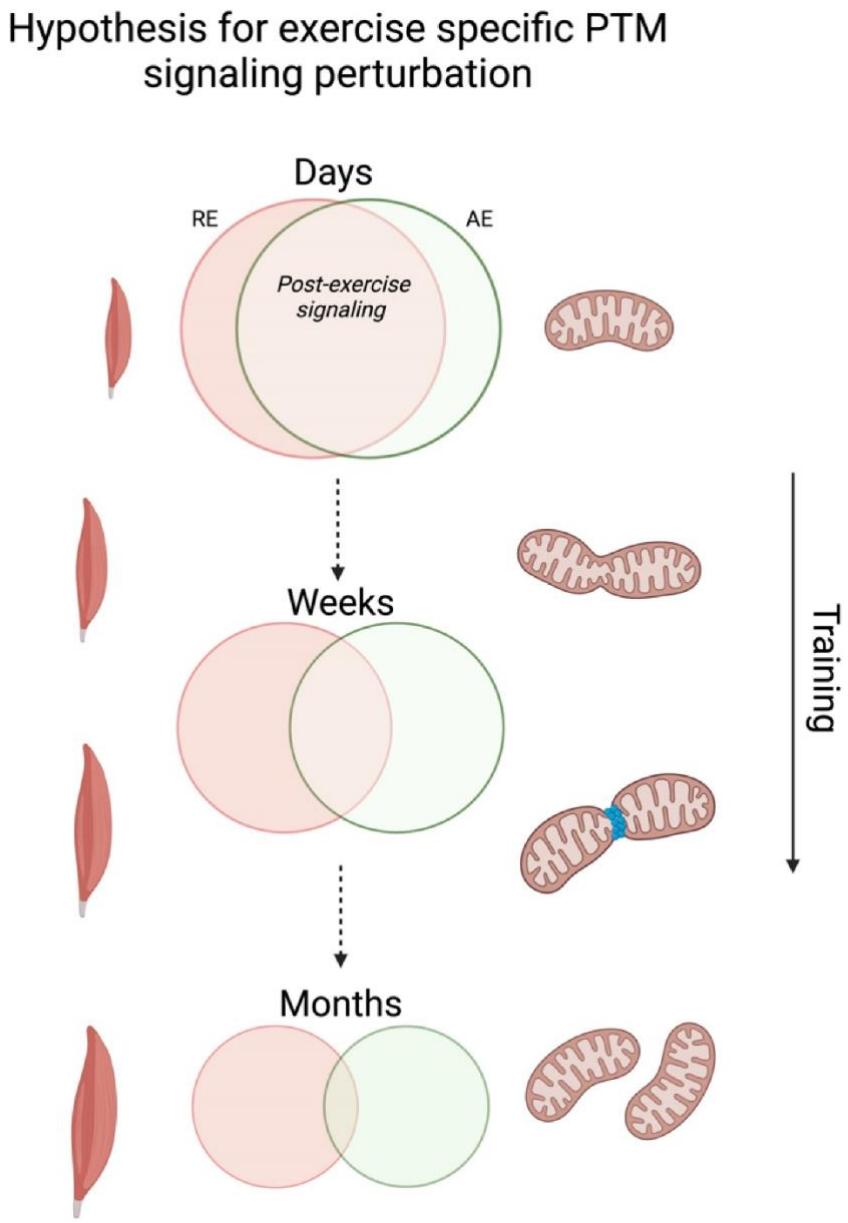


Figure 1.3. Exercise perturbation in unaccustomed muscle elicits a less specific signaling (PTM, transcription, and translation) response that becomes more refined with chronic training. With repeated exercise of a specific modality, the molecular response to exercise has a lesser but more efficient PTM cascade to induce exercise-specific changes in muscle protein content and phenotype.

The molecular responses to acute exercise in an untrained state may largely consist of a 'generic stress' response to the unaccustomed perturbation. Hence, only over time, with repeated exercise bouts, is muscle able to adapt to the stimuli, whereby the extent of the perturbation is refined and potentially targeted towards efficient adaptation for improved muscle function and substrate utilization specific to the exercise stimulus (Egan and Sharples 2023). Therefore, a preparatory shift in the muscle proteome and phosphoproteome should be apparent in the early phase of training.

Differences should exist between the proteomes of untrained, trained, and highly trained individuals that could aid the interpretation of differences in muscle signaling in these states. In line with these hypotheses, Damas et al. (2016) (Damas, Phillips et al. 2016) demonstrated that the acute exercise-induced increase in protein synthesis only correlated with hypertrophy after an initial attenuation of damage had occurred (Damas, Phillips et al. 2016). We propose that a significant overlap of exercise-induced signaling patterns exists in response to the initial exposures to exercise, regardless of modality. With chronic exposure (i.e., training), skeletal muscle transcriptional and translational programs are refined and initiate signaling pathways that drive phenotypic changes (Wilkinson, Phillips et al. 2008, Kumar, Atherton et al. 2009). Acute signaling can, therefore, change as an individual becomes more accustomed to the stress of exercise training. For example, in untrained individuals, the acute signaling response of canonical signaling pathways, including AMPK, ERK1/2, and p38, is attenuated after only 10 days of intensified aerobic exercise, such that acute exercise no longer elicits a significant elevation in phosphorylation (Benziane, Burton et al. 2008).

The degree of post-exercise signaling, and transcription does not necessarily equate to a proportional change in protein content or function. There exist some relationships between mRNA and protein expression that have been established (Perry, Lally et al. 2010); still, the acute expression does not correlate well with protein translation/ content or accurately predict phenotypic outcomes of training in humans (Arkinstall, Tunstall et al. 2004, Broholm, Mortensen et al. 2008, Egan and Zierath 2013, Makhnovskii, Zgoda et al. 2020). More recent omic studies have substantially expanded on the discordant relationship between genes and proteins (Emanuelsson, Arif et al. 2024). The discordance between mRNA expression and protein content may be related to (i) post-transcriptional processing and transcript stability, (ii) translational capacity and efficiency, and (iii) protein degradation. Discordant responses between PTM exercise signaling, mRNA abundance, protein synthesis, and long-term adaptation (typically assessed as change in muscle protein content) are not uncommon in exercise literature (Mitchell, Churchward-Venne et al. 2014, Damas, Phillips et al. 2016, Perry and Hawley 2018, Kuang, McGinley et al. 2022, Bishop, Hoffman et al. 2023, Egan and Sharples 2023). Therefore, more omic studies that encompass transcriptomic, phosphoproteomic, proteomic, and proteome dynamics are required to unravel the relationship between acute stimuli and phenotypic outcomes.

Lastly, changes in signal transduction following AE or RE elicit divergent elevation of protein synthesis despite commonly regulated signaling pathways. For example, AE and RE have demonstrated similar phosphorylation status of AMPK T^{172} and FAK $Y^{576/577}$ and eIF4E S^{209} but elicited differential responses in synthesis rates when assessing myofibrillar and mitochondrial protein fractions (Wilkinson, Phillips et al. 2008). Therefore, studies that better relate the acute (transient) and chronic (adaptation) changes in signaling to protein-specific dynamics and muscle

function are required to improve our understanding of the mechanisms of adaptation with exercise training. We hypothesize that there are key yet uncharacterized differences in the signaling and subsequent regulation of protein-specific synthesis and degradation that drive unique adaptation to AT and RT. These differences in molecular responses can only be detected with multi-timepoint/ omic methods that consider both key differences and similarities in the transient PTM responses to acute exercise, as well as changes in proteome dynamics over longer periods of training.

Funding. No specific sources of funding were used for this work. SMP thanks The Canada Research Chairs program for their support. SMP reports non-financial support from Enhanced Recovery, other from Exerkine and personal fees from Nestle Health Sciences and Optimal Nutrition outside the submitted work. In addition, SMP has patents licensed to Exerkine but reports no financial gains.

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CHAPTER 2:

Phosphoproteomic Investigations in Humans to Probe Critical Pathways in Resistance Exercise Adaptation.

Chapter 2: Phosphoproteomic Investigations in Humans to Probe Critical Pathways in Resistance Exercise Adaptation

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Keywords

Protein turnover, Protein signaling, Kinase

Abstract

It is well known that aerobic exercise training enhances skeletal muscle mitochondrial protein synthesis and biogenesis leading to enhanced oxidative capacity; however, it minimally impacts myofibrillar protein synthesis and does not enhance hypertrophy. In contrast, resistance exercise enhances myofibrillar protein synthesis leading to hypertrophy, but does not markedly enhance oxidative capacity. Although the induction of these distinct muscular adaptations is well known, the signaling events underpinning them remain unresolved. To better characterize these signaling events, 16 young healthy subjects (n=4 pilot study and n=12 follow-up study) performed a unilateral bout of aerobic or resistance exercise with biopsies taken Pre, immediately post (0h) and 3 hours following recovery. Muscle tissue (cohort 1: n=4) was subjected to deep phosphoproteomic analyses, identifying nearly 13000 individual phosphosites and unique clusters specifically associated with aerobic and resistance exercise. The cluster associated with resistance exercise contained >2500 sites and demonstrated an immediate (0h) change in phosphorylation that was largely sustained at 3h post-exercise. Additionally, bioinformatic- and literature-based predictions suggested that this effect was mediated by prolonged activation of a MKK3/6 → p38 → MK2 signalling pathway. Follow-up studies (cohort 2: n=12) were performed in both humans (data shown) and mice (data not shown), and the outcomes support the theory that prolonged activation of the MKK3/6, p38, and MK2 signalling axis is a resistance exercise-specific phenomenon. We also demonstrate that the activation of signalling through MKK3 is robustly correlated with the increase in myofibrillar protein synthesis that occurs after a bout of resistance exercise in humans ($r^2 = 0.60$, $p<0.01$). Collectively, these data suggest that MKK3/6, p38, and MK2 signalling axis is one of the core components of a signalling pathway that drives the hypertrophy-promoting effects of resistance exercise in humans.

Chapter 2 Introduction

Skeletal muscle is a highly plastic tissue capable of adapting to changes in contractile activity manifested through exercise. Importantly, by changing the nature of the exercise stimulus, it is possible to direct the response of the type of skeletal muscle proteins that are being synthesized leading eventually to a change muscle phenotype. For example, prolonged and repeated contraction using lower loads in skeletal muscle, such as aerobic exercise training (AE), results in an increase in the expression of mitochondrial genes (Perry, Lally et al. 2010), proteins (Holloszy 1967, Perry, Lally et al. 2010, Egan, O'Connor et al. 2013), and ultimately enhanced mitochondrial content (Gibala, Little et al. 2006). The result is a shift towards an oxidative muscle fibre phenotype with improved fatigue resistance (Egan and Zierath 2013). Loading of skeletal muscle with higher loads, such as resistance exercise (RE) training, also stimulates the transcription of genes and accrual of new muscle proteins (Mitchell, Churchward-Venne et al. 2012, Nader, von Walden et al. 2014) by enhancing muscle protein synthesis (MPS) (Pilegaard, Ordway et al. 2000, Damas, Phillips et al. 2016); however, the genetic (transcriptomic) and protein responses are largely associated with the myofibrillar protein fraction, and regular RE leads to muscle fibre hypertrophy, increased fibre cross-sectional area and increased force-generating capacity(Lim, Nunes et al. 2022, Roberts, McCarthy et al. 2023). Regardless of key differences, the signalling mechanisms that delineate how AE and RE exercise differentially regulate myofibrillar and mitochondrial protein synthesis remain unclear.

A key to exercise-induced adaptation is the post-translational modification of proteins. One important post-translational modification is phosphorylation. Phosphorylation is the addition of a

phosphate group to a target protein by a protein kinase using ATP and Mg as cofactors (McGlory, White et al. 2014) to serine (S), tyrosine (Y) or threonine (T) residues. The addition of this phosphate can enhance or downregulate the protein's catalytic activity, binding status or cellular location, resulting in a biological downstream effect. There are over 500 documented kinases in the human 'kinome'. We (Wilkinson, Phillips et al. 2008, McGlory, White et al. 2014, MacInnis, Haikalis et al. 2016) and others (Coffey, Zhong et al. 2006, Dreyer, Fujita et al. 2006) often assess the phosphorylation status of single or multiple kinases/proteins in response to exercise to gain a mechanistic understanding of how exercise alters the skeletal muscle phenotype. However, current methods for this quantification are largely semi-quantitative (Western blotting) and are biased in terms of which proteins are studied (*i.e.,* targeted analysis). Our understanding of how skeletal muscle adaptation is influenced by phosphorylation is, therefore, limited. Developments in mass spectrometry (Hoffman, Parker et al. 2015, McCloy, Parker et al. 2015) have enabled a wider interrogation of the phosphorylation status of proteins in response to exercise. Indeed, one study using advanced liquid chromatography-mass spectrometry revealed 1,004 unique exercise-regulated phosphorylation sites on 562 proteins in human skeletal muscle biopsy samples obtained before and after a bout of aerobic-type exercise (Hoffman, Parker et al. 2015). This seminal study significantly advanced our knowledge regarding how AE exercise impacts phosphorylation signaling. Yet, no measurements of endpoint phenotype (i.e., MPS) were made in this study (Hoffman, Parker et al. 2015) and, therefore, conclusions on the relationship between exercise regulation of phosphorylation sites and phenotypic responses – synthesis/turnover of proteins – could not be directly linked. Furthermore, an examination of the phosphoproteomic responses to RE was performed by (Blazev, Carl et al. 2022), who compared phosphopeptide responses following RE, AE, and sprint exercise, identifying a total of 5486

phosphosites on 1573 proteins significantly regulated immediately post-exercise or in recovery (3h) by at least one exercise modality. However, only 222 phosphosites (4.0%) were annotated with a known function and 202 (3.5%) with a known upstream kinase. Nonetheless, these authors (Blazev, Carl et al. 2022) also did not make any measurements of endpoint phenotypic responses in MPS.

Although different training modes lead to varied outcomes, the specific signalling mechanisms underpinning the transition to AE- or RE-trained muscle phenotypes are not well understood. Chronic muscle adaptations to exercise (*i.e.* changes in protein content) develop over weeks to months and cannot yet be accurately predicted from the immediate molecular responses of an acute exercise session. Understanding the mechanisms linking the acute muscle responses to chronic changes in hypertrophy and metabolic phenotype is crucial for optimizing training programs and lifestyle recommendations for exercise performance and health. The discovery and validation of predictive molecular markers for desirable muscle phenotype changes could improve exercise prescriptions, but challenges remain.

We aimed to conduct a global phosphoproteomic analysis of skeletal muscle using advanced mass spectrometry following both AE and RE exercises in humans. We also measured rates of MPS for the 3 hours following the exercise to understand which kinases were active in promoting and maintaining phenotypic adaptations. Additionally, we used targeted (Western blotting) analysis to confirm hypotheses generated from 'omic' data on RE-specific signalling pathways during recovery from RE. We hypothesized that the phosphorylation of some proteins would be unique to RE and would be related to MPS.

Methods

Ethics. The study (NCT04263714) was approved by the Hamilton Integrated Research Ethics Board (HIREB #2196) and conformed to the standards for the use of human subjects in research as outlined by the Canadian Tri-Council Policy (TCPS 2 2022) on the ethical use of human subjects in research (https://ethics.gc.ca/eng/policy-politique_tcps2-eptc2_2022.html) and the declaration of Helsinki. Each participant was informed of the purpose of the study, experimental procedures, and potential risks before written consent was obtained. Informed consent was obtained from all subjects prior to beginning the protocol.

Participants. Six females and ten males were recruited in two cohorts to participate in this study. Participants were recruited in two cohorts due to covid delays/restrictions: pilot cohort 1 (n=4) and cohort 2 (n=12). Participants were healthy, between the ages of 19-23, and had recreational experience in aerobic and resistance exercise but were not regularly performing exercise training. The sample size was determined with MPS as the primary outcome measure, using a previous, male-only study characterizing MPS responses between the two exercise modalities and phosphoproteomic data. We recruited equal numbers of males and females in cohort 2 with the hypothesis that there would be no sex-based differences (we did initially test for sex-based in the analysis differences but observed none; all $p>0.7$) and that exercise would induce a marked differential shift in MPS per our pilot data.

Study Overview. In a within-subject repeated measures, with time and condition (RE versus AE) design, 16 participants (n=10 male, n=6 female) visited the laboratories at the Department of Kinesiology on 6 separate occasions. On the first visit, participants were screened, and informed

consent was obtained. Participants were then assessed for body composition using dual-energy x-ray absorptiometry (DXA) after an overnight fast (~10 h), followed by familiarization with RE and AE equipment. Participants' legs were randomly assigned to the RE or AE group (see "Exercise protocols" below). On visits 2 and 4, participants completed 1-repetition maximum testing. On visits 3 and 5, participants completed a VO_2 peak test on a cycle ergometer. These tests were used to determine the intensity of AE and RE exercise load on the day of the experimental trial (visit 6). For 3 days before visit 6, participants refrained from strenuous exercise and consumed a controlled diet with all their meals provided by the study investigators. On visit 6, participants arrived at the laboratory at approximately 0630 after an overnight fast. Catheters were placed in the antecubital veins on each arm for: venous blood sampling and for the infusion of L-[*ring*-$^{13}C_6$] phenylalanine. At 0700, baseline blood samples were drawn, and then participants received a priming dose of the stable isotope (2μmol/kg) before initiating a constant tracer infusion of 0.05μmol/kg/min. Participants rested on a bed for the duration of visit 6 (excluding exercise protocols), and blood samples were taken every 20-30 min with evacuated heparinized tubes. After 180 min of rest, a muscle biopsy was taken from the vastus lateralis of a randomly selected leg to determine fasted-state MPS. Upon completion of the biopsy, participants began the unilateral AE and RE protocol in a randomized order, with biopsies taken from the leg immediately upon exercise completion. Upon completion of exercise protocols, participants rested on a bed with biopsies taken from each leg 180 minutes after completion of each exercise modality. Incorporation of the L-[*ring*-$^{13}C_6$] phenylalanine into the muscle between the immediate post-exercise and 3h muscle biopsy allowed us to measure the synthesis of the myofibrillar and sarcoplasmic proteins following each exercise modality.

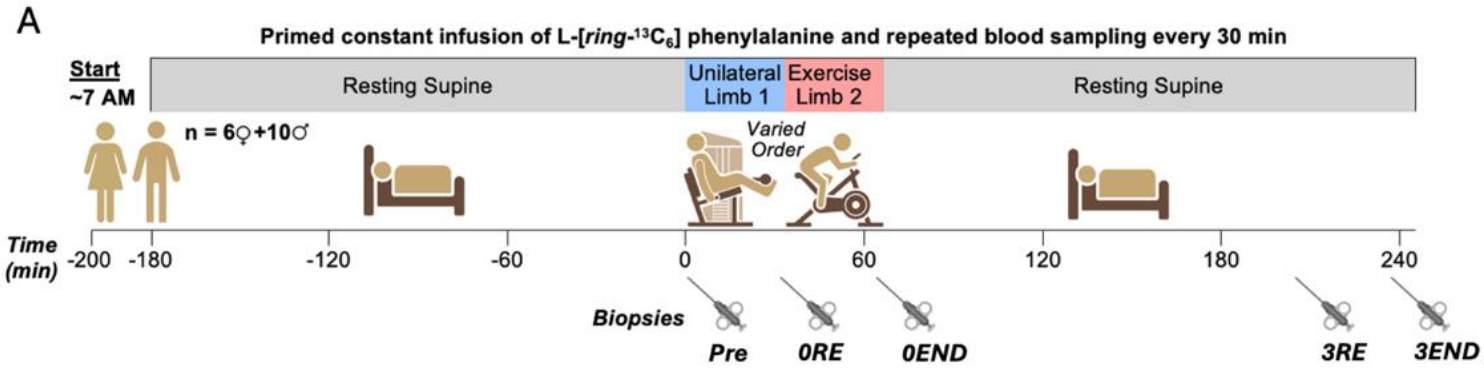


Figure 2.1: Experimental trial (visit 6) overview.

1-Repetition Maximum. After a brief warm-up, a specific warm-up of the given exercise was performed at approximately 50% of the participant's estimated 1RM. The load was progressively increased by approximately 5-20% for each repetition until a true 1RM was achieved, as previously described (McGlory, White et al. 2014). Three to 5 min of rest was given between each attempt. A successful attempt required the participant to move the load with the correct form throughout the full range of motion.

VO_2 Peak. Subjects completed double-leg and single-leg incremental peak oxygen uptake (VO_2peak) tests on a cycle ergometer (Velotron, RacerMate; Seattle, WA) as previously described (Thomas, Brown et al. 2022). A metabolic cart with an on-line gas collection system (Moxus modular oxygen uptake system, AEI Technologies, Pittsburgh, PA) measured oxygen consumption (VO_2) and carbon dioxide production (VCO_2) data and heart rate was monitored continuously with a heart rate monitor (Polar A3, Lake Success, NY). The test began with a 2-min warm-up at 50 watts (W), after which the power was increased by 1 W every two (bilateral) or four (unilateral) seconds until volitional exhaustion or the point at which pedal cadence fell

below 60 rpm. VO_2 peak was defined as the highest oxygen consumption achieved over a 30s period. Maximal workload (Wmax) was the highest power output achieved during the test. The subject was deemed to have reached VO_2 peak if their HR was within 5bpm of age-predicted maximal HR; the respiratory exchange ratio was >1.2, and a plateau was reached in their oxygen consumption.

Visit 6 Exercise Protocols. Randomized order unilateral: i) RE: 3 sets × 10 reps at 80% 1RM leg press followed by the same leg extension, with 2 min recovery between sets and exercises and ii) AE: 40 min @ 65% single leg peak power.

Diet. Three days before visit 6 (experimental trial), all food was provided to the participants as frozen meals (Supplier: Heart to Home meals) to ensure minimum protein intake >1.2 g/kg. Each participant's energy requirement was determined using the Harris-Benedict equation for BMR and multiplied by an activity factor of 1.55, corresponding to moderately active individuals.

Biopsies. All biopsies were taken after administration of 1% xylocaine local anesthesia with the use of a 5-mm Bergström needle that was adapted for manual suction. Muscle tissue samples were freed from any visible connective and adipose tissue, rapidly frozen in liquid nitrogen to measure MPS and phosphor-signalling events and stored at −80° C for further analysis.

Analytic Methods. Muscle samples (~30–50 mg) were homogenized on ice in buffer [10 μL/mg 25 mM Tris-HCl pH7.2 0.5% vol:vol Triton X-100 and protease/phosphatase inhibitor cocktail tablets (Complete Protease inhibitor Mini-Tabs, Roche; and PhosSTOP, Roche Applied Science)]

and centrifuged at 2250g for 10 min at 4°C to separate the sarcoplasmic and myofibrillar enriched protein fractions. The supernatant was removed for sarcoplasmic protein analysis, and the myofibrillar pellet was retained (McKendry, Lowisz et al. 2024). Doubly distilled water (500 μL) was added to the myofibrillar pellet, which was vortexed and centrifuged (250g for 10 min at 4°C). The supernatant was removed and discarded; the remaining pellet was incubated in 0.3NaOH for 30 min at 50°C to solubilize the myofibrillar protein fraction. The samples were vortexed and centrifuged at 11200g for 5 min at 4°C, supernatant (myofibrillar protein) was removed, and the collagen pellet was discarded. This step was repeated, and supernatants were pooled. Myofibrillar and sarcoplasmic proteins were precipitated in 1 mL 1M perchloric acid and centrifuged at 700g for 10 min at 4°C. The supernatant was discarded, and the fraction was washed twice with 70% ethanol. Ethanol was removed, and the sample was placed in 1mL 1M activated Dowex H+ (50XW8 Cation exchange resin) and 1mL 1M HCl. The protein–enriched pellets were hydrolyzed at 110 °C for 72 h to release their respective amino acids (AAs). Following hydrolysis, the AAs were purified by ion exchange chromatography on Dowex H+ resin using 2M NH_4OH. Following isolation and purification of myofibrillar and sarcoplasmic protein fractions, samples were dried under nitrogen, reconstituted in 0.1m HCl, and analyzed for myofibrillar and sarcoplasmic L-[*ring*-$^{13}C_6$] phenylalanine enrichment analysis per previous protocols (Brook, Stokes et al. 2022, Lim, Janssen et al. 2024).

Plasma L-[*ring*-$^{13}C_6$] phenylalanine enrichment was determined as previously described (Glover, Oates et al. 2008). Muscle samples were analyzed as previously described (Glover, Oates et al. 2008, McGlory, Wardle et al. 2016) for L-[*ring*-$^{13}C_6$] phenylalanine enrichment, to be used as the precursor for calculating the MPS rate using the precursor-product equation:

E_b represents the enrichment of bound myofibrillar protein, E_{ic} is the average intracellular enrichment between two biopsies, and t is the tracer incorporation time in h. Participants were 'tracer naïve' (i.e., they had not previously participated in a study protocol where L-[*ring*- $^{13}C_6$] phenylalanine was infused). Thus, a pre-infusion blood sample was used to calculate resting myofibrillar and sarcoplasmic MPS (McGlory, Wardle et al. 2016). Myofibrillar and sarcoplasmic enrichments of L-[*ring*- $^{13}C_6$] phenylalanine were measured as previously described (Churchward-Venne, Burd et al. 2012).

Mass Spectrometry and Analysis. Tissue lysis and centrifugation: Frozen muscles were homogenized in 1 mL of buffer A [40 mM Tris (pH 7.5), 1 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, one PhosSTOP tablet (Roche) per 10 ml, and one Complete Mini EDTA-Free Protease Inhibitor Cocktail Tablet (Roche) per 10 ml]. Samples were homogenized with a Polytron (PT 1200 E) for 20 s and then centrifuged at 6,000 x G for 1 min to remove bubbles and confirm complete homogenization. The homogenate was then separated into soluble and insoluble fractions, as detailed previously (Potts, McNally et al. 2017). A 100 ml aliquot of sample was saved for western blotting, and the remainder was reserved for MS analysis.

Protein Digestion and Peptide Desalting. The total amount of protein in each sample was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), and proteins were precipitated by bringing the original sample solution to a 90% concentration of MeOH by

volume. The sample was then centrifuged at 12,000 x G for 5 min, the supernatant was removed, and then the remaining protein precipitate was resuspended in 8 M urea, 50 mM Tris (pH 8.0), 10 mM TCEP, and 40 mM chloroacetamide and incubated for 30 min with shaking to completely reduce and alkylate the proteins. The sample was diluted to a concentration of 1.5 M urea with 50 mM Tris (pH 8.0) and digested with trypsin (1:50 enzyme:protein ratio) at 37°C for 15 h. The enzymatic digestion was quenched by acidifying the sample to pH < 2 with 10% trifluoroacetic acid (TFA). Strata-X desalting columns (Phenomenex) were prepared by flowing 1 mL of 100% acetonitrile (ACN) over the column, followed by 1 mL of 0.1% TFA. Individual samples were then spun down, and the acidic supernatant was collected and gravity-filtered through the Strata-X columns. The bound peptides were washed with 1 mL 0.1% TFA, followed by elution into a fresh tube using 500 ml of 40% ACN and 0.1% TFA, and finally, an additional elution of 300 mL of 80% ACN and 0.1% TFA. Eluted peptides were dried down by vacuum centrifugation. A Pierce Quantitative Colorimetric Peptide Assay (Thermo Fisher Scientific) was performed to determine peptide concentrations prior to TMT labelling.

TMT Labeling. This investigation comprised 2, 10-plex and 2, 11-plex TMT experiments, the preparation and processing of which were carried out following established methodologies (Potts, McNally et al. 2017). In summary, tryptic proteome digests were tagged with tandem mass tags (Thermo Scientific, Waltham, MA), then combined in equal proportions for each 16-plex trial. Phosphopeptide enrichments were performed by chelation with titanium dioxide immobilized to magnetic beads (MagReSyn, Resyn Biosciences) according to manufacturer guidelines. The remaining samples were preserved for protein quantification. The enriched and unenriched samples were both separated via semipreparative, high pH reverse phase

chromatography. The resulting fractions were dried and reconstituted in 0.2% formic acid and analyzed with LC-MS/MS using an LTQ-OT-IT tribrid mass spectrometer (Orbitrap Fusion Lumos, Thermo Fisher Scientific, Waltham, MA) with upfront nano-LC separation. MS1 survey scans were collected in the orbitrap mass analyzer (60K resolution, AGC – 1e6, 50 ms max injection time). HCD fragmentation was performed on isolated precursor ions. The resulting product ions were also analyzed in the orbitrap (60K resolution, AGC - 2e5, 118 ms max injection time, 1.8 Da isolation window). Monoisotopic precursor selection and dynamic exclusion (60 s) were enabled.

Thermo RAW files were searched with the COMPASS software suite against a target-decoy database of *Homo sapiens* proteins downloaded from Uniprot (Wenger, Phanstiel et al. 2011). Fixed modifications included TMT labelling at lysines and N-termini and carbomidomethylation of cysteine residues. Methionine oxidation was included as a variable modification. The searches for phopshopeptide-enriched samples also included variable phosphorylation of serines (S), threonines (T), and tyrosines (Y), along with corresponding neutral losses. Search results were filtered to a false discovery rate of 1% at both peptide and protein levels.

Phosphorylation sites were considered localized if they received a localization score >0.75 using the PhosphoRS algorithm embedded in COMPASS (Taus, Köcher et al. 2011). Phosphopeptide intensities were normalized to the total reporter ion intensity at the protein level and the protein mean-normalized fold change. The significance of differential expression was assessed via moderated T-test (Ritchie, Phipson et al. 2015), with multiple hypothesis testing conducted using Benjamini-Hochberg correction.

Western Blot Analysis. Frozen muscle samples were homogenized with a Polytron for 30 s in ice-cold buffer A (described above) or buffer B [40 mM Tris (pH 7.5), 1 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 25 mM b-glycerophosphate, 25 mM NaF, 1 mM Na3VO4, 10 mg/ml leupeptin, and 1 mM PMSF]. The resulting protein concentrations were determined using a DC protein assay kit (Bio-Rad). Equivalent amounts of protein were then dissolved in Laemmli buffer, boiled for 5 min, and subjected to electrophoretic separation by SDS-PAGE. Proteins were then transferred to a PVDF membrane at 300 mA for 1 hour 45 min and subsequently blocked with 5% powdered milk in tris-buffered saline (TBST) containing 0.1% Tween 20 for 1 hour. After 30 min of washing in TBST, the membranes were incubated overnight at 4 degrees C with the primary antibody dissolved in a 1% bovine serum albumin (BSA)-TBST solution. The next day the membranes were washed for 30 min with TBST and then probed with a peroxidase-conjugated secondary antibody dissolved in 5% powdered milk-TBST for 1 hour at room temperature. After 30 min washing in TBST, the blots were developed using a film processor or with a UVP Autochemi system (Analytik Jena AG) along with either a regular enhanced chemiluminescence (ECL) reagent (Pierce) or ECL-prime (Amer- sham). Once the appropriate images were collected, the membranes were stained with Coomassie blue to verify equal loading in all lanes. Images were quantified using ImageJ software (U.S. NIH).

Antibody	Source Species	Vendor	Product #	Dilution
Phospho-Acetyl-CoA Carboxylase (Ser79) Antibody	Rabbit	Cell Signaling	3661	1;1000
Acetyl-CoA Carboxylase (C83B10) mAb	Rabbit	Cell Signaling	3676	1;1000
Phospho-p70 S6 (389)	Rabbit	Santa Cruz	sc-11759	1;3000
p70 S6 Kinase (49D7) Rabbit mAb	Rabbit	Cell Signaling	4171S	1;1000
Phospho-S6 Ribosomal Protein (Ser240/244) (D68F8) XP(tm)	Rabbit	Cell Signaling	5364S	1;300
S6 Ribosomal Protein (5G10) Rabbit mAb	Rabbit	Cell Signaling	2217	1;1000
Phospho-MKK3 (Ser189)/MKK6 (Ser207) (22A8) Rabbit mAb	Rabbit	Cell Signaling	9236P	1;1000
MKK6 (D31D1) Rabbit mAb	Rabbit	Cell Signaling	8550S	1;1000
MKK3b Rabbit mAb	Rabbit	Cell Signaling	9238S	1;1000
Phospho-SEK1/MKK4 (Ser257) (C36C11) Rabbit mAb	Rabbit	Cell Signaling	4514P	1;1000
MKK4/SEK1 (5C10) Rabbit mAb	Rabbit	Cell Signaling	3346S	1;1000
Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP® Rabbit mAb	Rabbit	Cell Signaling	#4511	1;1000
p38α MAPK Antibody	Rabbit	Cell Signaling	#9218	1;1000
p38β MAPK (C28C2) Rabbit mAb	Rabbit	Cell Signaling	#2339	1;1000
p38γ MAPK Antibody	Rabbit	Cell Signaling	#2307	1;1000
p38 MAPK Antibody D13E1	Rabbit	Cell Signaling	#8690S	1;1000
Phospho-MAPKAPK-2 (Thr222) (9A7) Rabbit mAb	Rabbit	Cell Signaling	3316	1;1000
Phospho-MAPKAPK-2 (Thr334) (27B7) Rabbit mAb	Rabbit	Cell Signaling	3007T	1;1000
MAPKAPK-2 (D1E11) Rabbit mAb	Rabbit	Cell Signaling	12155	1;1000
Pho-Ser59-αB- Crystallin Polyclonal antibody	Rabbit	Enzo Life Sciences Inc	ADI-SPA-227-D	1;1000
αB- Crystallin monoclonal antibody	Mouse IgG1	Enzo Life Sciences Inc	ADI-SPA-222-D	1;100
Phospho-HSP27 (Ser82) (D1H2F6) XP® Rabbit mAb	Rabbit	Cell Signaling	9709	1;1000
HSP27 (E1J4D) Rabbit mAb	Rabbit	Cell Signaling	50353S	1;1000

Table 2.1: Antibody table for Western blotting analyses

Quantification and Statistical Analyses. The present study used a within-subject, repeated-measure design. A significance level of $p < 0.05$ was chosen, and multiple comparisons were corrected for using Benjamini-Hochberg controlling the false discovery rate. Benjamini-Hochberg corrected p-value (q-value) < 0.05 and ± 1.5-fold change (FC) from Pre was chosen for identifying significantly regulated phosphopeptides. MPS (sarcoplasmic and myofibrillar) was (initially) assessed using a two-way analysis of variance, which showed no main or interaction effects for sex, so the data were collapsed across sex and analyzed as a single group. Further analysis of MPS used a one-way repeated measures analysis of variance. A one-way mixed ANOVA was employed when datasets contained an outlier as defined by the median absolute deviation method with a 'very conservative' threshold of 3 (Leys, Ley et al. 2013). Following significant terms, relevant preplanned pair-wise comparisons were further tested post hoc using the Bonferroni post hoc test. Significance was set at $p < 0.05$. Statistical analyses of MPS and Westerns were completed using IBM SPSS Statistics for Mac version 21 (IBM SPSS Statistics for Mac, Version 21.0; IBM Corporation). Graphical representations of the data are as box and whisker plots, with the box representing the inter-quartile range, the line in each box indicating the median, the cross in each box denoting the mean, and the whiskers indicating the maximum and minimum values.

Results

Subject Characteristics. To characterize the differences in phosphorylation signalling and protein synthesis in relation to exercise type (AE vs. RE), we recruited two cohorts of healthy, untrained young individuals to measure protein synthesis and phosphorylation signalling following aerobic and resistance exercise. Cohort 1 (n=4 male participants, age 21±1; BMI, 26.1±2.6 kg/m^2) and

cohort 2 (n=6 male and n=6 female, age 21±2; BMI, 25.3±4.1 kg/m^2) each completed an acute session of unilateral aerobic (40 min at 65% peak watts) and resistance (3x10 reps at 80% 1RM leg press and extension) exercise in the overnight fasted state. All participants completed both unilateral exercises in randomized order on the same trial day.

A) Subject Characteristic (Cohort 1)	n=4 (M)
Age (years)	21 ± 1
Height (cm)	180 ± 2
Weight (kg)	84.4 ± 10.4
BMI (kg· m^{-2})	26.1 ± 2.6
Unilateral Work Peak (watts)	199 ± 6
Unilateral 1RM Leg Press (kg)	178 ± 42
Unilateral 1RM Leg Extension (kg)	78 ± 15

B) Subject Characteristic (Cohort 2)	n=12 (n=6 F, n=6 M)
Age (years)	21 ± 2
Height (cm)	173 ± 7
Weight (kg)	75.6 ± 14.2
BMI (kg· m^{-2})	25.3 ± 4.1
Unilateral Work Peak (watts)	172 ± 29
Unilateral 1RM Leg Press (kg)	102 ± 35
Unilateral 1RM Leg Extension (kg)	29 ± 9

Tables 2.2 and 2.3: Subject characteristics for (A) cohort 1 (n=4) and (B) cohort 2 (n=12)

Muscle Protein Synthesis. We first investigated protein synthesis to phenotypically characterize the muscle response following AE and RE. Both AE and RE induce an increase in myofibrillar protein synthesis (myoPS) ($p<0.001$), but the magnitude of the increase that occurred in response to RE was greater ($p<0.001$). Both AE and RE induced a comparable increase in sarcoplasmic protein synthesis (sarcoPS) from Pre ($p<0.001$), with no difference ($p>0.05$) between modalities. AE caused myoPS to be elevated 51 ± 30 % for 0-3 hours following exercise. RE caused myoPS to be elevated 146 ± 53% for 0-3 hours following exercise. AE resulted in an elevation in sarcoPS 123 ± 115% for 0-3 hours following exercise. RE caused sarcoPS to be elevated 82 ± 51% for 0-3 hours following exercise.

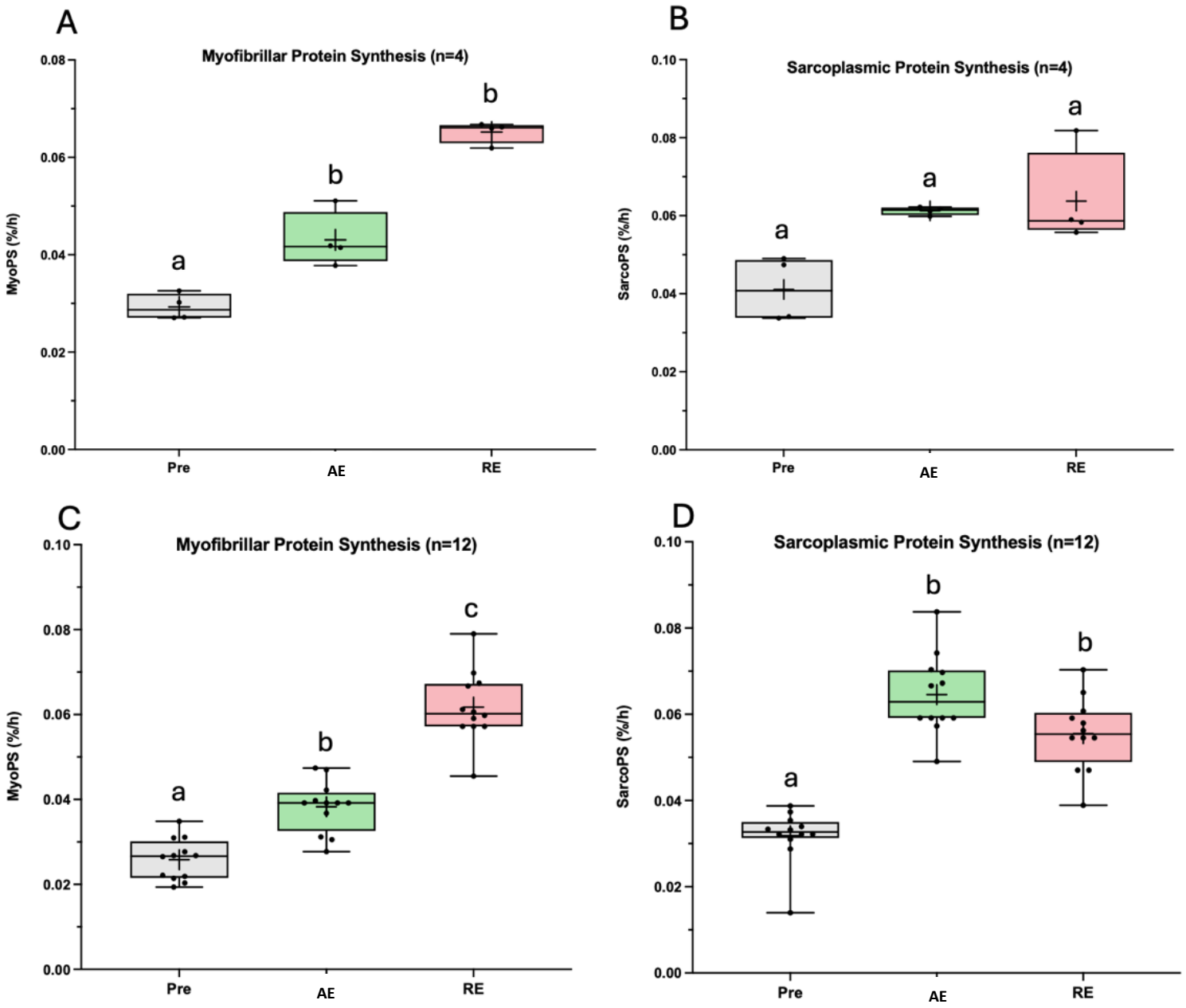
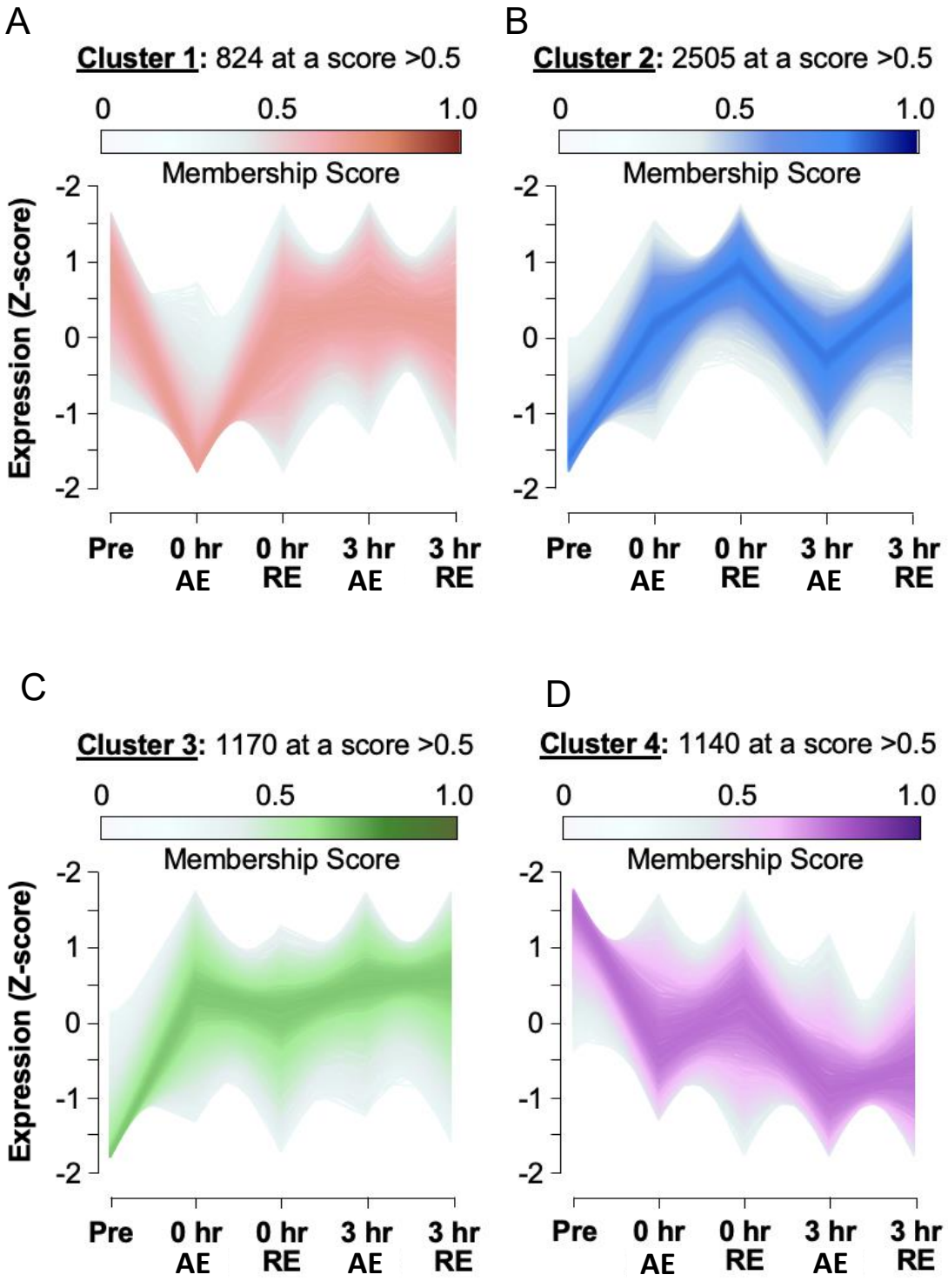


Figure 2.2. Myofibrillar (myoPS) and sarcoplasmic (sarcoPS) protein synthesis rates (%/h) for the 0-to-3-hour period immediately following exercise for cohort 1, n=4 (**A-B**) and cohort 2, n=12 (**C-D**), values in the graphs are presented as individual points in box plots, range min to max. The data was analyzed with one-way repeated measures (RM) ANOVA. Means with dissimilar letters are significantly different ($p<0.05$).

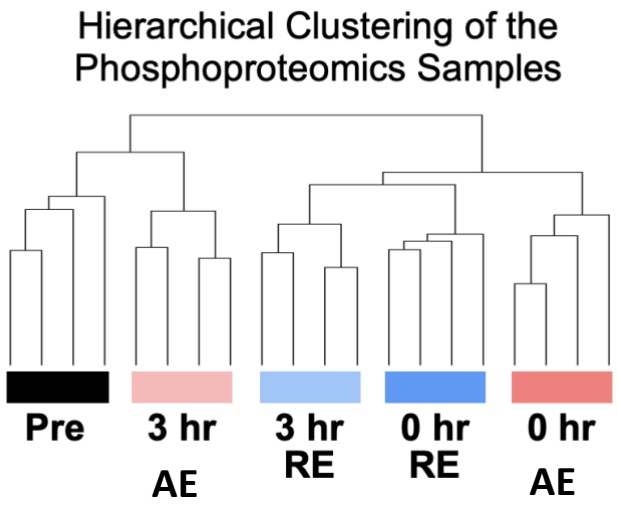
Phosphorylation of Proteins Following Exercise. Altogether, the LC-MS/MS analysis paired with PhosR reliably identified 12899 phosphosites on 3102 proteins, detectable in each participant for each condition (Pre, 0AE, 0RE, 3AE, 3RE). Only 1 protein was quantified as having changed in abundance 3 hours post AE; supporting any change in phosphopeptide abundance with exercise was the result of phosphorylation. We detected 1035 phosphopeptides that were significantly ($q < 0.05$ and $x > |1.5\ FC|$) regulated immediately following exercise vs. Pre. Of these 1035 phosphopeptides, 845 and 419 are regulated immediately post AE and RE, respectively, with 229 phosphopeptides that are commonly regulated between AE and RE. At 3 hours post-exercise, 494 phosphopeptides were significantly regulated ($q < 0.05$ and $x > |1.5\ FC|$) vs. Pre. Of these 494 phosphopeptides, 279 and 327 were phosphorylated at 3 hours post AE and RE, respectively, with 112 phosphopeptides that are commonly regulated between AE and RE. Importantly, we found 85 phosphopeptides that were significantly regulated immediately post RE and remained significantly elevated at 3 hours during recovery. Of these 85 phosphopeptides, 12 were unique to RE only; TTN_(T^{11532}), RCSD1_(S^{268});(S^{284}), SYNM_(T^{598}), HSPB1_(S^{83}), PALLD_(S^{1104}), LIMCH1_(S^{437}), ACTN2_(S^{761}), STAC3_(S^{9}), SUGT1_(T^{265}), ARHGAP1_(S^{51}), FBXO6_(S^{284}), STMN1_(S^{38}). The early and sustained activation of these phosphopeptides may be indicative of their role in signalling for synthesis and degradation of proteins that alter phenotype towards a more RE-specific phenotype (i.e. hypertrophy). Unbiased non-targeted approaches are useful for hypothesis generation, leading to the identification of protein kinase networks that may have important implications in supporting the mechanisms or outcome of an intervention, i.e. growth/atrophy, protein turnover or signalling. The 12 unique phosphopeptides identified here, and others: CRYAB_(S^{19}), HSPB1_(S^{83}), MAPK1_(T^{181}), MAPK3_(T^{202}:Y^{204}), WNK1_(S^{1978}) were therefore postulated to be worth further investigation.

Interestingly, a larger proportion of the identified phosphopeptides were found to be upregulated immediately following RE (302 up:117 down) in comparison to AE (321 up: 524 down). The contrast in phosphopeptide responses between exercise types is also prevalent at 3 hours post-exercise, with a greater amount of phosphopeptides found to be upregulated in recovery (3h) following RE (182 up:145 down) in comparison to AE (63 up: 216 down).

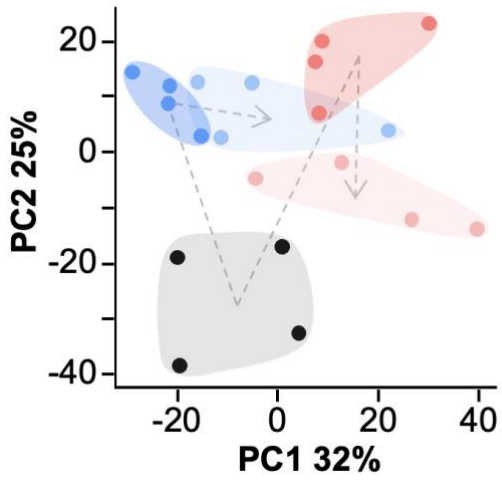
Mfuzz Clustering. Phosphoproteomic data from each subject in cohort 1 underwent unsupervised hierarchical clustering and principal component analysis (PCA). At the subject level, the phosphoproteomic data clusters according to the experimental condition (Ex), which illustrates the distinct impacts that each exercise type had on the phosphoproteome. PCA analysis revealed a large perturbation of the phosphoproteome at both 0AE and 0RE. The large perturbation was maintained at 3RE, whereas a partial return toward the pre-exercise state was observed at 3AE. Soft clustering of the phosphoproteomic data revealed four major clusters of phosphopeptides that were uniquely affected by AE and RE. Specifically, clustering led to the identification of a phosphopeptide group that appeared to be negatively regulated by AE (cluster 1 - pink), as well as a very large cluster of phosphopeptides (cluster 2 – blue) that showed a prolonged elevation in phosphorylation preferentially after RE. Cluster 1 was comprised of 824 phosphopeptides with a membership score ≥0.5, of which 269 were significant ($q<0.05$) at 0AE vs. Pre. Cluster 2 was comprised of 2505 phosphopeptides with a membership score ≥0.5, of which 201 and 54 were significant ($q<0.05$) at 0RE and 3RE, respectively, versus pre-exercise. Cluster 2 gene ontology (GO) Terms consisted of positive regulation of muscle growth and contraction and protein tyrosine kinase activity.


A
Cluster 1: 824 at a score >0.5
0
0.5
1.0
Membership Score
Expression (Z-score)
-2
1
0
-1
-2
Pre
0 hr AE
0 hr RE
3 hr AE
3 hr RE
B
Cluster 2: 2505 at a score >0.5
0
0.5
1.0
Membership Score
-2
1
0
-1
-2
Pre
0 hr AE
0 hr RE
3 hr AE
3 hr RE
C
Cluster 3: 1170 at a score >0.5
0
0.5
1.0
Membership Score
Expression (Z-score)
-2
1
0
-1
-2
Pre
0 hr AE
0 hr RE
3 hr AE
3 hr RE
D
Cluster 4: 1140 at a score >0.5
0
0.5
1.0
Membership Score
-2
1
0
-1
-2
Pre
0 hr AE
0 hr RE
3 hr AE
3 hr RE

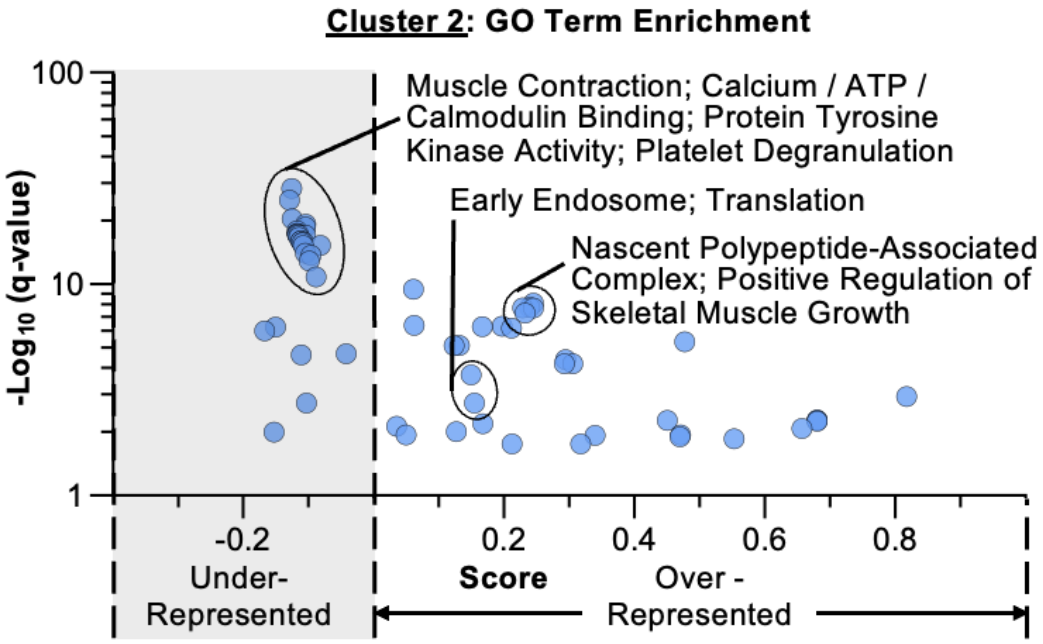
E

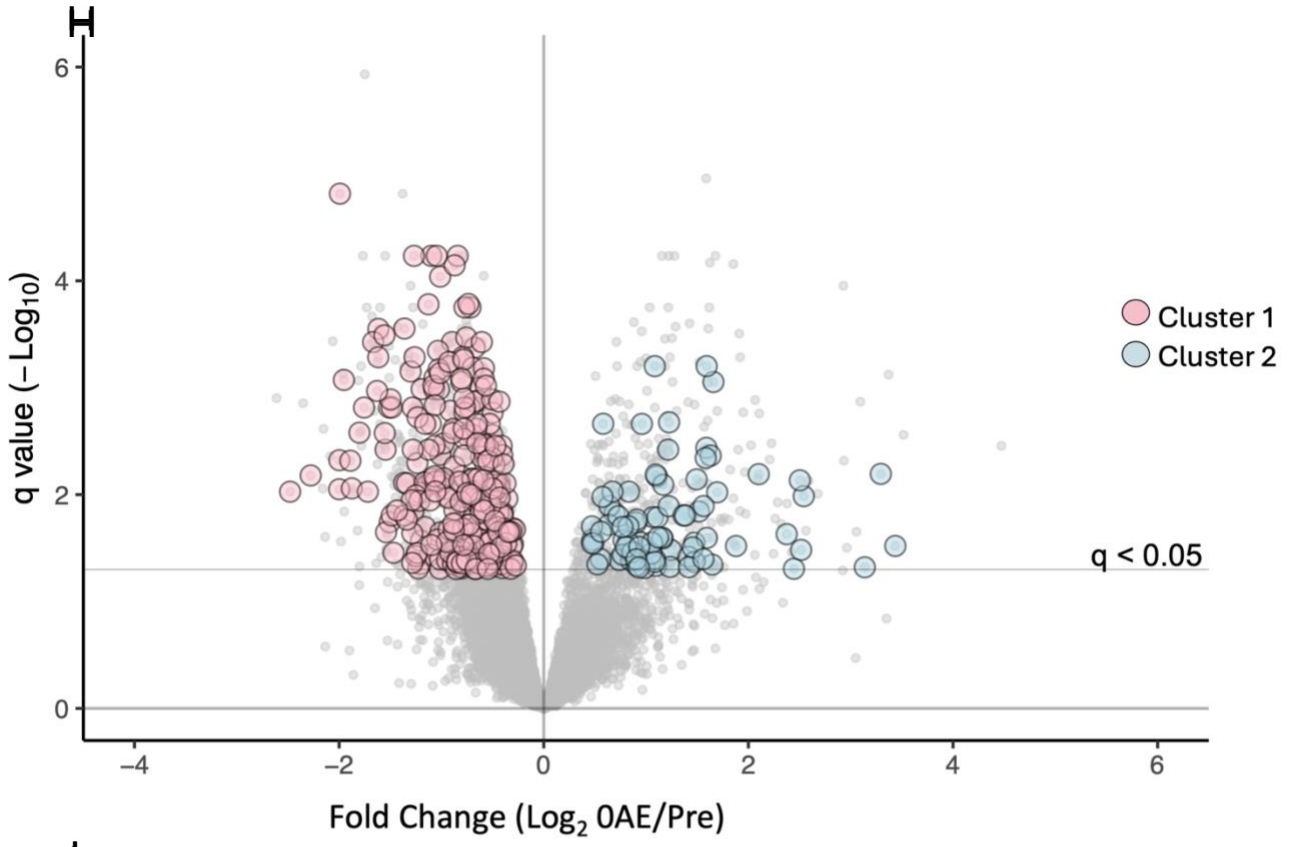

Hierarchical Clustering of the Phosphoproteomics Samples
Pre
3 hr AE
3 hr RE
0 hr RE
0 hr AE

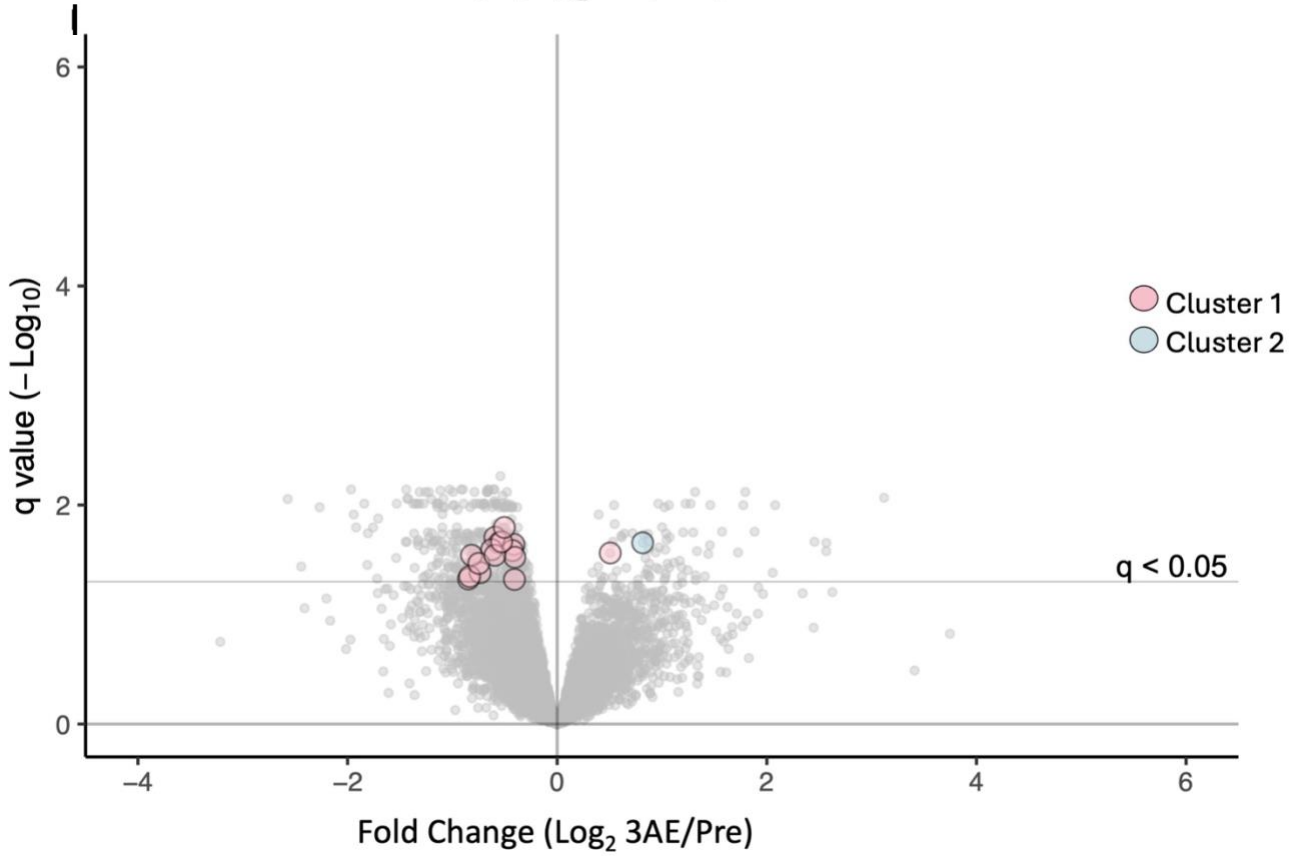
F

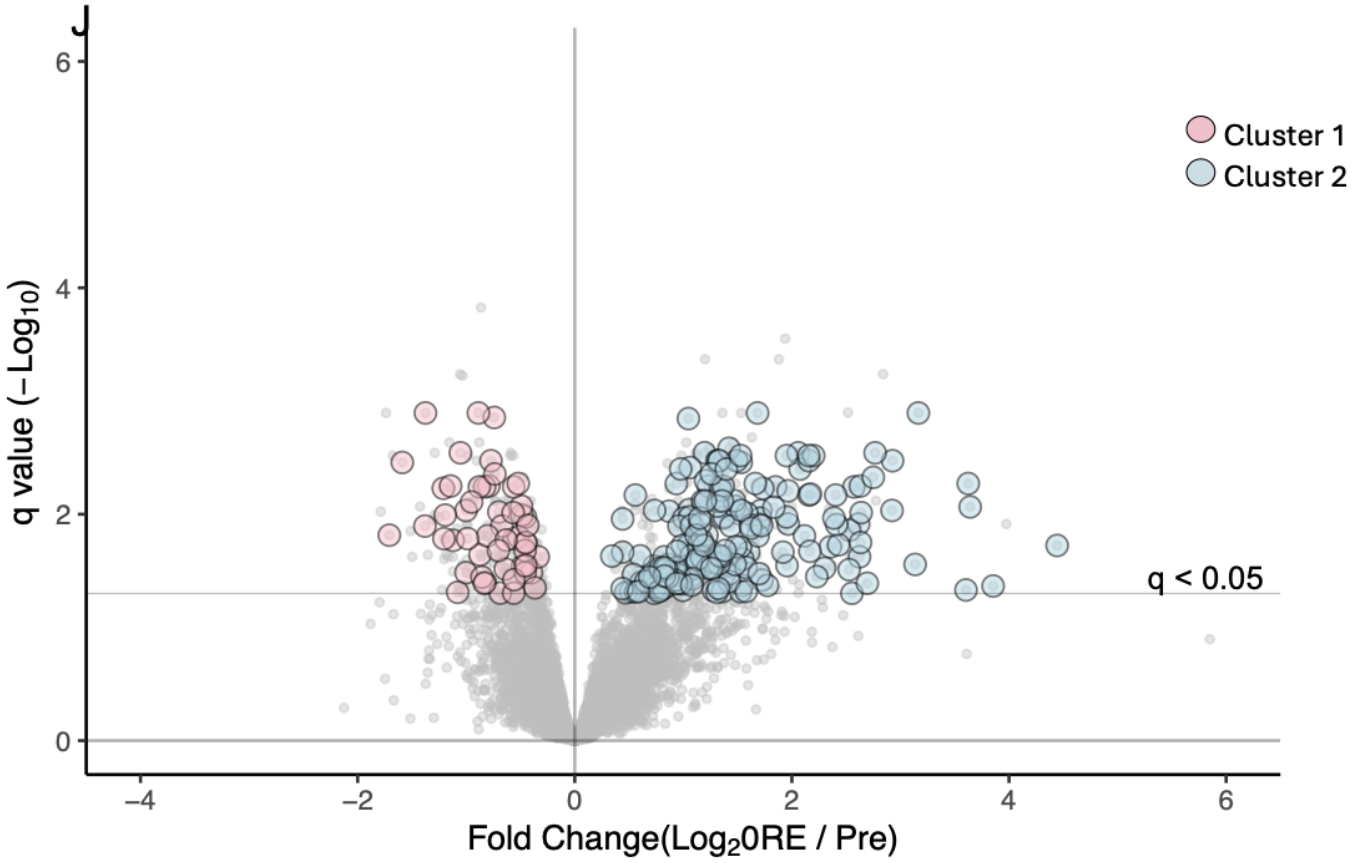

20
0
-20
-40
PC2 25%
-20
0
20
40
PC1 32%

G


Cluster 2: GO Term Enrichment
100
10
1
-Log10 (q-value)
Muscle Contraction; Calcium / ATP / Calmodulin Binding; Protein Tyrosine Kinase Activity; Platelet Degranulation
Early Endosome; Translation
Nascent Polypeptide-Associated Complex; Positive Regulation of Skeletal Muscle Growth
-0.2
0.2
0.4
0.6
0.8
Under-Represented
Score
Over - Represented


H
q value (−Log10)
Fold Change (Log2 0AE/Pre)
Cluster 1
Cluster 2
q < 0.05


I
q value (−Log10)
Fold Change (Log2 3AE/Pre)
Cluster 1
Cluster 2
q < 0.05


J
Cluster 1
Cluster 2
q < 0.05
q value (−Log10)
Fold Change(Log20RE / Pre)

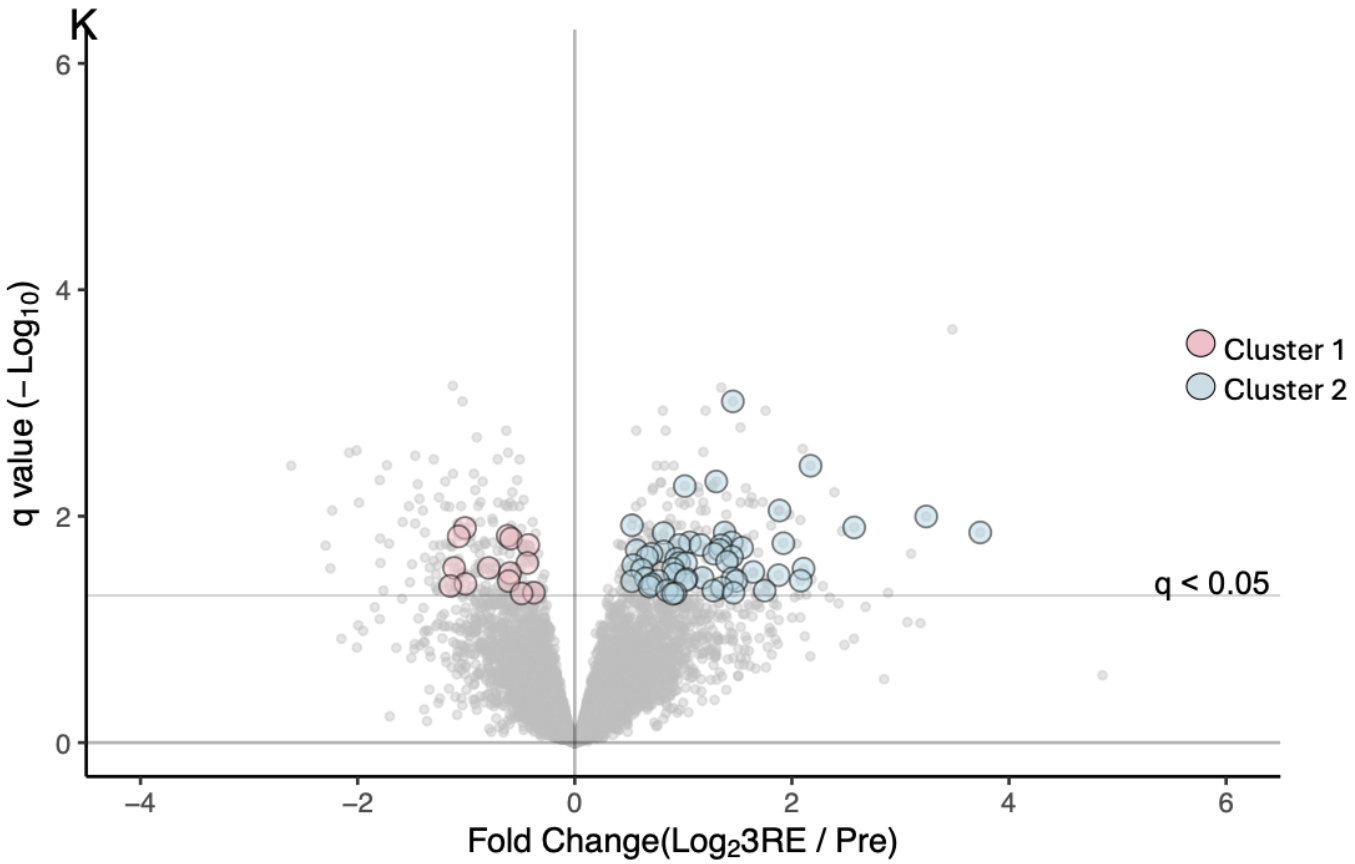

K
Cluster 1
Cluster 2
q < 0.05
q value (−Log10)
Fold Change(Log23RE / Pre)

Figure 2.3: Clustering analysis (A-D) Soft clustering of the phosphoproteomic data revealed four major clusters of phosphopeptides that were affected by exercise. In B, phosphopeptides that had a membership score of ≥ 0.5 for cluster 1 are highlighted in pink, while those with a membership score of ≥ 0.5 for cluster 2 are highlighted in blue. (E) Unsupervised hierarchical clustering of the phosphoproteomic data from each of the subjects (n=4). (F) Principal component analysis of the phosphoproteomic data from each of the subjects. Dots represent the values for each subject and the color of the dot indicates the experimental condition (as described in E). (G) 1D enrichment analysis for GO terms was performed with the cluster 2 membership score for all phosphopeptides. All GO terms were assigned a rank-based score between -1 and 1 with a negative score indicating underrepresentation of the term, and a positive score indicating overrepresentation. In the graphs the score for each term was plotted against the $-\text{Log}_{10}$ of its respective FDR corrected p-value (q). Individual dots indicate GO terms with a q-value of < 0.05. GO terms of interest are highlighted in the graph. (H-K) Volcano plots from the phosphoproteomic analyses for the two conditions (AE and RE) and time points (0 and 3hr). Significantly ($q<0.05$) regulated phosphopeptides are colored from cluster 1 (pink) and cluster 2 (blue).

GO Terms Protein-Associated Phosphopeptides. GO Terms for proteins associated with significantly upregulated phosphopeptides at 0 and 3RE. The top 5 (FDR q-value) ranked GO terms for 0 and 3RE both included actin cytoskeletal organization, cytoskeletal organization, actomyosin structure organization and actin filamin-based process. These GO terms align with the cluster 2 phosphopeptides, supporting the role of RE in inducing a positive regulation of skeletal muscle hypertrophy.

Fold Change Ranked Phosphopeptides and Venn Diagrams. Significantly regulated phosphopeptides at 0- and 3-hours following AE and RE were sorted into Venn diagrams in order to characterize the timing and exercise mode specificity of the phosphopeptide response. These analyses revealed that immediately post- exercise, 229 phosphopeptides were common between AE and RE. Furthermore, 3 hours post-exercise, 112 sites were commonly regulated between AE and RE. The commonly regulated phosphopeptides represent a relatively large proportion of the exercise response at 0 and 3 hours (27% of all 0AE & 55% of all 0RE) (40% of all 3AE & 34% of all 3RE). In contrast, many phosphopeptides regulated following exercise were exclusive to one modality (AE or RE). In total, following AE, 584 phosphopeptides were significantly different from Pre ($q<0.05$ and $x>|1.5FC|$) at 0- and/or 3-hours post-exercise. These 584 phosphopeptides represent ~61.4% of the total exercise induced phosphorylation detected following AE. Similarly, following RE, 294 phosphopeptides were significantly different from Pre ($q<0.05$ and $x>|1.5FC|$) at 0- and/or 3-hours post-exercise. These 294 phosphopeptides represent ~44.5% of the total exercise-induced phosphorylation detected following RE. Interestingly, 85 phosphopeptides were common between 0 and 3 hours post RE. These phosphopeptides demonstrated a rapid (0hours post-exercise) and prolonged (3hours post-

exercise) response to RE. Of those 85 phosphopeptides, 12 were exclusively regulated following RE (does not overlap with AE). The phosphopeptides exclusive to RE are TTN_(T^{11532}), RCSD1_(S^{268});(S^{284}), SYNM_(T^{598}), HSPB1_(S^{83}), PALLD_(S^{1104}), LIMCH1_(S^{437}), ACTN2_(S^{761}), STAC3_(S^{9}), SUGT1_(T^{265}), ARHGAP1_(S^{51}), FBXO6_(S^{284}) and STMN1_(S^{38}). Additionally, 173 sites were common between 0 and 3 hours post AE. Of those 173 phosphopeptides, 57 were exclusively regulated following AE and did not overlap with RE.

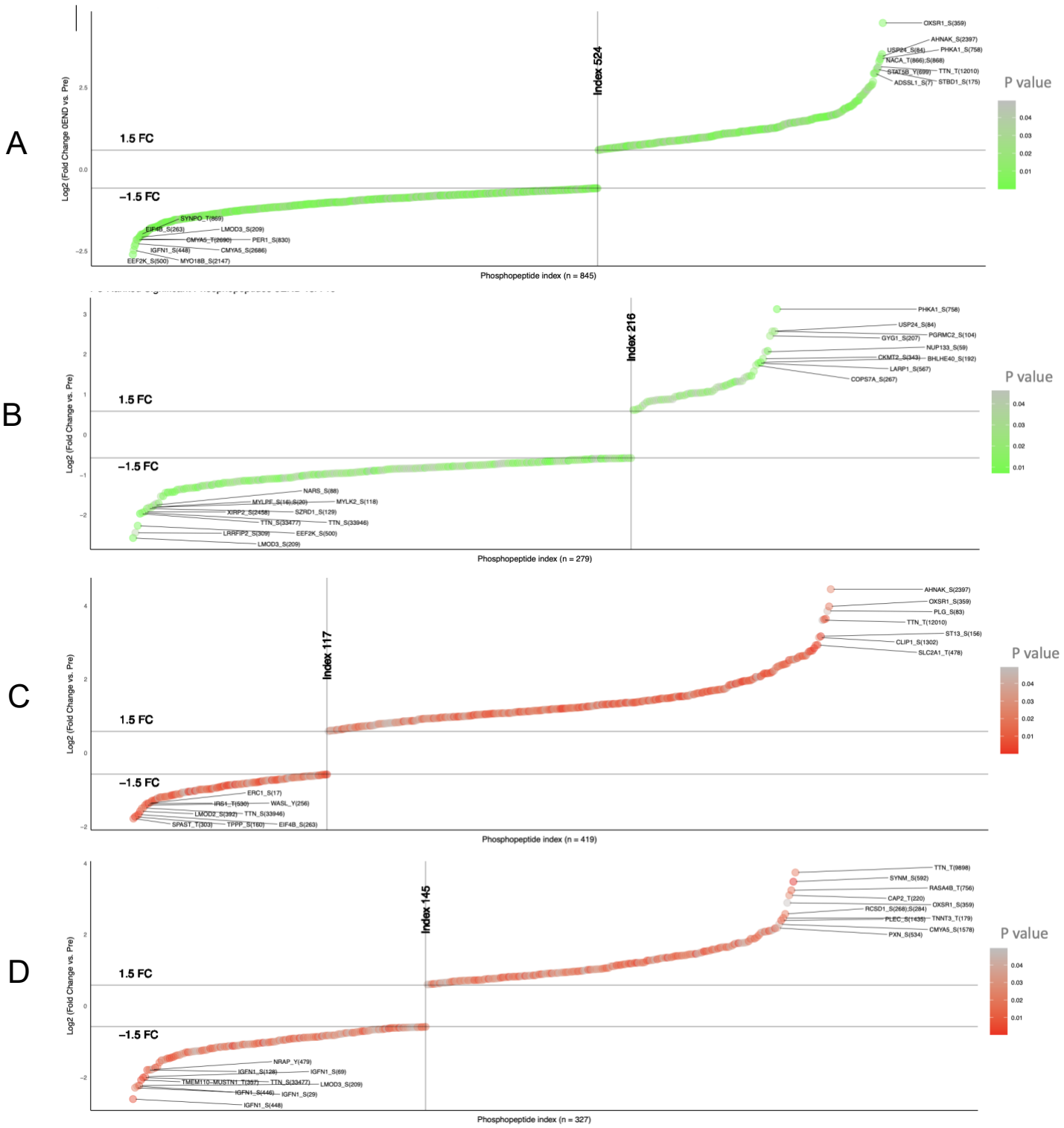
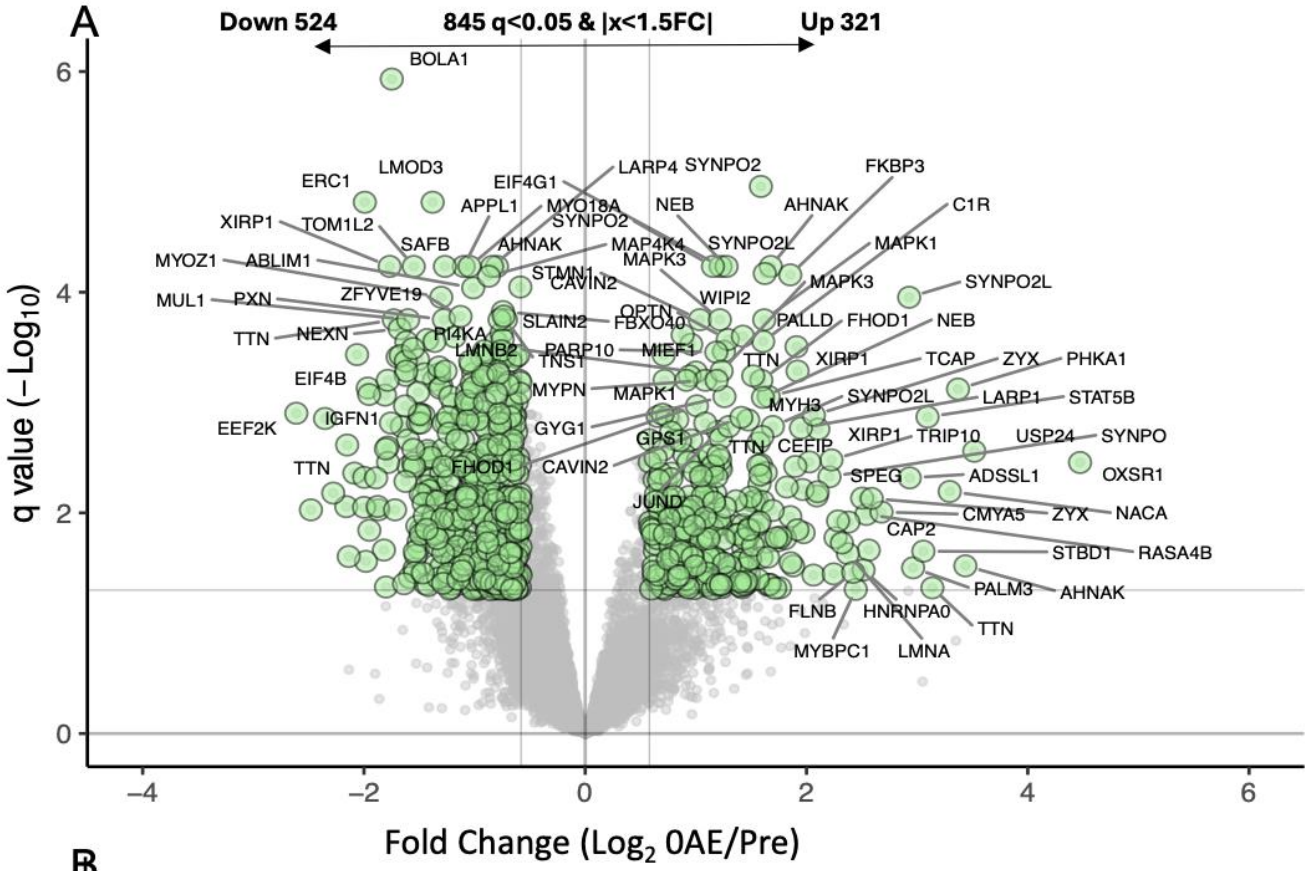
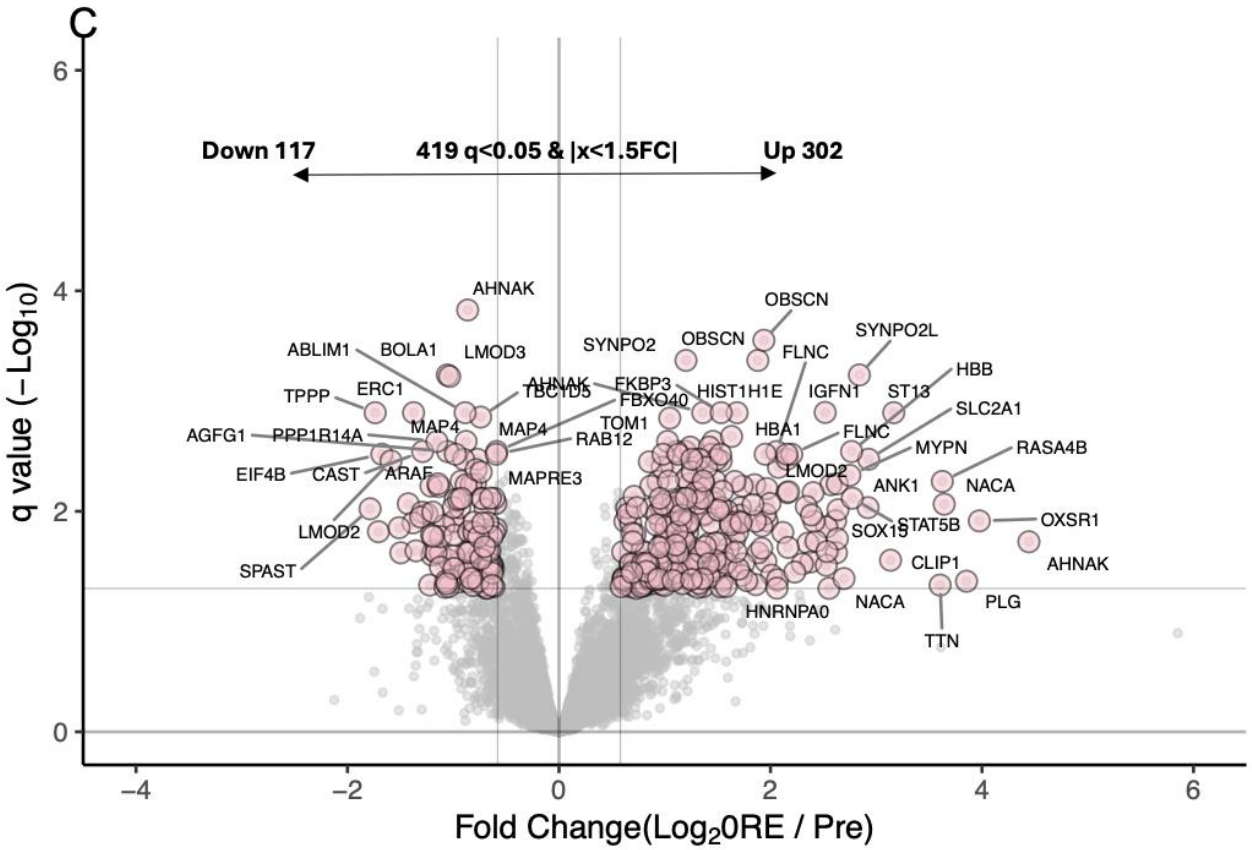


Figure 2.4: Ranked significant phosphopeptides vs. Pre (q<0.05 & x >|1.5 FC|). Significant phosphopeptides ranked as Log2 FC vs. Pre at 0AE (**A**), 0RE (**B**), 3AE (**C**), 3RE (**D**). Level of significance represented in color, n=4.


A
Down 524
845 q<0.05 & |x<1.5FC|
Up 321
q value (−Log10)
Fold Change (Log2 0AE/Pre)
BOLA1
LMOD3
ERC1
EIF4G1
LARP4
SYNPO2
FKBP3
APPL1
MYO18A
NEB
AHNAK
C1R
XIRP1
TOM1L2
SYNPO2
SAFB
AHNAK
MAP4K4
SYNPO2L
MAPK1
MYOZ1
ABLIM1
MAPK3
STMN1
CAVIN2
MAPK3
SYNPO2L
MUL1
PXN
ZFYVE19
WIPI2
OPTN
SLAIN2
FBXO40
PALLD
FHOD1
NEB
TTN
NEXN
PI4KA
LMNB2
PARP10
MIEF1
TTN
XIRP1
TCAP
ZYX
PHKA1
EIF4B
TNS1
MYPN
MAPK1
MYH3
SYNPO2L
LARP1
STAT5B
EEF2K
IGFN1
GYG1
GPS1
TTN
CEFIP
XIRP1
TRIP10
USP24
SYNPO
TTN
FHOD1
CAVIN2
SPEG
ADSSL1
OXSR1
JUND
CMYA5
ZYX
NACA
CAP2
STBD1
RASA4B
PALM3
AHNAK
FLNB
HNRNPA0
TTN
MYBPC1
LMNA
B
Down 216
279 q<0.05 & |x<1.5FC|
Up 63
q value (−Log10)
Fold Change (Log2 3AE/Pre)
ATAD1
PDHA1
AKT1S1
C1R
LARP1
CLU
LMOD3
TTN
PNISR
OBSCN
BHLHE40
UBA2
PHKA1
DENND1A
GYG1
MLX
CKMT2
GYG1
EEF2K
TTN
CLINT1
ALS2
KNG1
PXN
PGRMC2
LRRFIP2
PALLD
FABP3
SYNM
COPS7A
USP24
MYOM1
TTN
NEB
NUP133
CPNE3
TTN
DPYSL3

E

G


C
Down 117
419 q<0.05 & |x<1.5FC|
Up 302
q value (−Log10)
Fold Change(Log20RE / Pre)
AHNAK
OBSCN
SYNPO2L
ABLIM1
BOLA1
LMOD3
SYNPO2
OBSCN
FLNC
HBB
TPPP
ERC1
AHNAK
TBC1D5
FKBP3
FBXO40
HIST1H1E
IGFN1
ST13
SLC2A1
AGFG1
PPP1R14A
MAP4
MAP4
TOM1
RAB12
HBA1
FLNC
MYPN
RASA4B
EIF4B
CAST
ARAF
MAPRE3
LMOD2
ANK1
NACA
LMOD2
STAT5B
OXSR1
SOX15
SPAST
CLIP1
AHNAK
HNRNPA0
NACA
PLG
TTN

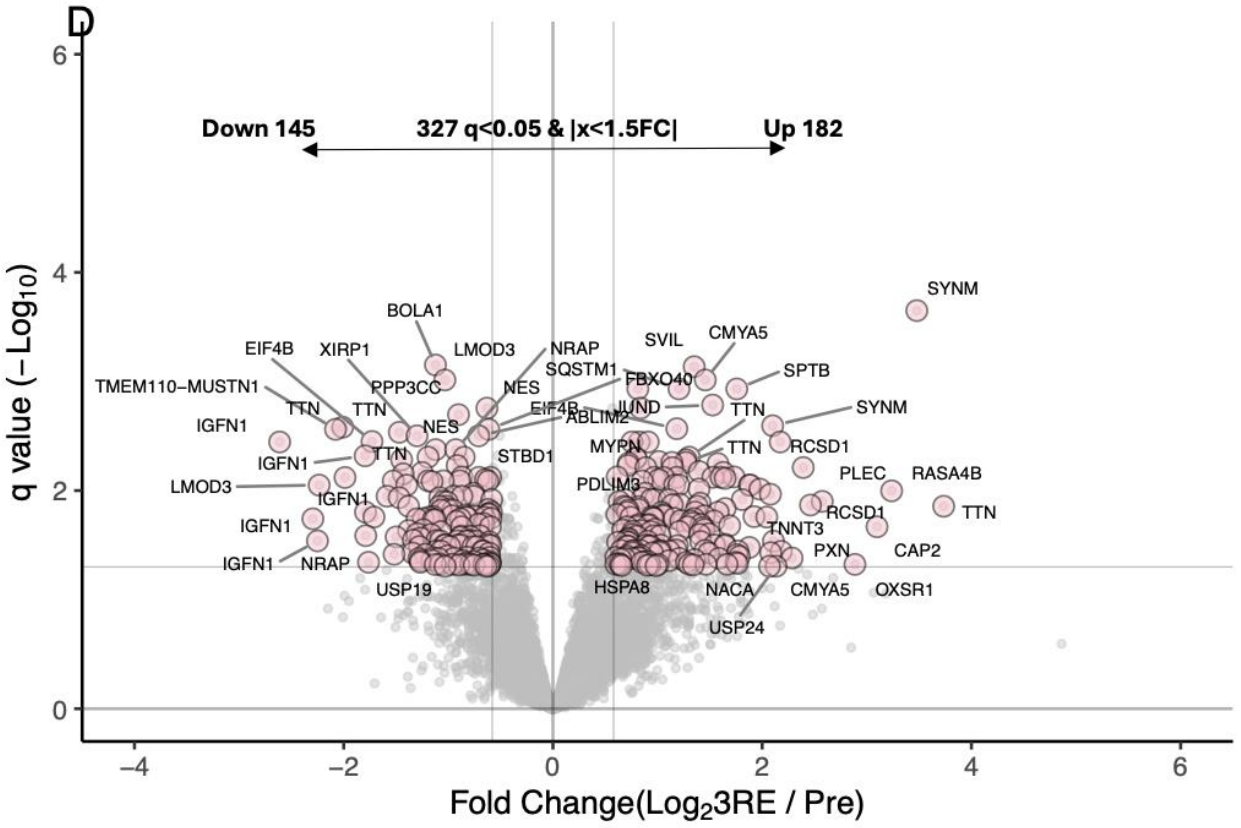

D
Down 145
327 q<0.05 & |x<1.5FC|
Up 182
q value (−Log10)
Fold Change(Log23RE / Pre)
SYNM
BOLA1
SVIL
CMYA5
EIF4B
XIRP1
LMOD3
NRAP
SQSTM1
FBXO40
SPTB
TMEM110−MUSTN1
PPP3CC
NES
EIF4B
JUND
TTN
SYNM
IGFN1
TTN
TTN
NES
ABLIM2
MYPN
TTN
RCSD1
IGFN1
TTN
STBD1
PLEC
RASA4B
LMOD3
PDLIM3
IGFN1
RCSD1
TTN
IGFN1
TNNT3
IGFN1
NRAP
PXN
CAP2
USP19
HSPA8
NACA
CMYA5
OXSR1
USP24

Figure 2.5: Significantly regulated phosphosites (q<0.05) & x >|1.5 FC|) (**A-D**) Significantly regulated phosphopeptides with a q-value of < 0.05 and greater than 1.5 FC vs. Pre. (**E-H**) Venn diagrams of significantly regulated phosphopeptides in response to AE and RE that are common and uniquely regulated by each exercise modality at 0 and 3 hours. (**I-J**) GO Term biological process Top 5 (FDR ranked) pathway enrichment analysis of proteins uniquely phosphorylated for significantly regulated phosphopeptides (q<0.05) & x >|1.5 FC|) upregulated vs. Pre at 0 and 3 hours post RE, GO data generated from String-db.org, n=4.

Western Blot Results and Correlations with MPS. Phosphoproteomic analyses of cohort 1 (n=4) revealed potential kinase-substrate signalling pathways that may be associated with RE and the subsequent rise in myoPS. We chose several of these kinases to undergo targeted Western blotting analyses in cohort 2 (n=12) to characterize their role in elevating protein synthesis in the 0-to-3-hour period following RE and AE. Simple linear regression analyses of the % change (from Pre) in the ratio of phosphorylated to total protein (P:T protein) and myoPS revealed several significant ($p < 0.05$) relationships, providing evidence for the role of the predicated kinase-substrate signalling pathways found to be significantly regulated in cohort 1 following RE. Specifically, the ratio of phosphorylated-to-total (P:T) protein immediately following RE in phosphopeptides p70_(T^{389}), MK2_(T^{222})S, MK2_(T^{334})S, MKK3_(S^{189}), MKK4_(S^{257}) and MKK6_(S207) all demonstrated significant ($p <0.05$) relationships with myoPS (% change from Pre). Furthermore, the ratio of P:T at 3 hours following RE in phosphopeptides MK2_(T^{222})S, MK2_(T^{222})L, HSPB1_(S^{82}) and MKK3_(S^{189}) all demonstrated significant ($p <0.05$) relationships with myoPS (% change from Pre).

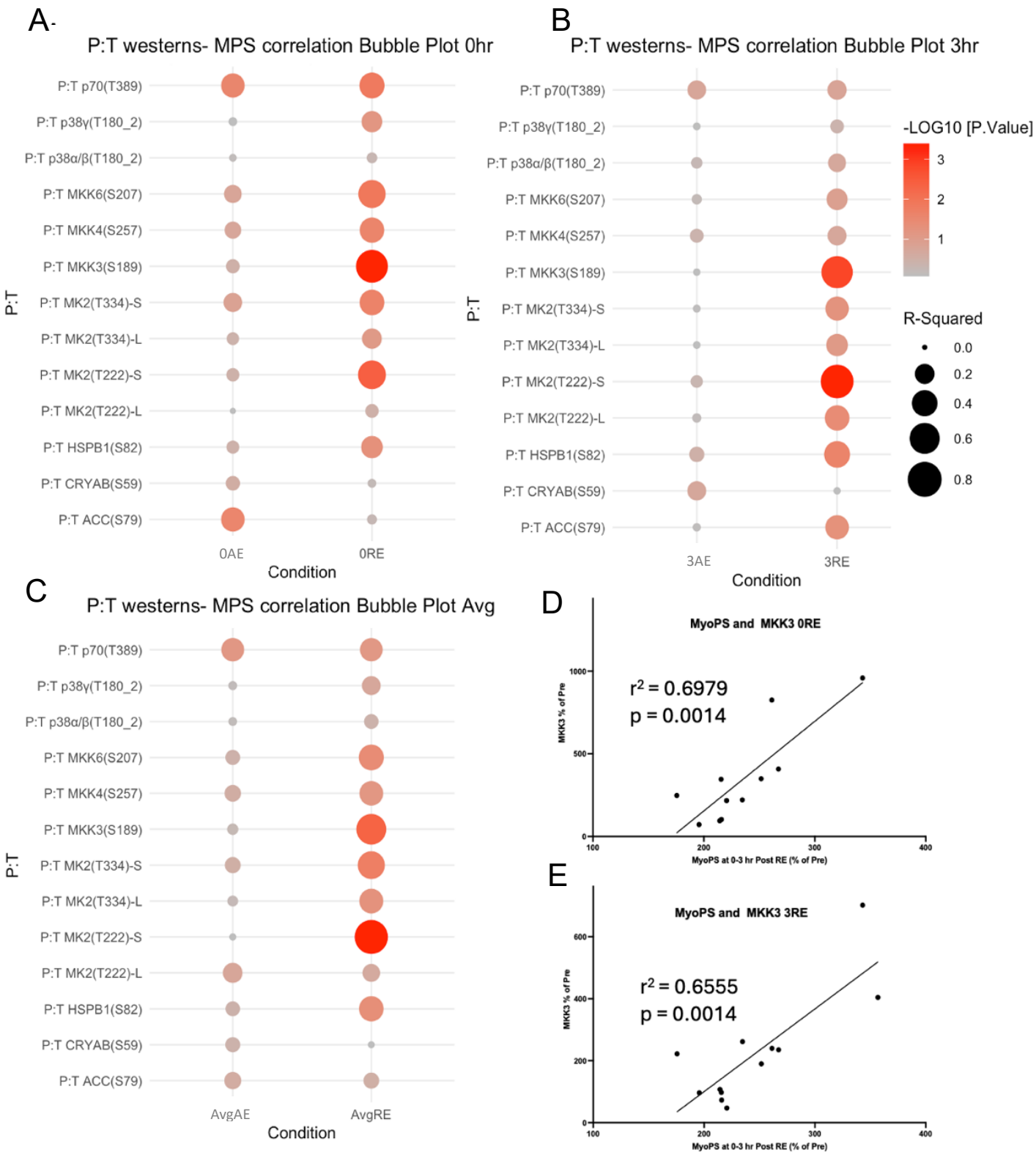


Figure 2.6: Summary of linear regression analysis of MyoPS and P:T protein following targeted analysis (Western) (**A**) 0hours, (**B**) 3 hours and (**C**) 0- and 3-hours average, following exercise, (n=12). (**D, E**) Linear regression and scatter plot of P:T MKK3(S189) % of Pre plotted against the 0-3hr myoPS post RE as a % of Pre, (n=12).

Discussion

We utilized phosphoproteomics and protein synthetic responses to assess the perturbation in muscle homeostasis and signalling events following AE and RE. A primary aim was to characterize the changes that occurred in phosphorylation signalling in relation to the change in protein fractions being synthesized between 0- and 3-hours post-exercise. Specifically, we hypothesized that there would be several phosphopeptides predominantly regulated by RE and associated with the post-exercise rise in myoPS. As such, participants performed unilateral AE and RE type exercises in randomized order following an overnight fast. RE and AE caused myoPS to be elevated 146 ± 53% and 51 ± 30%, respectively, for the 0–3-hour time period following exercise. RE and AE also induced an elevation in sarcoPS by 82 ± 51% and 123 ± 115%, respectively, for the 0–3-hour time period following exercise. An advantage of our study is the unilateral design that allowed for within-subject comparisons of each exercise modality without the introduction of confounding variables such as diet or other daily lifestyle factors (i.e. circadian rhythm, activity status, hydration, etc.) on the day of, and leading up to, the experimental trial.

We identified 12899 phosphosites on 3102 proteins across each time point and condition (Pre, 0AE, 0RE, 3AE, 3RE), representing the largest compendium of exercise-regulated phosphorylation sites to date. We further performed downstream analyses to identify phosphopeptides common and unique to each exercise modality. Characterization of phosphopeptides regulated specifically by AE or RE may be important for understanding the distinct regulation of protein turnover and divergent phenotypic adaptation with chronic exercise. In the phosphoproteomic data, 85 phosphopeptides were found to be significantly regulated

following RE at both 0 and 3 hours, 12 of which were exclusive to RE (did not overlap with AE). The identification of phosphopeptides and associated kinases, which can be linked with a robust elevation in myoPS following RE but not AE, has the potential to reveal RE-specific signalling mechanisms underlying various exercise responses relevant to muscle adaptation and growth. To date, protein dynamics (turnover) occurring following exercise has largely been studied in the context of bulk synthesis and degradation. In future, measuring the turnover rates of individual proteins over a longer duration (days – weeks) in addition to regulation of phosphopeptides can advance our understanding of how acute signalling events influence exercise adaptation and better predict exercise training outcomes.

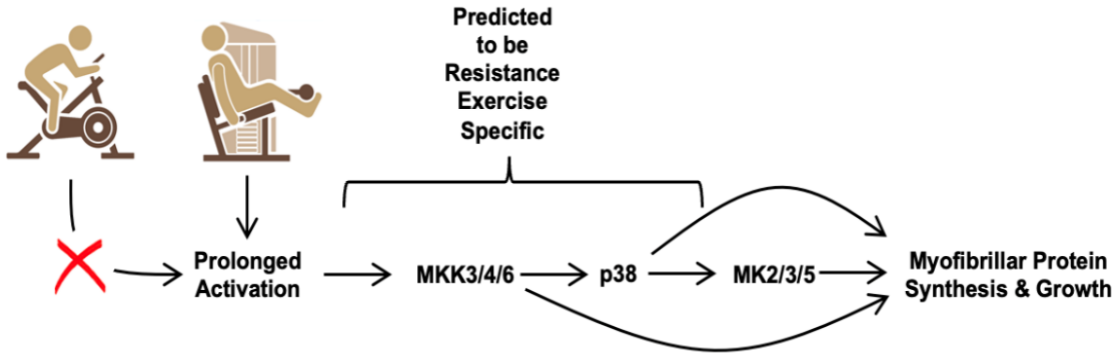


Fig 2.7: Overview of predicted RE specific pathway that induces an increase in myofibrillar protein synthesis and muscle cell hypertrophy.

Currently, several canonical phosphopeptides are implicated as primary mediators in the signalling and mechanisms of adaptation to exercise (Coffey, Zhong et al. 2006). Yet, the mechanisms that link muscle contraction and the metabolic perturbations as a result of exercise to signalling and downstream outcomes (i.e. changes in protein content/ function) are largely

unknown. For example, protein kinases such as p70, AKT, mTOR, AMPK and p38 MAPK are all implicated as having important roles in signalling for adaptation during recovery from exercise. However, current knowledge of their role in muscle signalling does not explain how different phenotypes emerge from persistent training of either AE or RE. Therefore, other key kinase-substrate relationships must exist that underlie the dichotomous outcomes of AE and RE training. These key kinases may be found by examining phosphopeptides regulated primarily by one modality of exercise (i.e. AE or RE). Our investigation revealed 294 phosphosites that demonstrated a significant regulation of phosphorylation following RE but not AE ($q<0.05$ vs. Pre at 0 or 3 hours and does not overlap with AE). Specifically, phosphopeptides such as CRYAB_(S^{19}), BAG3_(T^{62}), EIF4B_(S^{500}), IGFN1_(S^{787}), WNK1_(S^{1978}) and HSPB1_(S^{83}) all demonstrated a RE specific response and could therefore contribute toward mechanisms of signalling transcription or translation that induces dichotomous protein expression for different training modalities, eliciting unique (hypertrophic) muscle phenotypes.

In alignment with the current data, Blazev et al. (2022) found HSPB1_(S^{82}) to likely be regulated (thought to be involved in intracellular trafficking) and classified as a top 25 canonical phosphosite following exercise (AE, sprint and RE) (Blazev, Carl et al. 2022). Similarly, PRKACA (protein kinase A catalytic subunit) potentiation during recovery from RE was also observed in the current data set, as well as Blazev (2022) (Blazev, Carl et al. 2022). Activation of PRKACA may be involved in the regulation of lipid and glucose metabolism and is a component of the signal transduction mechanism of certain GPCRs (Stratakis 2018). Additionally, kinase prediction analyses in both the current data set and Blazev et. al (2022) identified >10 proteins that are commonly regulated in recovery from RE between the datasets. Of these proteins, MK2

(MAPKAPK2), MK3 (MAPKAPK3) and MK5 (MAPKAPK5), in particular, were identified as being well aligned with the post-RE rise in myoPS. Also consistent with previous phosphoproteomics kinase activity predictions are the prolonged activation of MK2_(T^{222}) and MKK3_(S^{189}), which suggests these kinase-substrate relationships are likely important in the regulation of muscle protein synthesis and adaptation with exercise training. Unpublished data from murine exercise models support the hypothesis-generating work of cohort 1 and the targeted analyses of cohort 2, demonstrating a novel and key potential pathway in the RE-induced regulation of muscle proteins. The murine model data provided to us from Prof. T. Hornberger's lab were able to recapitulate our human-generated results. The murine data supported the notion that divergent exercise types elicit unique metabolic perturbance and signalling, inducing dichotomous protein synthesis and phenotypic outcomes (data not shown).

Clustering analysis from our pilot cohort led to the identification of 4 unique clusters that responded to AE and RE. Of interest were clusters 1 and 2. Cluster 1 comprised 824 phosphopeptides (membership score >0.5) and tended to respond negatively immediately following AE. Cluster 2, comprised of 2505 phosphopeptides (cluster 2), responded positively following RE and was persistently elevated (0 and 3 RE). Interestingly, in cluster 2, there is an over-representation of phosphorylation events occurring on proteins that are annotated with GO terms for translation initiation and the positive regulation of skeletal muscle growth. This observation is noteworthy because it suggests that the phosphorylation events in this cluster are likely involved with stimulating the increase in myoPS and hypertrophy in response to chronic RE. Characterization of clusters/families of kinases that are regulated uniquely following AE versus RE could lead to the identification of key proteins responsible for muscle adaptation.

In addition to investigating the specificity of the phosphorylation response to exercise type, we found the regulation of phosphopeptides post-exercise is largely generic in our untrained (recreationally active) participants, with many common points of regulation shared between AE and RE in the recovery period post-exercise. In the phosphoproteomic data, for example, the following were found to be significantly regulated following both AE and RE (0 and 3h): VDAC_(S^{127}), voltage-dependant anion challenge (VDAC), which aids in the regulation of metabolic and energetic function of mitochondria. Similarly, BOLA1_(S^{81}) acts as a mitochondrial iron-sulphur (Fe-S) cluster assembly factor that facilitates (Fe-S) cluster insertion into a subset of mitochondrial proteins and may protect cells against oxidative stress. Also, EIF4B_(S^{263}) and EIF4G1_(S^{1160}) were commonly regulated and are involved in exercise-induced translation initiation. Together, these phosphopeptides may contribute toward acutely regulating common aspects of metabolism perturbed by AE and RE or contribute towards signalling for synthesis/ degradation of a commonly regulated protein.

We also performed Western blot analysis on several phosphopeptides predicted to be involved in the regulation of myoPS following RE by correlating the ratio of phosphorylated:total protein to the elevation in myoPS 0-3 hours following exercise. Several phosphopeptide ratios (phosphorylated:total) were significantly correlated ($p<0.05$) with the % change (from Pre) in myoPS following RE but not AE. MKK3_(S^{189}) in particular demonstrated robust associations with myoPS, r^2 =0.69 and r^2 =0.65 for 0RE and 3RE, respectively. Importantly, the post-exercise change in MKK3_(S^{189}) was more closely associated with myoPS than p70_(T389) (average 0-3 hours), which only demonstrated a low-modest (r^2 =0.30) but non-significant (p=0.06)

association with myoPS. The identification of phosphopeptides that are primarily associated with RE and demonstrate a significant relationship with the post-exercise rise in myoPS has potential to elucidate key previously unrecognized components in the signalling mechanisms of protein transcription and translation that are responsible for muscle hypertrophy.

Conclusion

In summary, our study led to the identification of a novel signalling network, primarily regulated by RE and associated with the post-exercise rise in myoPS. Figure 7 demonstrates an overview of the proposed novel pathway that is likely to be responsible for the RE-induced elevation of protein synthesis. Additionally, we are the first to demonstrate phosphopeptide signalling in response to both AE and RE in males and females. Lastly, we demonstrate the widespread phosphorylation (12899 sites) of peptides (3102 proteins) both shared and unique to AE and RE.

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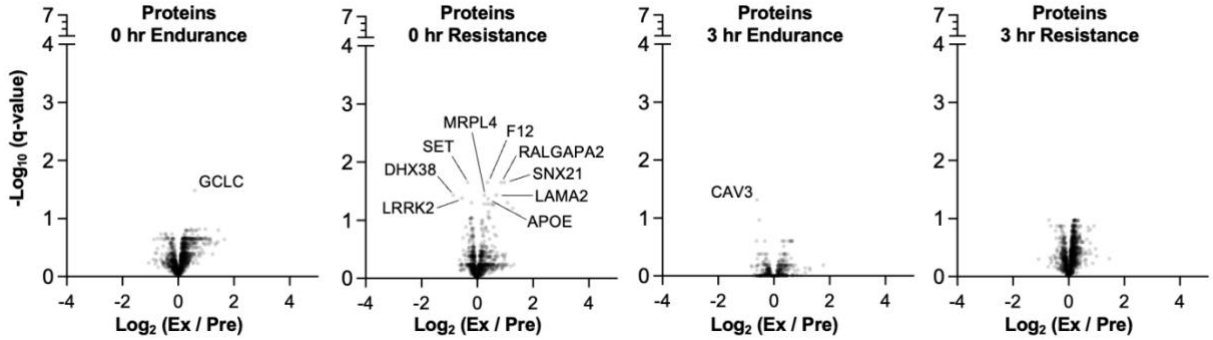
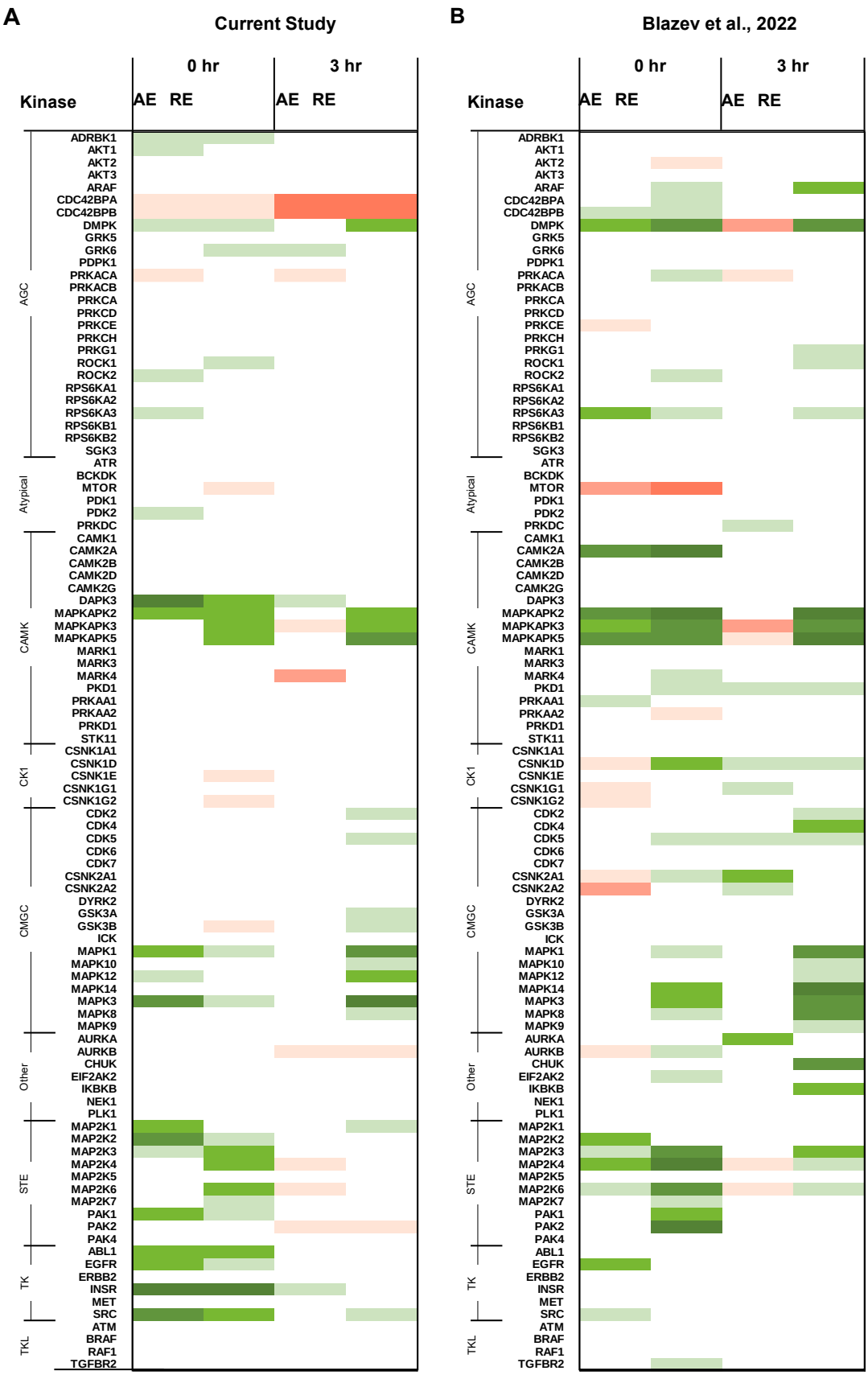
Chapter 2 Supplemental figures and info



Supplemental Figure 2.1: Volcano plots from the proteomic analyses and a list of the proteins that experienced a significant alteration in content at 0 hr or 3 hr post aerobic or resistance exercise, $q \leq 0.05$.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Current Study
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3 hr
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AKT1
AKT2
AKT3
ARAF
CDC42BPA
CDC42BPB
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GRK5
GRK6
PDPK1
PRKACA
PRKACB
PRKCA
PRKCD
PRKCE
PRKCH
PRKG1
ROCK1
ROCK2
RPS6KA1
RPS6KA2
RPS6KA3
RPS6KB1
RPS6KB2
SGK3
ATR
BCKDK
MTOR
PDK1
PDK2
PRKDC
CAMK1
CAMK2A
CAMK2B
CAMK2D
CAMK2G
DAPK3
MAPKAPK2
MAPKAPK3
MAPKAPK5
MARK1
MARK3
MARK4
PKD1
PRKAA1
PRKAA2
PRKD1
STK11
CSNK1A1
CSNK1D
CSNK1E
CSNK1G1
CSNK1G2
CDK2
CDK4
CDK5
CDK6
CDK7
CSNK2A1
CSNK2A2
DYRK2
GSK3A
GSK3B
ICK
MAPK1
MAPK10
MAPK12
MAPK14
MAPK3
MAPK8
MAPK9
AURKA
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MAP2K5
MAP2K6
MAP2K7
PAK1
PAK2
PAK4
ABL1
EGFR
ERBB2
INSR
MET
SRC
ATM
BRAF
RAF1
TGFBR2
AGC
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Blazev et al., 2022
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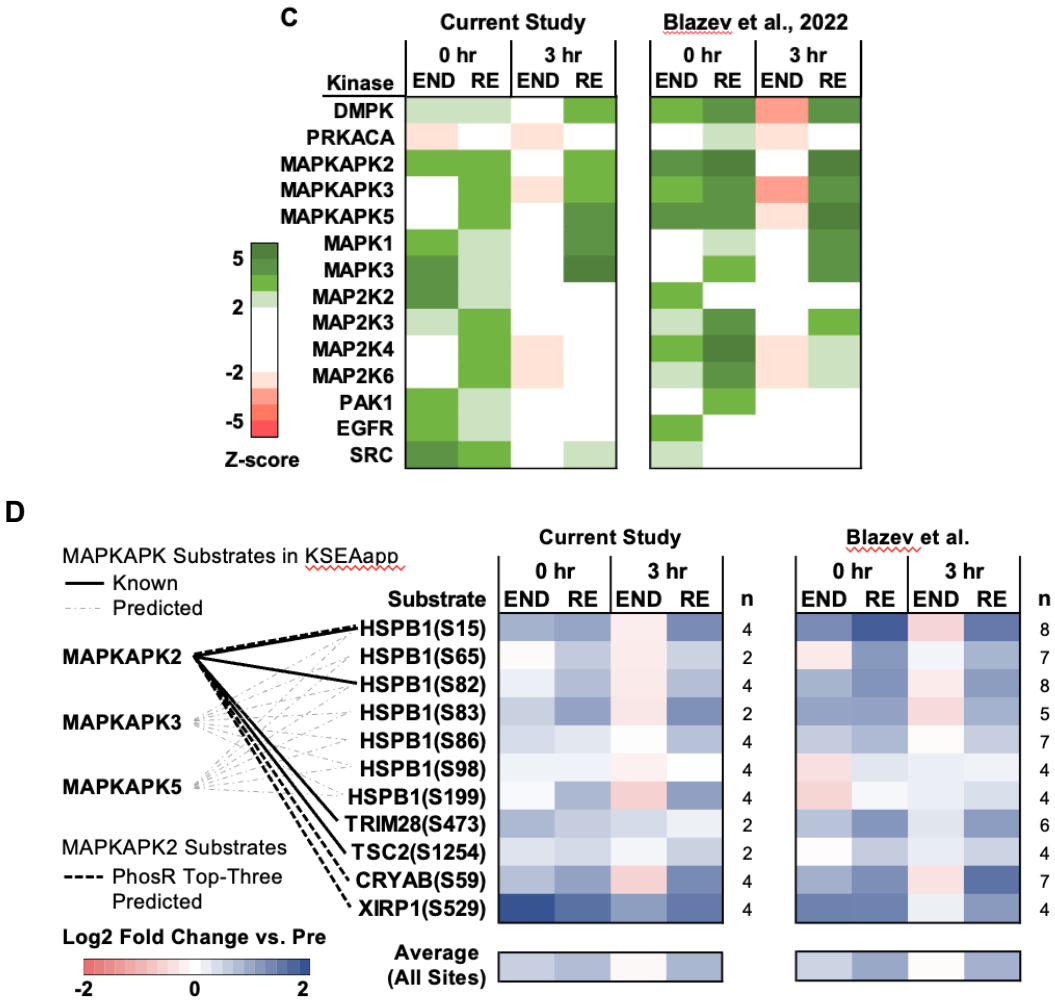
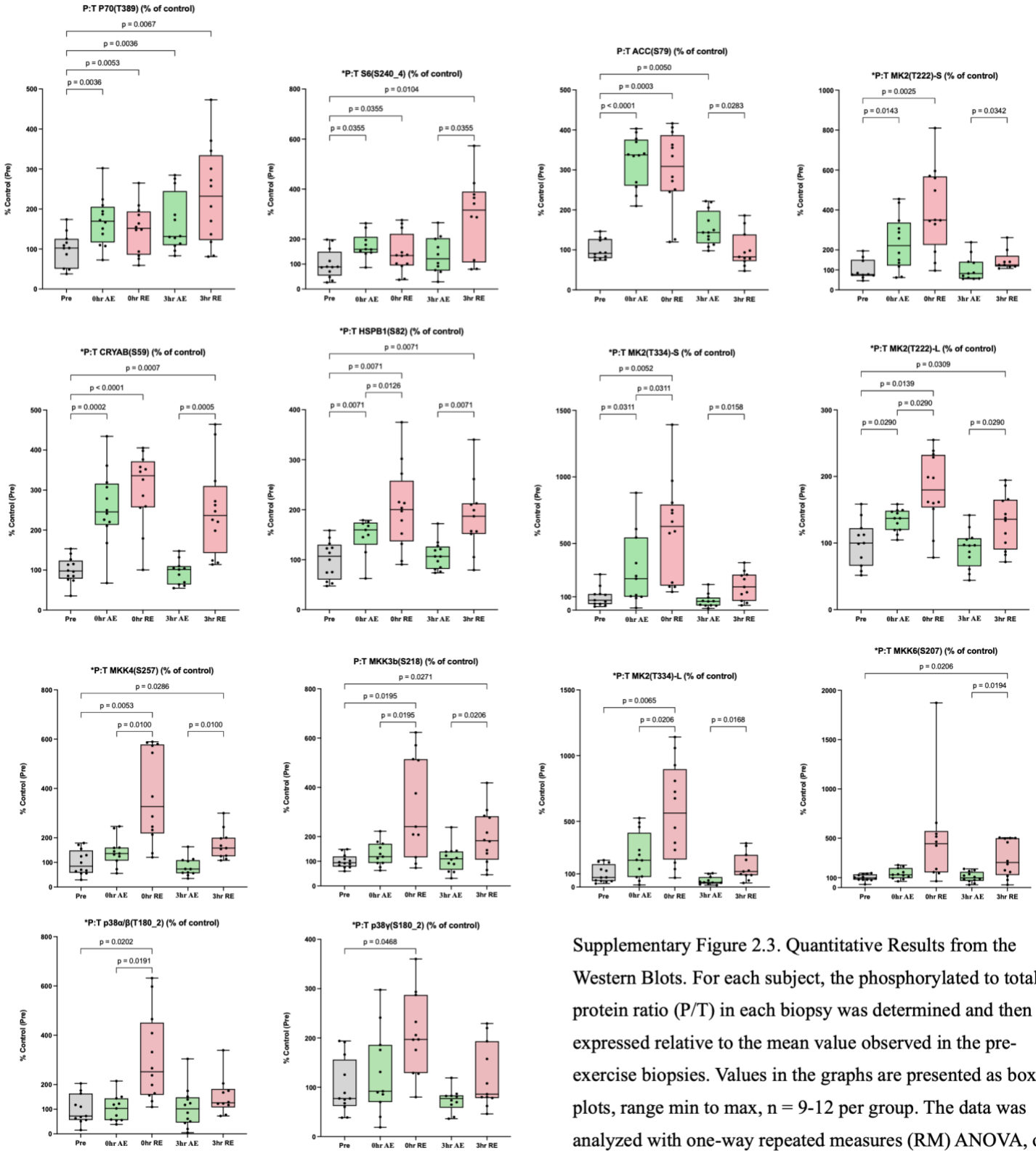
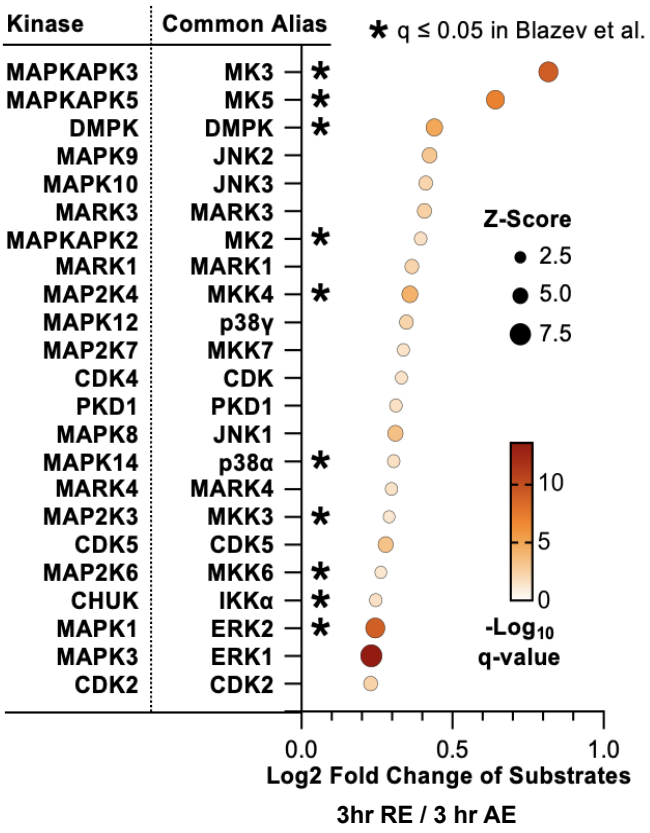


Supplemental Figure 2.2: (**A**) Heat map of Z-scores for the inferred alterations in kinase activity at 0 hr or 3 hr post aerobic (AE) or resistance (RE) exercise when compared to the pre-exercise state. Results from the current phosphoproteomic dataset (as shown in Figure 4) are compared with (**B**) results that were derived from identical processing of the phosphoproteomic dataset published by Blazev et al., 2022. The gene names for all kinases expressed in skeletal muscle that possessed at least 5 known and/or predicted substrates in the phosphoproteomic dataset are listed. Kinases are clustered according to their group (e.g., AGC) and then listed in alphabetical order. (**C**) Heat maps of the Z-scores for the kinases whose activity was inferred to be significantly altered ($q \leq 0.05$) in at least one condition in both the Blazev et al., 2022 dataset and the current dataset. (**D**) Reproducibly of the Aerobic and Resistance Exercise-Induced Changes in the Phosphorylation of Putative MAPKAPK Substrates. Heat maps of the mean exercise-induced change in the phosphorylation of the known and predicted substrates of the MAPKAPK's as derived from the current phosphoproteomic dataset and that of Blazev et al., 2022. Also shown is the number of subjects (n) that the mean values were obtained from in each dataset.



Supplementary Figure 2.3. Quantitative Results from the Western Blots. For each subject, the phosphorylated to total protein ratio (P/T) in each biopsy was determined and then expressed relative to the mean value observed in the pre-exercise biopsies. Values in the graphs are presented as box plots, range min to max, n = 9-12 per group. The data was analyzed with one-way repeated measures (RM) ANOVA, or one-way mixed ANOVA as indicated above each graph.



Supplementary figure 2.4. Prediction and Validation of a Signaling Pathway that is Activated Specifically by RE. Multivariable plot with Z-scores, q-values, and the mean differences in the phosphorylation of the known and predicted substrates for each kinase that was inferred by KSEAapp to have a significant difference in activity at 3 hr post aerobic (3 hr AE) vs. 3 hr post resistance (3 hr RE) exercise. The list includes the gene name of each kinase as well as it's common alias. Also highlighted with a * are kinases that were inferred to have significant ($q \leq 0.05$) differences in activity at 3 hr AE vs. 3hr RE as determined from the phosphoproteomic dataset published by Blazev et al., 2022

CHAPTER 3

The dynamic proteomic response to aerobic and resistance training in human skeletal muscle.

Chapter 3 – The dynamic proteomic response to aerobic and resistance training in human skeletal muscle.

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Keywords

Protein turnover, Aerobic, Resistance, Exercise

Abstract

Exercise induces numerous changes in skeletal muscle phenotype dependent on the loading pattern of the exercise stimulus. We sought to determine the divergent changes in the skeletal muscle proteome induced earlier and later in a training program using both resistance (RE) and aerobic exercise training (AE), in parallel, in men and women. We investigated muscle adaptations to AE training versus RE training during the early untrained versus trained state using novel deuterium oxide labelling and proteomic techniques. In a within-subject longitudinal design, 14 (8F/6M) healthy individuals (20± 1y, body mass: 70±10 kg) completed 10-wks of thrice weekly unilateral resistance (RT: 3 sets × 10-12 reps 80% 1-repetition maximum (RM) leg press and leg extension) and unilateral aerobic (AT: 4 × 5 min one-legged cycling at 65% W_{max}) training. Biopsies were taken at the start and end of a 1-week free-living period prior to training (Baseline), the 1st week of training (acute – AC), and the 10th week of training (chronic – CR). Participants consumed deuterium oxide across all 3 study periods. Muscle samples (n = 14 individuals, 6M/8F) were analyzed by liquid chromatography-tandem mass spectrometry. Significant differences ($p<0.05$) were investigated using bioinformatic analyses, which allowed for the quantification of 41 and 53 protein synthesis rates after 1 and 10 weeks of training, respectively, to complement the analysis of 474 protein abundances. Our data illustrate the common and unique networks of protein regulation following AE and RE. Specifically we highlight the influence both AE and RE have on mitochondrial proteins, likely aimed at improving the metabolic function and possibly underpinning an increase in aerobic capacity. In both AE and RE, we identified changes in protein abundance that do not align with individual protein synthetic rates, suggesting that targeted degradation of certain proteins may be an adaptive feature of the shared response to aerobic and resistance training. To date, this work

represents the most in-depth analysis of protein-specific fractional synthesis rates in human muscle in vivo in response to differential forms of exercise training. This study generates new insight into training mode-specific muscle adaptations by measuring protein-specific changes in abundance in combination with synthesis rates.

Introduction

Skeletal muscle is a highly plastic tissue capable of adapting to changes in nutritional intake and contractile activity. For instance, resistance exercise (RE) results in muscle hypertrophy (an increase in the radial cross-sectional area of the muscle), which is the result of stimulation of the rates of muscle protein synthesis (MPS) more so than those of muscle protein breakdown (MPB) which is also stimulated (Rennie, Edwards et al. 1982, Atherton, Etheridge et al. 2010, Morton, McGlory et al. 2015). By changing the nature of the exercise stimulus, it is possible to direct the focus on the type of skeletal muscle proteins that are being synthesized and the phenotypic outcome (i.e. aerobic versus strength). For example, we know that prolonged and repetitive lower-load dynamic loading of skeletal muscle (*i.e.,* aerobic exercise or aerobic – AE – training) results in an increase in the expression of mitochondrial genes (Perry, Lally et al. 2010), proteins (Holloszy 1967, Perry, Lally et al. 2010, Egan, O'Connor et al. 2013), and ultimately enhanced mitochondrial content (Gibala, Little et al. 2006). The result is a shift towards an oxidative muscle phenotype and improved fatigue resistance (Egan and Zierath 2013).

In contrast, RE training also stimulates the transcription of genes and accrual of new muscle proteins (Mitchell, Churchward-Venne et al. 2012, Nader, von Walden et al. 2014); however, these genes and proteins are largely associated with the myofibrillar protein fraction and regular RE leads to muscle hypertrophy and increased force-generating capacity (Campos, Luecke et al. 2002, Wilkinson, Phillips et al. 2008). However, during the early stages of exercise training, particularly in training-naïve participants, there is a significant increase in the expression of genes common to both modalities of exercise (Fluck and Hoppeler 2003, Vissing and Schjerling 2014). It is only with sustained exercise training that there may occur a ‘fine tuning’ of the

transcriptome, the protein synthetic response, and thus the proteome that gives rise to divergent hypertrophic and oxidative phenotypes (Fluck and Hoppeler 2003, Mahoney and Tarnopolsky 2005).

The temporal pattern of the exercise-induced changes in gene transcripts and protein abundance with respect to AE and RE training is now becoming clearer. Typically, the molecular biological dogmatic model is that a transient increase in the expression of genes following an acute aerobic exercise session provides the mRNA template that, once translated, precedes the respective increases in protein content and presumably functional adaptation observed during exercise training (Perry, Lally et al. 2010, Egan, O'Connor et al. 2013). Exercise-induced mitochondrial biogenesis, for example, is a characteristic feature of AE training (Talanian, Galloway et al. 2007, Egan, O'Connor et al. 2013, Egan and Zierath 2013) and is underpinned by the coordinated up-regulation of both mitochondrial and nuclear transcripts that encode for proteins involved in electron transport chain (Scarpulla 2002, Scarpulla 2006, Scarpulla 2008), and lipid metabolism (Gilde, van der Lee et al. 2003) proteins that are synthesized and new mitochondria are formed. However, recent reports have identified a poor correlation between changes in mRNA abundance and the proteins encoded by those mRNA, which highlights that transcription may not be the locus of regulation for some proteins depending on their function (Makhnovskii, Zgoda et al. 2020). Others have also questioned the gene-to-mRNA-to-protein scheme (central dogma of molecular biology), citing that it does not consider the rate or relative contribution of the different processes involved in the transfer of biological information between DNA, RNA and protein (Bishop, Hoffman et al. 2023). A lack of correlation between mRNA abundance and protein content may be due to the synergistic activation of multiple pathways in response to

exercise. For example, an acute bout of AE exercise activates sensors of cellular stress (Yu, Stepto et al. 2003, Perry, Lally et al. 2010), decreases methylation on certain gene promoter regions (Barres, Yan et al. 2012), stimulates (de)phosphorylation of mediators of translation initiation (Wilkinson, Phillips et al. 2008), and increases the expression of the various gene transcripts (PGC-1α, NRFs, TFAM) (Perry, Lally et al. 2010, Egan and Zierath 2013). In the context of RE, our understanding of the molecular mechanisms orchestrating the molecular adaptive response is less comprehensive compared to AE and is thus deserving of further study.

The use of traditional stable isotope methodologies and proxy markers of translation initiation (e.g. eIFs and p70S6K) has allowed inferences to be made on the transient changes in mixed-protein synthesis rates following a single session of exercise. Stable isotope labelling using deuterated water (D_2O) *in vivo* has been combined with peptide mass spectrometry, leading to the generation of data on the dynamic proteome under free-living conditions (Camera et al., 2017 and Hesketh et al., 2020). The use of D_2O allows for profiling of protein abundance and dynamics (synthesis and degradation) on a protein-by-protein basis. This simultaneous measurement of protein abundance and synthesis data also allows the calculation of an estimated contribution of the role of breakdown on changes in protein content. The dynamic proteomic method offers a novel insight into the protein abundance-synthesis-degradation relationship within skeletal muscle and its response to exercise (see Chapter 1 of this thesis).

The mechanisms that dictate how discordant exercise training stimuli affect common and unique protein-specific changes in human skeletal muscle over both shorter and longer-term periods remain unclear. To date, studies have not examined the proteomic responses to RE and AE

training within the same group of individuals, which would be the most efficient means by which to examine responses with a similar genetic template (i.e., DNA). To investigate the common and unique protein adaptive responses that occur following AE and RE training, techniques previously used (Camera et al., 2017) were optimized to analyze protein abundance and synthesis responses. We utilized a unilateral exercise model, as previously described (MacInnis, McGlory et al. 2017) utilized previously in our lab (Wilkinson, Phillips et al. 2008), alongside stable isotope labelling with D_2O, which allowed for the measurement of protein-specific abundance and synthesis rates at rest in the early (1st week – AC) and later (10th week – CR) phases of training. We aimed to detail the protein-specific synthesis rates and abundance changes in human muscle that underpin the acute and chronic responses to AE and RE. By integrating these analyses, we aimed to characterize how different training modalities produce distinct muscle phenotypes, ultimately elucidating novel mechanisms and proteins necessary for exercise-specific adaptation.

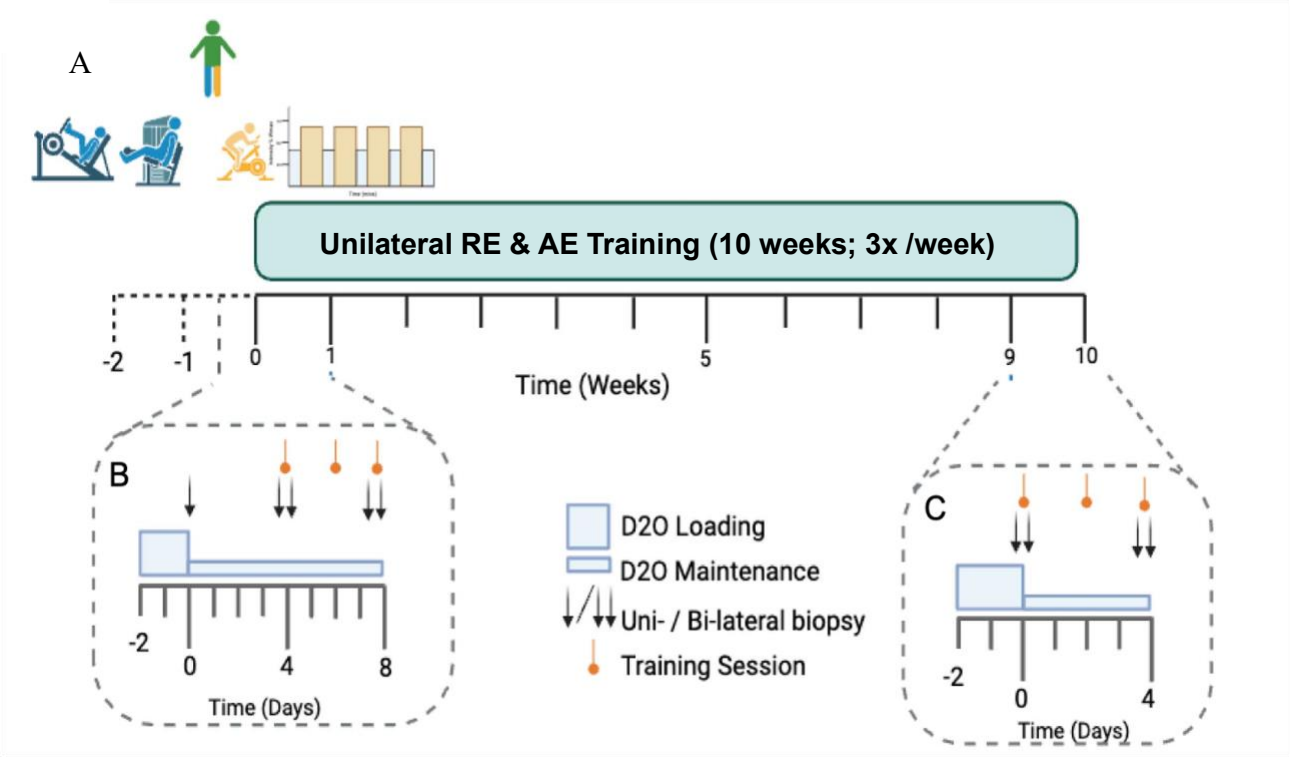


Figure 3.1. Experimental design of unilateral resistance and aerobic training across 10-weeks. **A)** Participants (n = 14; 8F/6M) were recruited and legs were randomized to either resistance (RE) or aerobic (AE) exercise. Once randomized, physiological assessments (i.e. body composition, ultrasound measurements and unilateral exercise tests) were completed pre-training. **B)** Seventy-two hours after exercise testing participants underwent a unilateral vastus lateralis biopsy (day 0) and began D_2O consumption. Participants consumed a standardized diet and refrained from exercise until after the bilateral biopsy on day 4 (baseline measurements) then performed unilateral RE and AE training on days 4, 6, and 8. Three hours after completing the training on day 8, bilateral biopsies were collected (acute response – AC – measurements). D_2O consumption was ceased during weeks 2-8 of training. **C)** Participants then repeated physiological assessments as well as 6 days of D_2O consumption and bilateral biopsies on days 0 and 4 across the final week of exercise (chronic responses – CR – measurements) in line with the acute measurement period.

Methods

Experimental Design. Before participation, participants gave their written and informed consent to the experimental procedures approved by the Hamilton Integrated Research Ethics Board (HIREB #15317). The study conformed to the standards for the use of human subjects in research as outlined by the Canadian Tri-Council Policy (TCPS 2 2022) on the ethical use of human subjects in research and the declaration of Helsinki. We utilized a within-subject repeated measures design of unilateral exercise training; 14 participants (n = 8 females, n = 6 males) were recruited to participate in this study. Participants were healthy, recreationally active, and between the ages of 18-24.

A schematic of the protocol is shown in Figure 3.1.

Participants' legs were randomly assigned to undertake resistance (RE) or aerobic (AE) exercise. Vastus lateralis biopsies (Bx) were collected at the start (unilateral Bx on day 0) and end (bilateral Bx on day 4) of the baseline period. Participants refrained from moderate-vigorous exercise across the baseline period. Immediately following the end of the baseline period, participants began a 10-week unilateral exercise intervention that required training thrice weekly using unilateral lower-limb resistance and aerobic exercise in the contralateral limb. Dynamic proteomic responses to training were quantified over the first 5 days of training (acute study period; bilateral biopsies on day 8) and the final 5 days of training (chronic study period) with bilateral biopsies collected at the start (day 0) and end (day 4) of week 10. Prior to the final biopsies of the acute and chronic study periods, participants laid supine and bilateral biopsies were collected 3 h following the final exercise bout. Biosynthetic labelling of proteins with D_2O was initiated via 2 days of loading doses (days -2 and -1 of baseline and chronic study period), and 5 days of labelling was conducted throughout the baseline, acute, and chronic study periods

via administration of maintenance doses of D_2O. Throughout the D_2O labelling periods, saliva samples were collected daily (Figure 3.1). Anthropometric and physiological testing was completed >72 hours before the initiation of D_2O labelling in the baseline, free-living period and chronic study period. As well as in the place of a regular training session in week 5 to adjust exercise prescription if required. During each measurement period, participants were provided with a standardized weight-maintaining diet containing >1.4 g $\cdot$ $kg^{-1} \cdot d^{-1}$ protein.

Physiological assessments. Body composition and leg lean mass were assessed using dual-energy X-ray absorptiometry (DEXA) scanning. Subjects were fasted (~10 h) and had not completed any exercise during the 48 h before the scan was taken pre-training prior to the initiation of the baseline-acute chronic measurement period (Figure 1). The DEXA was calibrated prior to each participant's arrival using a three-compartment Universal Whole-Body DEXA Phantom (Orthometrix Inc. Universal whole body DXA phantom – Oscar Jr., Naples, Florida). Following DEXA, participants performed 1-repetition maximum unilateral (RE limb only) strength tests on quadriceps press and extension. Participants were instructed on proper lifting technique and carried out a warm-up set of 10 repetitions at ~ 50% 1RM. Participants provided feedback on the difficulty of each set using an adapted Borg Scale (CR-10) (Buckley and Borg 2011), and the load was progressively increased by ~ 5-20% for each repetition until a true 1RM was achieved, as previously described (McGlory, White et al. 2014); participants were given 3 min rest between each attempt. A successful attempt required the participant to move the load with the correct form through the full range of motion. Participants also completed a unilateral (AE limb only) incremental exercise test to exhaustion on a cycle ergometer (Velotron, RacerMate; Seattle, WA) as previously described (Thomas, Brown et al. 2022). Heart rate was

monitored continuously with a heart rate monitor (Polar A3, Lake Success, NY). The test began with a 2 min warm-up at 50 watts (W), followed by incremental increases in resistance (1 W every 4 s) until volitional exhaustion or the point at which cycling cadence fell below 60 rpm. Maximal workload (W_{max}) was recorded as the highest power output achieved during the last 30 s of the test.

Unilateral training protocol. The training period consisted of participants visiting the lab 3 times weekly to complete supervised exercise sessions. On each visit, subjects completed unilateral aerobic and resistance training in a counterbalanced, randomized order. Resistance exercise consisted of 3 sets × 10-12 reps at 80% 1RM leg press followed by 3 sets × 10-12 reps at 80% 1RM leg extension, with 2 min recovery between sets and exercises. Aerobic exercise consisted of 2.5 min warm-up at 40% W_{max} followed by 4 bouts of 5 min cycling at 65% W_{max} with 2.5 min recovery (40% W_{max}) between sets, followed by 2.5 min of 'cooldown' at 40% W_{max}. Throughout each training session, participants were periodically monitored for heart rate (Polar A3, Lake Success, NY) and RPE (Borg CR10).

In vivo stable isotope labelling. We attempted to apply a similar experimental design as used in rodents to dose the D_2O, consisting of a 'loading phase' and a 'maintenance phase' of D_2O provision. However, due to the greater body weight of humans, orally administered loading doses were administered over 2 days. Enrichment of D_2O was then maintained via oral administration of smaller 'maintenance' doses of D_2O. Labelling was initiated on day -2 (BL) of each measurement period. D_2O loading (days -2 and -1) consisted of participants consuming 10 doses of 0.66ml · kg body mass^{-1} of 99.8 atom % deuterium oxide (D_2O; Sigma-Aldrich)

approximately 1 hour apart each day (equating to D_2O loading of 13.2 ml · kg body mass^{-1}). D_2O enrichment was then maintained by consumption of 1 daily dose of 0.66ml.kg body mass^{-1} D_2O during each measurement period. Body water enrichment was measured in saliva samples by cavity ring-down spectroscopy using a Liquid Water Isotope Analyzer with an automated injection system (Los Gatos Research, Mountain View, CA). The water phase of the saliva was injected six times, and the average of the last three measurements was used for data analysis as described previously (McKendry, Shad et al. 2019).

Saliva enrichment. Saliva deuterium enrichments were provided as a ratio where:

Atom percent values were calculated as previously described (Wilkinson, Franchi et al. 2014) :

Where AR is the absolute ratio constant for deuterium (0.00015595) based on the Vienna Standard Mean Ocean Water (VSMOW) standard in addition to 3 lab-made standards to ensure a working linear range. The sample APE was calculated by subtracting background deuterium enrichment (at time = 0) from each sample and multiplying the result by 375 to account for sample dilution.

Muscle collection. Muscle biopsies were obtained from the vastus lateralis under local anaesthesia (1% xylocaine) using the Bergström needle (Bergstrom 1975) that was adapted for manual suction and utilized per our previous protocols. Biopsied muscle tissue was quickly blotted to remove any visible fat and connective tissue before being frozen in liquid nitrogen and then stored at −80° C pending further analysis.

Muscle processing. Muscle samples (~35 mg) were pulverized in liquid nitrogen, then homogenized on ice in 10 volumes of 1% Triton X-100, 50 mM Tris, pH 7.4, that contained complete protease inhibitor (Roche Diagnostics, Lewes, United Kingdom) using a PolyTron homogenizer. Samples were incubated on ice for 15 min, then centrifuged at 1000 *g*, 4°C, for 5 min. Supernatants that contained soluble/sarcoplasmic proteins were decanted and stored on ice, and the myofibrillar pellet was resuspended in 0.5 ml of homogenization buffer, then centrifuged at 1000 *g*, 4°C, for 5min. The washed myofibrillar pellet was solubilized in 0.5 ml of 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, pH 8.5, and cleared by centrifugation at 12,000 *g*, 4°C, for 45 min. Protein concentrations of both the myofibrillar fraction and sarcoplasmic fraction were measured by using the Bradford assay (Sigma-Aldrich, Poole, Dorset, UK). Aliquots that contained 100 μg protein were precipitated in 5 volumes of acetone for 1h at −20°C.

Tryptic digestion was performed using the filter-aided sample preparation method (Wiśniewski, Zougman et al. 2009). Aliquots containing 100 μg protein were precipitated in acetone and resuspended in 40 μL UA buffer (8 M urea, 100 mM Tris, pH 8.5). Samples were transferred to filter tubes and washed with 200 μl of UA buffer. Proteins were incubated at 37 °C for 15 min in UA buffer containing 100 mM dithiothreitol, followed by incubation (20 min at 4 °C) protected

from light in UA buffer containing 50 mM iodoacetamide. UA buffer was exchanged with 50 mM ammonium bicarbonate, and sequencing-grade trypsin (Promega, Madison, WI, USA) was added at an enzyme-to-protein ratio of 1:50. Digestion was allowed to proceed at 37 °C overnight then peptides were collected 100 µL 50 mM ammonium bicarbonate containing 0.2 % trifluoroacetic acid. Samples containing 4 µg of peptides were de-salted using C18 Zip-tips (Millipore) and resuspended in 20 µl of 2.5 % (v/v) ACN, 0.1 % (v/v) formic acid (FA) containing 10 fmol·µg^{-1} yeast alcohol dehydrogenase (ADH1; MassPrep, Waters Corp., Milford, MA).

Liquid chromatography-mass spectrometry. Peptide mixtures were analyzed using an Ultimate 3000 RSLC nano liquid chromatography system (Thermo Scientific) coupled to a Q-Exactive orbitrap mass spectrometer (Thermo Scientific). Samples were loaded onto the trapping column (Thermo Scientific, PepMapTM 100, 5 µm C18, 300 µm X 5 mm), using ulPickUp injection, for 1 minute at a flow rate of 25 µL/min with 0.1 % (v/v) TFA and 2% (v/v) ACN. Samples were resolved on a 110 cm analytical column (µPAC Column; Thermo Fisher) using a gradient of 97.5 % A (0.1 % formic acid), 2.5 % B (79.9 % ACN, 20 % water, 0.1 % formic acid) to 50 % A 50 % B over 90 min at a flow rate of 300 nl/min. The data-dependent selection of the top-10 precursors selected from a mass range of m/z 300-1600 was used for data acquisition consisted of a 70,000-resolution full-scan MS scan at m/z 200 (AGC set to 3e^6 ions with a maximum fill time of 240 ms). MS/MS data were acquired using quadrupole ion selection with a 3.0 m/z window, HCD fragmentation with a normalized collision energy of 30 and in the orbitrap analyzer at 17,500-resolution at m/z 200 (AGC target 5e^4 ion with a maximum fill time of 80 ms). To avoid repeated selection of peptides for MS/MS, the program used a 30 s dynamic exclusion window.

Progenesis protein profiling. Progenesis Quantitative Informatics for Proteomics (QI-P; Nonlinear Dynamics, Waters Corp., Newcastle, UK) was used for label-free quantitation, consistent with previous studies (Burniston, Connolly et al. 2014, Camera, Burniston et al. 2017, Hesketh, Sutherland et al. 2020). Log-transformed MS data were normalized by inter-sample abundance ratio, and relative protein abundances were calculated using nonconflicting peptides only. In addition, abundance data were normalized to the 3 most abundant peptides of yeast ADH1 to obtain abundance estimates in fmol · μg^{-1} protein. MS/MS spectra were exported in Mascot generic format and searched against the Swiss-Prot database (2022_08) restricted to 'Homo Sapiens (20,371 sequences) using a locally implemented Mascot server (v.2.8 www.matrixscience.com). The enzyme specificity was trypsin with 2 allowed missed cleavages, carbamidomethylation of cysteine (fixed modification), deamidation of asparagine and glutamine (variable modification) and oxidation of methionine (variable modification). M/Z error tolerances of 10 ppm for peptide ions and 0.6 Da for fragment ion spectra were used. The Mascot output (XML format), restricted to non-homologous protein identifications, was recombined with MS profile data in Progenesis. Protein abundance data was collected for unique peptides at an identification score of < 1% false-discovery rate.

Measurement of protein synthesis rates. Protein synthesis rates were calculated by using mass isotopomer distribution analysis that was consistent with previous work (Camera, Burniston et al. 2017). Mass isotopomer abundance data were extracted from MS only spectra by using Progenesis Quantitative Informatics (Nonlinear Dynamics). Peak picking was performed on ion features with +1, +2, or +3 charge states within an analysis window of 15–105 min and 350–

1500 *m/z.* The abundance of M_0–M_3 mass isotopomers were collected over the entire chromatographic peak for each nonconflicting peptide that was used for label-free quantitation in the Progenesis Quantitative Informatics for proteomics analysis. Mass isotopomer information was exported from Progenesis Quantitative Informatics and processed in R version (2023.06.2+561) (Team 2020) according to published methods (Lam, Wang et al. 2014). In brief, the incorporation of deuterium into newly synthesized protein causes a decrease in the abundance of the monoisotopic (M_0) peak relative to the abundances of the M_1, M_2, and M_3 isotopomers that contain 1, 2, or 3 heavy isotopes (*e.g.,* ^{13}C, ^{2}H, ^{15}N, etc.). Throughout the experiment, changes in mass isotopomer distribution follow a nonlinear biexponential pattern as a result of the rise-to-plateau kinetics in precursor enrichment—body water D_2O enrichment measured in saliva samples and the rise-to-plateau kinetics of D_2O-labeled amino acids into newly synthesized protein; therefore, a machine learning approach was taken that employed the Nelder-Mead method to optimize for the rate of change in the relative abundance of the M_0 peak. The rate of change in mass isotopomer distribution is also a function of the number of exchangeable H sites, and this was accounted for by referencing each peptide sequence against standard tables that reported the relative enrichment of amino acids by tritium in mice (Commerford, Carsten et al. 1983) or deuterium in humans (Price, Holmes et al. 2012) to give the synthesis rate (k) for each peptide. Peptide synthesis rates across each protein were averaged to give a synthesis rate for that protein in each participant

Estimation of protein breakdown rates. The rate of change in the abundance of a protein is dependent on the difference between its rate of synthesis k_S and the rate of breakdown

(degradation) k_d. We assumed each of these was constant and used first-order kinetics that followed the standard formula:

Where is the abundance at time t, and is the abundance at time . By using protein abundance data at times t and , the net rate of change in abundance can be calculated by rearranging the above to give:

Converting differences in abundance between BL, Pre and Post for each testing period (1 and 10 weeks) to rates of change in abundance enables the rate of breakdown of each protein to be calculated as the difference between its rate of synthesis and its rate of change in abundance. Table 3 summarizes the mean abundance, synthesis, and degradation data for each protein that was investigated in the myofibrillar fraction.

Statistical and Bioinformatic Analysis. A one-way analysis of variance was applied to investigate the physiological responses to training across the training period. Where possible, 2-way ANOVA was used to investigate differences between exercise mode on physiological data (e.g. leg lean mass). Where appropriate, significant ($p < 0.05$) differences in physiological data between means were analysed using Tukey's post-hoc analysis.

For protein abundance data, prior to statistical analysis, data were filtered so that only values quantified in all sampling time points (BL, AC: Pre and Post; and CR: Pre and Post) and conditions (AE and RE) in all n = 14 individuals (i.e. successfully quantified in n = 135 muscle samples). Altogether, relative protein abundance was reliably determined for 474 proteins in the myofibrillar fraction. Due to the unilateral BL biopsy, within our analysis of protein abundance data (expressed as femtomole per μg protein, i.e. $fmol \cdot \mu g^{-1}$) the BL sample was excluded so that we could determine differences in protein abundance, which used paired t-tests (given the exploratory nature of our work) to assess significance across both time (i.e. AC_Pre vs. AC_Post, AC_Pre vs. CR_Post) and condition (AE and RE). For fractional synthesis rate, prior to statistical analysis, data were filtered so that only values quantified in all sampling time points (BL, Week1- Pre and Post, Week10- Pre and Post) and conditions (AE and RE) in at least 50% (n = 7-14 individuals), that had a complete time-course for within-subject comparison of individual proteins' fractional synthetic rate (FSR).

The labelling periods within our design allow for the quantification of baseline protein fractional synthesis rates (unilaterally) and the protein-specific FSR response to 1 week (acute) and 10 weeks (chronic) RE and AE training. Mixed-protein FSR was assessed by calculating the abundance normalized average FSR from individual proteins (median FSR weighted by abundance) quantified in all time points in n =7-14 participants. Altogether 39 proteins FSR were reliably detected in n=7-14 participants for both the acute and chronic periods that allowed for within-subject comparison across time and condition. Paired t-tests were applied to compare individual synthesis rates after acute and chronic RE and AE to baseline turnover rates. Statistical significance was set at $p < 0.05$ and, where appropriate, assessed for multiple

comparisons by calculating false-discovery rates (q-values; (Storey and Tibshirani 2003)). Dynamic proteomics data (protein-specific abundance and FSR) identified as exhibiting a significant response to training were used for bioinformatic analysis to understand the effect of time (i.e. conserved responses to AE and RE) or interactions (i.e. differential response of AE and RE over time) on the muscle dynamic proteome response.

Subject Characteristic (n=14)	Baseline	Week 5	Post
Age (years)	21 ± 1	ND	ND
Height (cm)	169 ± 9	ND	ND
Weight (kg)	70.1 ± 10.4	ND	69.8 ± 17.2
BMI (kg· m^{-2})	24.2 ± 4.1	ND	24.1 ± 4.0
Unilateral Work Peak (W)	145 ± 31	160 ± 36**	165 ± 40**
Unilateral 1RM Leg Press (kg)	91 ± 49	138 ± 46**	158 ± 51**
Unilateral 1RM Leg Extension (kg)	31 ± 14	39 ± 13**	44 ± 12**
AE Limb Lean Mass (g)	8932 ± 2145	ND	9122 ± 2150
RE Limb Lean Mass (g)	8859 ± 2174	ND	9248 ± 2276*

Table 3.1. Physical and physiological characteristics were measured at baseline, after 5 weeks (mid-training) and after 10 weeks. Data shown for Work Peak are only from the AE leg, and data for 1RM are only for the RE leg. Data are presented as means ± standard deviation. *$p<0.05$; **$p<0.005$ from Baseline. ND – not determined.

Results

Physiological response to unilateral training. Fourteen (8F/6M) young (20 ± 1 years), healthy, recreationally active individuals were recruited and completed a 10-week (3 sessions per week) unilateral aerobic (AE) and resistance (RE) exercise protocol. We report dynamic proteomic analysis of n = 14 participants. Participants' physiological data are reported in Table 1. All participants completed ≥ 90 % (27/30 possible training sessions) of the programmed training sessions. Within the 14 participants, 10 weeks unilateral RE significantly increased quadriceps press 1RM ($p < 0.0001$; mean diff = 67 ± 3 kg) and quadriceps extension 1RM ($p < 0.0001$; mean diff = 13 ± 2 kg) (Figure 2A and 2B), whereas AE significantly ($p = 0.0347$) increased unilateral W_{max} (+ 20 ± 7 W) (Figure 2C). Quadriceps press/ extension 1RM was not performed in the AE trained leg (nor was the W_{max} test performed in RE trained leg). Overall, 10 weeks of training resulted in a slight yet significant ($p = 0.0002$) increase in leg lean mass in the RE limb (Figure 2D). Ultrasound imaging of the *vastus lateralis* also demonstrated a modest yet significant ($p<0.05$) increase in CSA in the RE limb following 10 weeks of training (Figure 3A and 3B).

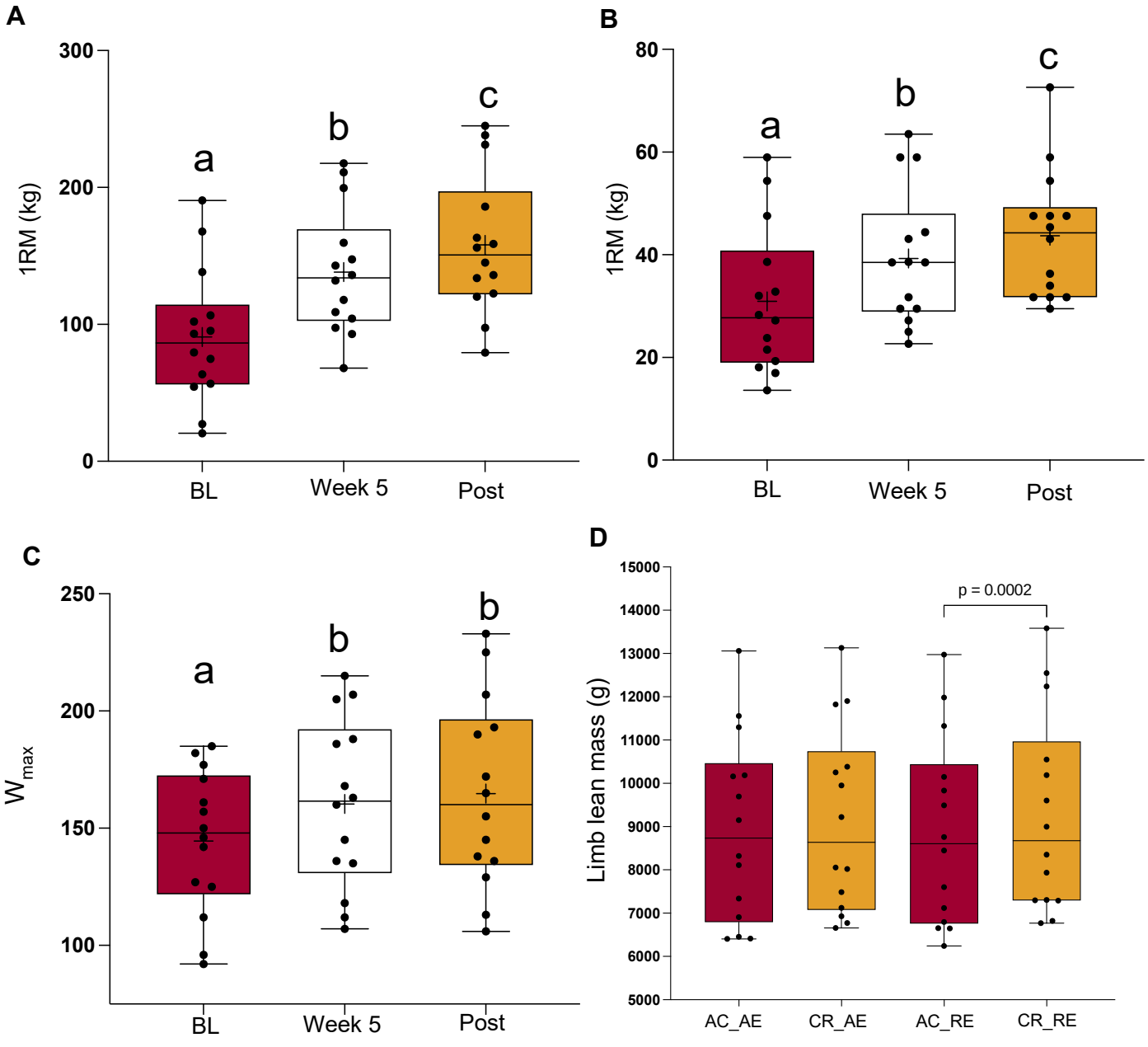


Figure 3.2. Physical and physiological characteristics measured at baseline (BL), after 5 weeks and after 10 weeks (Post). n=14 (6M/8F) **(A)** leg press (1RM), **(B)** leg extension (1RM), **(C)** VO_2 Ramp peak watts and **(D)** leg lean mass (g) as measured by DXA. Data are presented as box and whisker plots with upper and lower range (whiskers) and interquartile range (box), mean (+) and median (line in the box). Means in figures that do not share a letter are significantly different $p<0.05$.

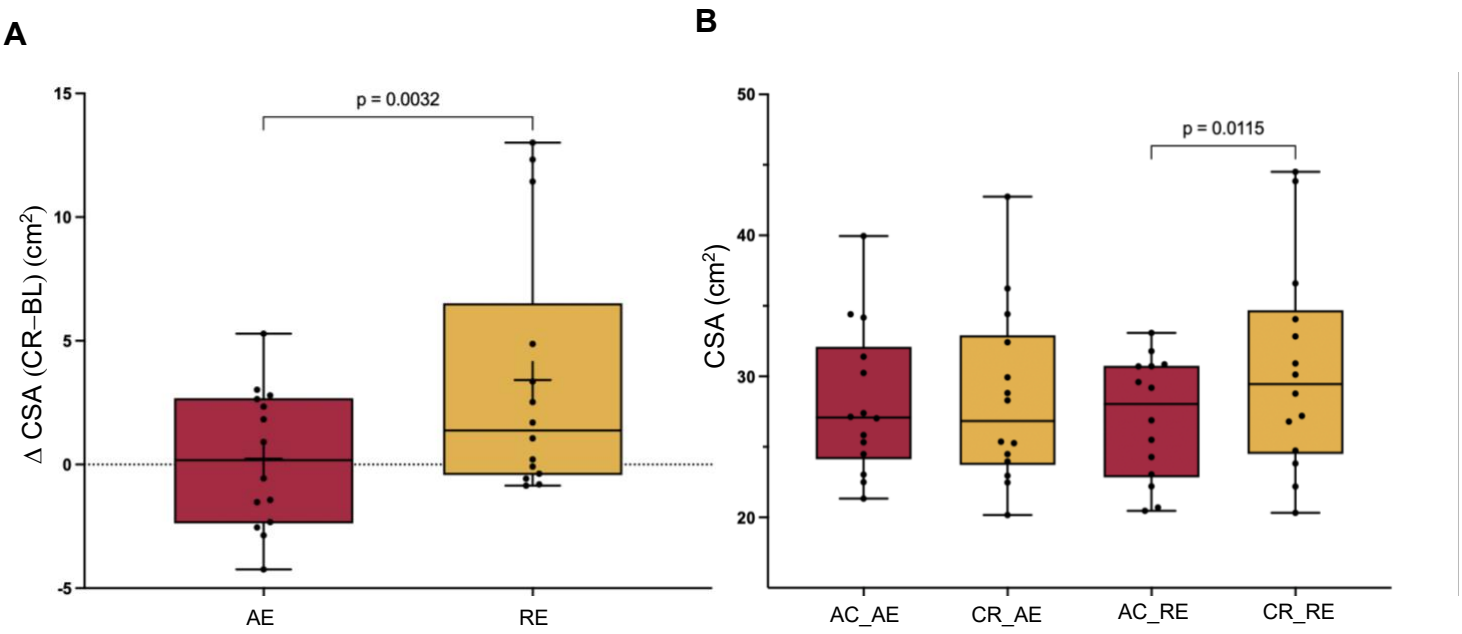


Figure 3.3. Ultrasound of the *vastus lateralis* measured at 50% the length of the femur, assessed at baseline and after 10 weeks of training. Data are presented as box and whisker plots with upper and lower range (whiskers) and interquartile range (box), mean (+) and median (line in the box). n=14 (6M/8F) (A) Delta CSA (cm^2) across 10 weeks training in the AE and RE limb. (B) CSA demonstrated as cm^2 for each limb pre- and post- training. Data presented as box and whisker plots, mean ± standard deviation, min to max.

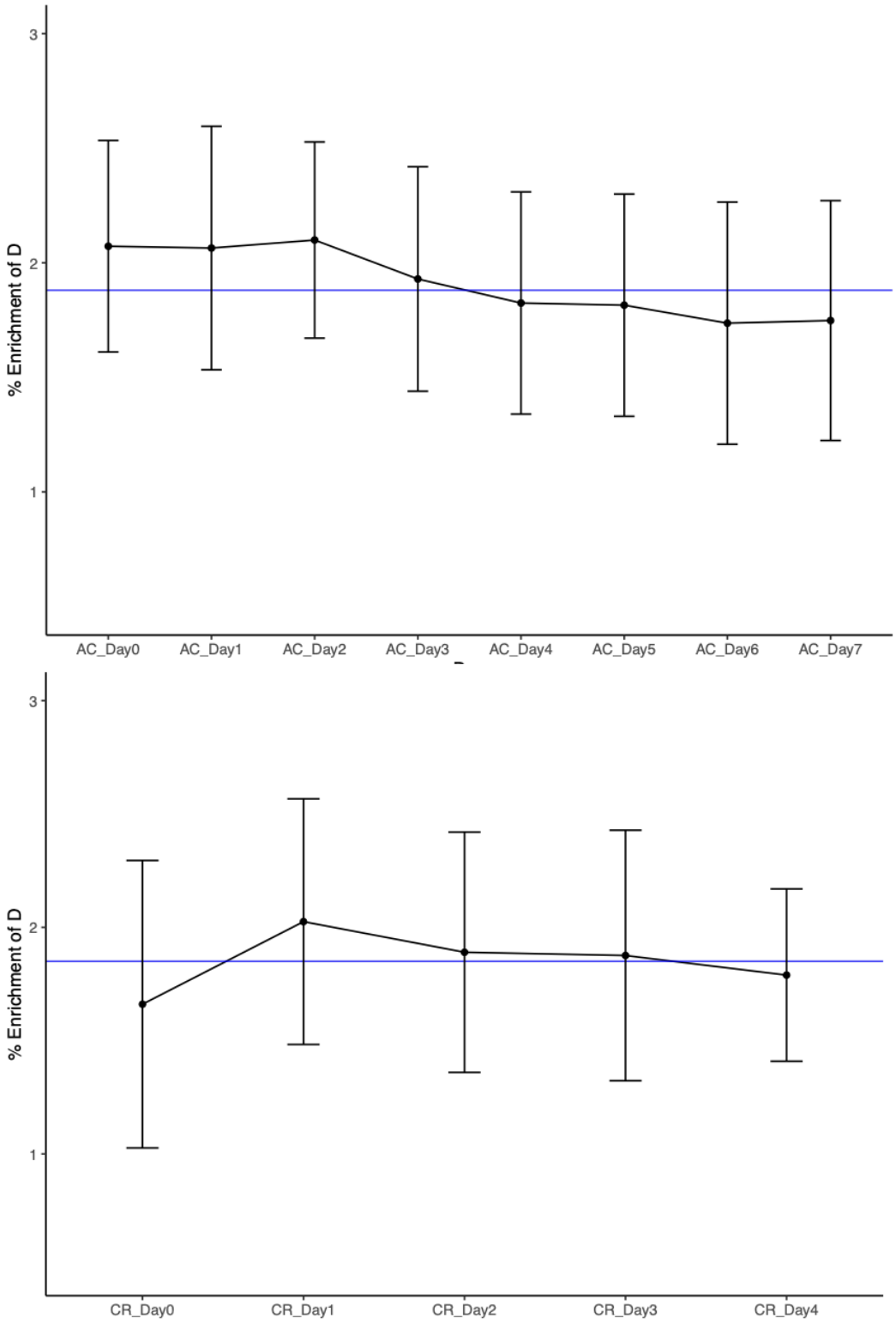


Figure 3.4. **(A)** Salivary deuterium (D_2O) enrichment across the acute (AC - week 1) and **(B)** chronic (CR - week 10) testing periods. Data presented means ± SD.

Proteomic analysis. Overall, 862 proteins were confidently identified with ≥1 unique peptide at a false-discovery rate of identification ≤ 1%. After data were filtered to remove missing values amongst biological replicates, the abundance of 474 proteins was measured in all muscle samples (n = 9) within each individual (n = 14). The dynamic range of the abundance (fmol·μg^{-1} of protein; fmol·μg^{-1}) of the proteins quantified spanned ~6.4 orders of magnitude from 0.00069 ± 0.00078 fmol·μg^{-1} (MYH16, myosin 16) to 1862 ± 533 (ACTS, Actin alpha skeletal muscle) (Figure 5A). The top 10 most abundant proteins included myosin light chain (MYL1; 537 ± 214 fmol·μg^{-1}), type IIa (MYH2; 466 ± 185 fmol·μg^{-1}) and type Ia (MYH7; 332 ± 147 fmol·μg^{-1}) myosin heavy chain (MYH). Analysis of the relative abundance of MYH at baseline indicated MYH7, 2, and 1 account for ~99.9 % of MYH proteins (from 9 isoforms quantified in total). MYH7 accounted for 50.5 ± 12.0 % of MYH protein profile within baseline biopsies across our participants. In comparison, MYH2 (IIa) and MYH1 (IIx) accounted for 11.6 ± 5.0 % and 37.9 ± 16.8 %, respectively (Figure 5B).

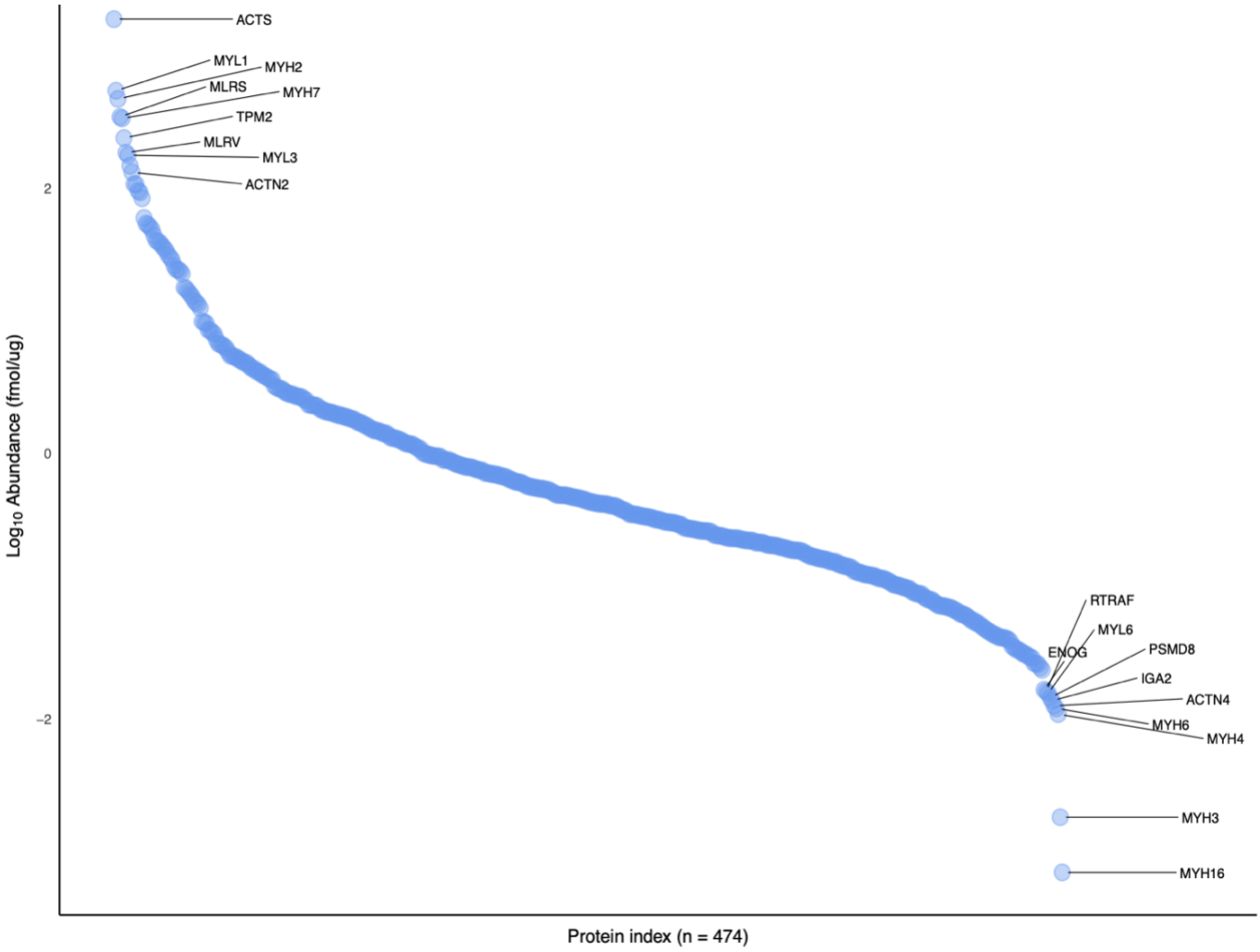
Statistical analysis of protein abundance data over time (BL, acute Pre and Post, chronic Pre and Post) in both the aerobic (AE) and resistance (RE) trained limb identified proteins that significantly changed in abundance following 1 week and 10 weeks of training. Following 1 week of AE (Figure 6A), 11 and 2 proteins significantly ($p<0.05$) increased and decreased abundance, respectively. Following 1 week of RE (Figure 7A), 9 and 5 proteins significantly ($p<0.05$) increased and decreased abundance, respectively. One protein (KLH40, Kelch-like protein 40) was significantly increased in both the AE and RE muscle following 1 week of exercise training. GO analysis indicated no significant enrichment in GO terms/ pathways associated with RE following 1 week of exercise training.

Following 10 weeks of AE, 80 and 3 proteins significantly ($p<0.05$) increased and decreased abundance, respectively (Figure 6B). Following 10 weeks of RE, 105 and 1 proteins significantly ($p<0.05$) increased and decreased abundance, respectively (Figure 7C). Gene ontology (GO) analysis of the proteins elevated in abundance following 10 weeks AE indicated strong networks of mitochondrial proteins (i.e. NDUA4, TUFM, PPIF, ATP5, NNTM and MPCP) involved in oxidative phosphorylation and energy metabolism. GO analysis of the proteins elevated in abundance following 10 weeks RE also indicated enrichment of proteins associated with the 'mitochondrion' (false discovery rate [FDR]=9.25e-8) and 'mitochondrial matrix' (FDR=3.61e-7). Some of the next top 10 ranked (FDR) cellular components for proteins with significant increases in abundance following 10 weeks RE were 'Contractile fibre' (FDR=1.80e-5), 'I band' (FDR=6.50e-4), 'myofibril' (FDR=2.94e-6) and Z disk (FDR=2.62e-5). Thirty proteins were common and significantly increased in both the AE and RE limb following 10 weeks of exercise training, which included proteins such as HADHA (Trifunctional enzyme subunit alpha, mitochondrial), ACAT1 (Acetyl-CoA acetyltransferase, mitochondrial) and CYC5 (Cytochrome c; Electron carrier protein). GO analysis of the proteins commonly elevated in abundance following 10 weeks EX (AE and RE) indicated cellular component enrichment of proteins associated with the 'hemoglobin complex' (n proteins=2; FDR=0.0451; p=2.96e-5) and biological process enrichment for 'fatty acid beta-oxidation' (n proteins=4; FDR=0.00051; p=3.28e-8) and 'carbon dioxide transport' (n proteins=2; FDR=0.0442; p=3.38e-5).

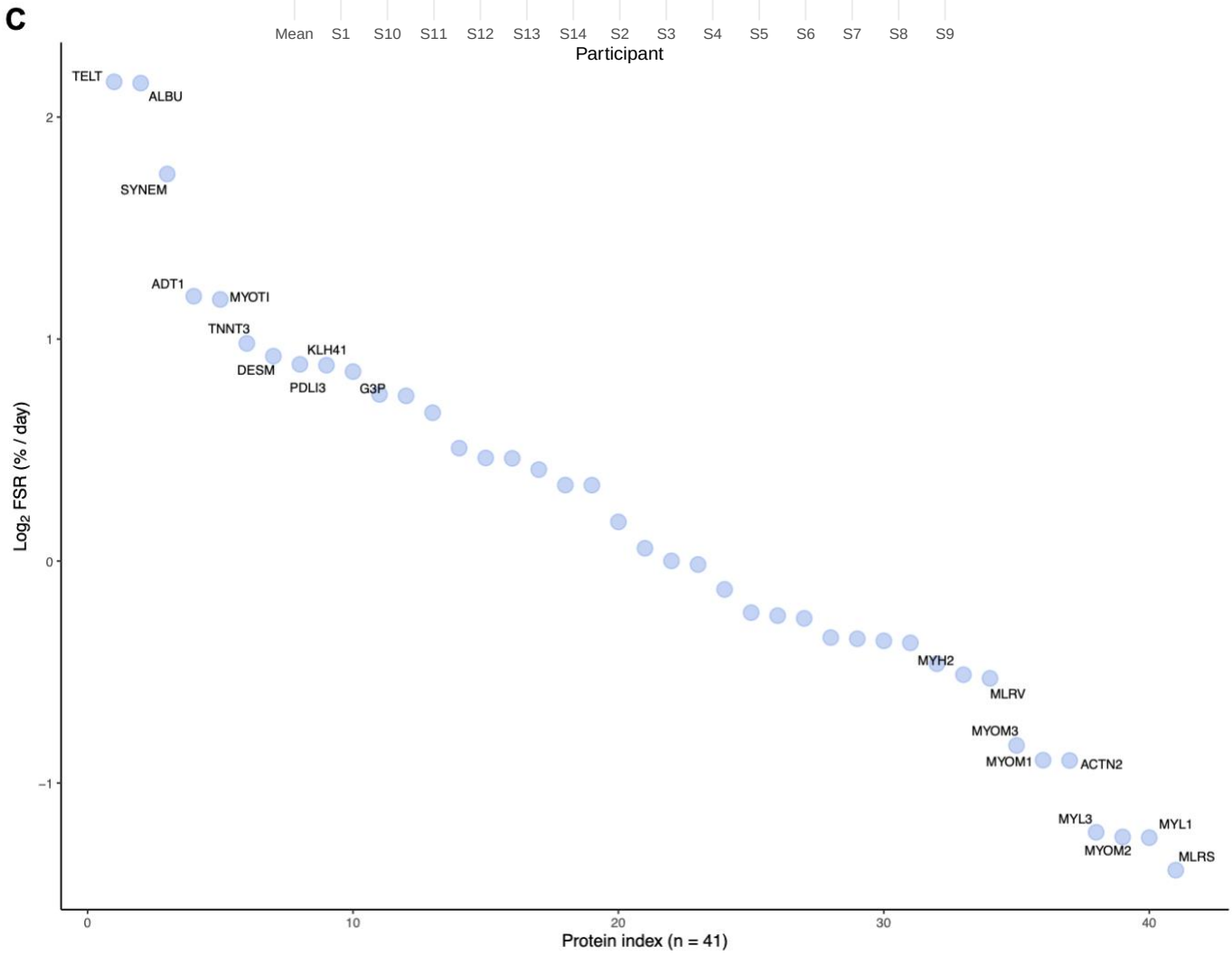
Deuterium oxide consumption resulted in an average body water enrichment of 1.88 ± 0.15 % across the baseline-acute period (AC days 0 – 7) and 1.85 ± 0.12 % across the chronic

measurement period (CR days 0-4) (Figures 3A and 3B). Dynamic proteome profiling quantified the fractional synthesis rate (FSR; % d^{-1}) of 507 proteins (in ≥ 1 participant). Protein FSR data was accepted for statistical analysis if quantified in ≥ 50% of participants (n = 7-14) across the baseline and first week of training (acute response) and/ or across the baseline and final week of training (chronic response). Analysis of 41 muscle protein turnover rates at baseline indicated our protein FSR data spanned from 0.381 ± 0.22 %/d (MLRS, myosin regulatory light chain 11) to 4.466 ± 1.73 %/d (TELT, Telethonin), median FSR of individual muscle proteins in the across baseline was 1.270 ± 0.97 %/d (Figure 5C).

A




B
100%
75%
50%
25%
0%
Proportion MYH %
Mean
S1
S10
S11
S12
S13
S14
S2
S3
S4
S5
S6
S7
S8
S9
Participant
Isoform
MYH1
MYH2
MYH7


C
TELT
ALBU
SYNEM
ADT1
MYOTI
TNNT3
KLH41
DESM
PDLI3
G3P
MYH2
MLRV
MYOM3
MYOM1
ACTN2
MYL3
MYL1
MYOM2
MLRS
Log2 FSR (% / day)
Protein index (n = 41)

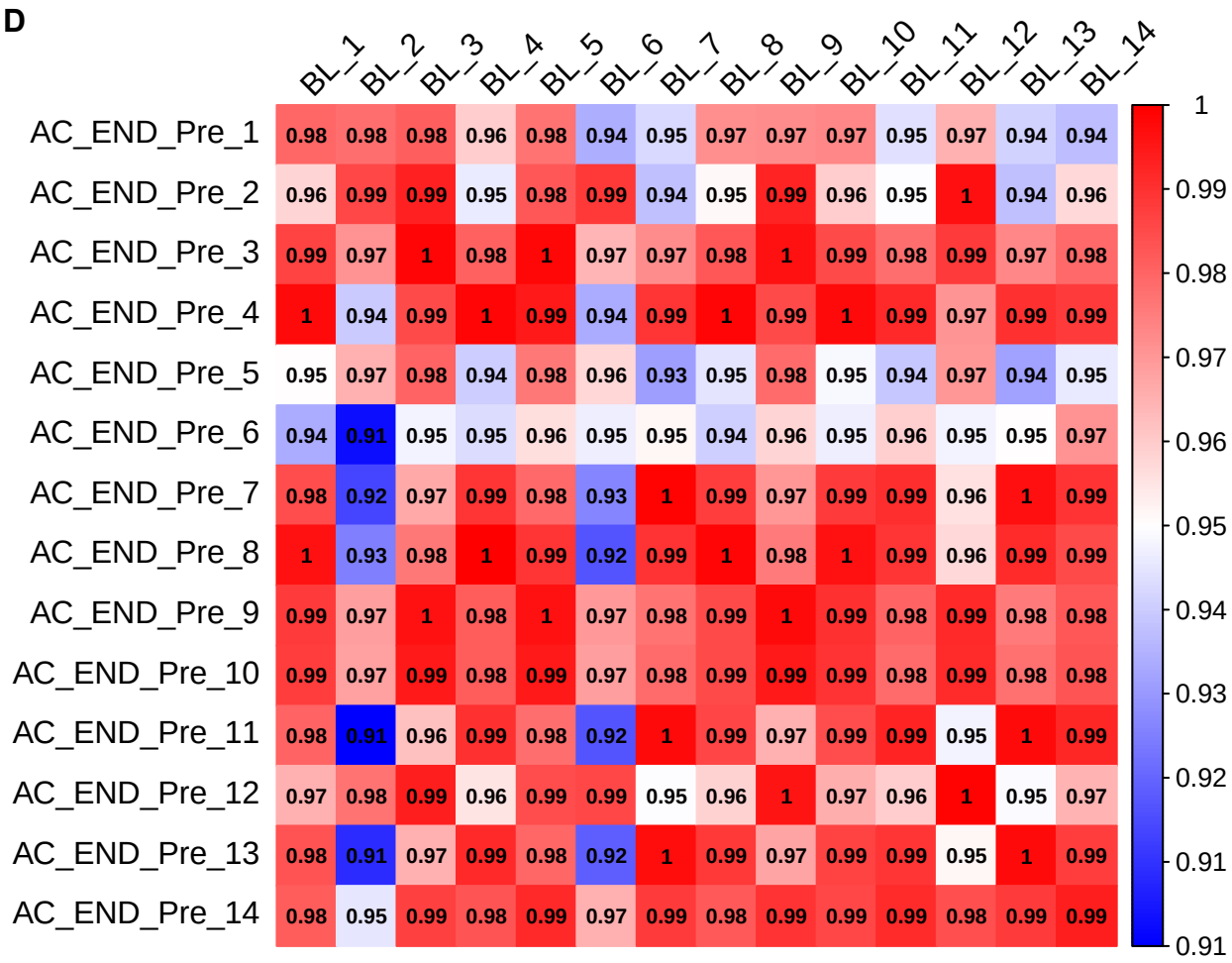
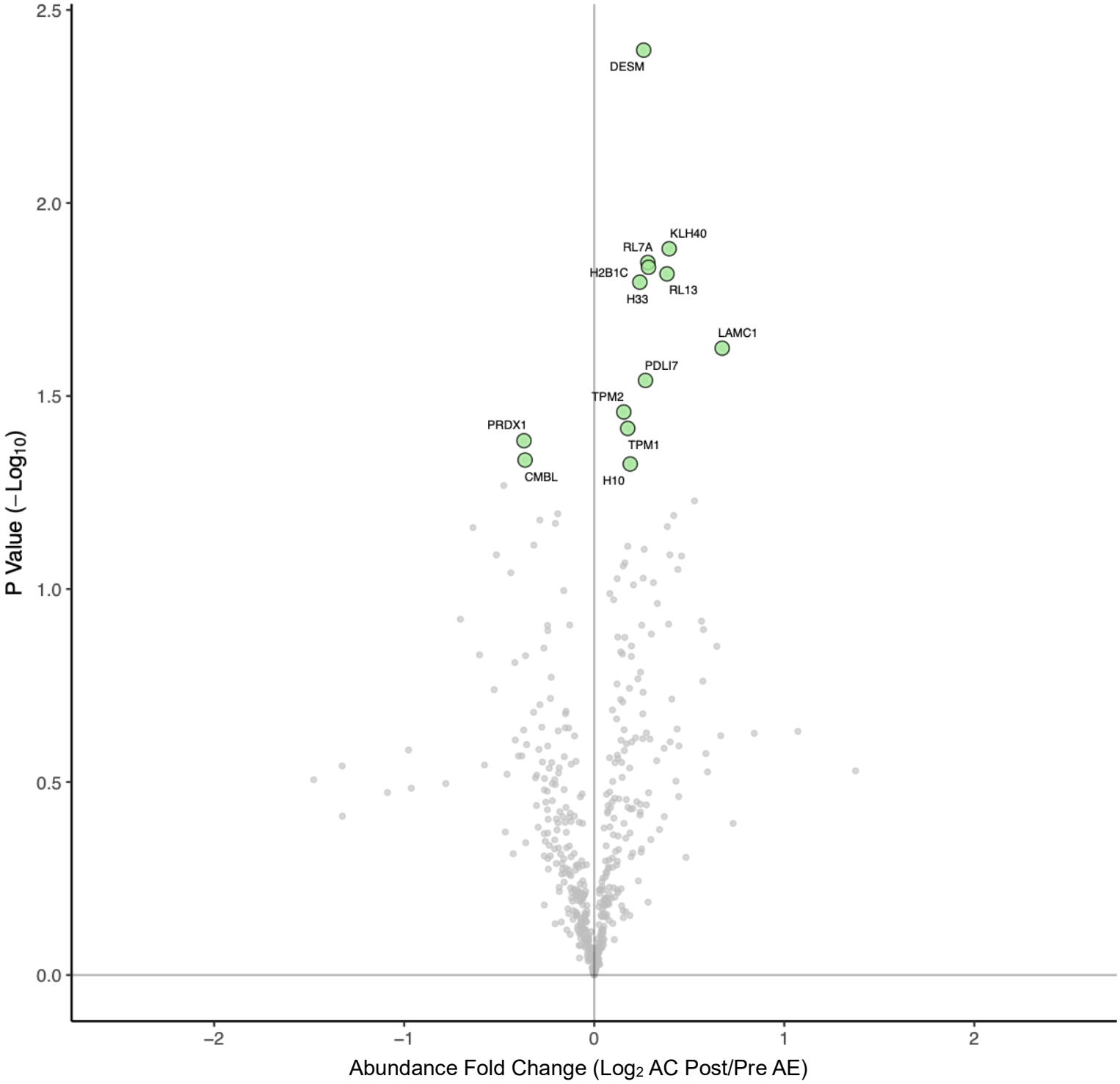
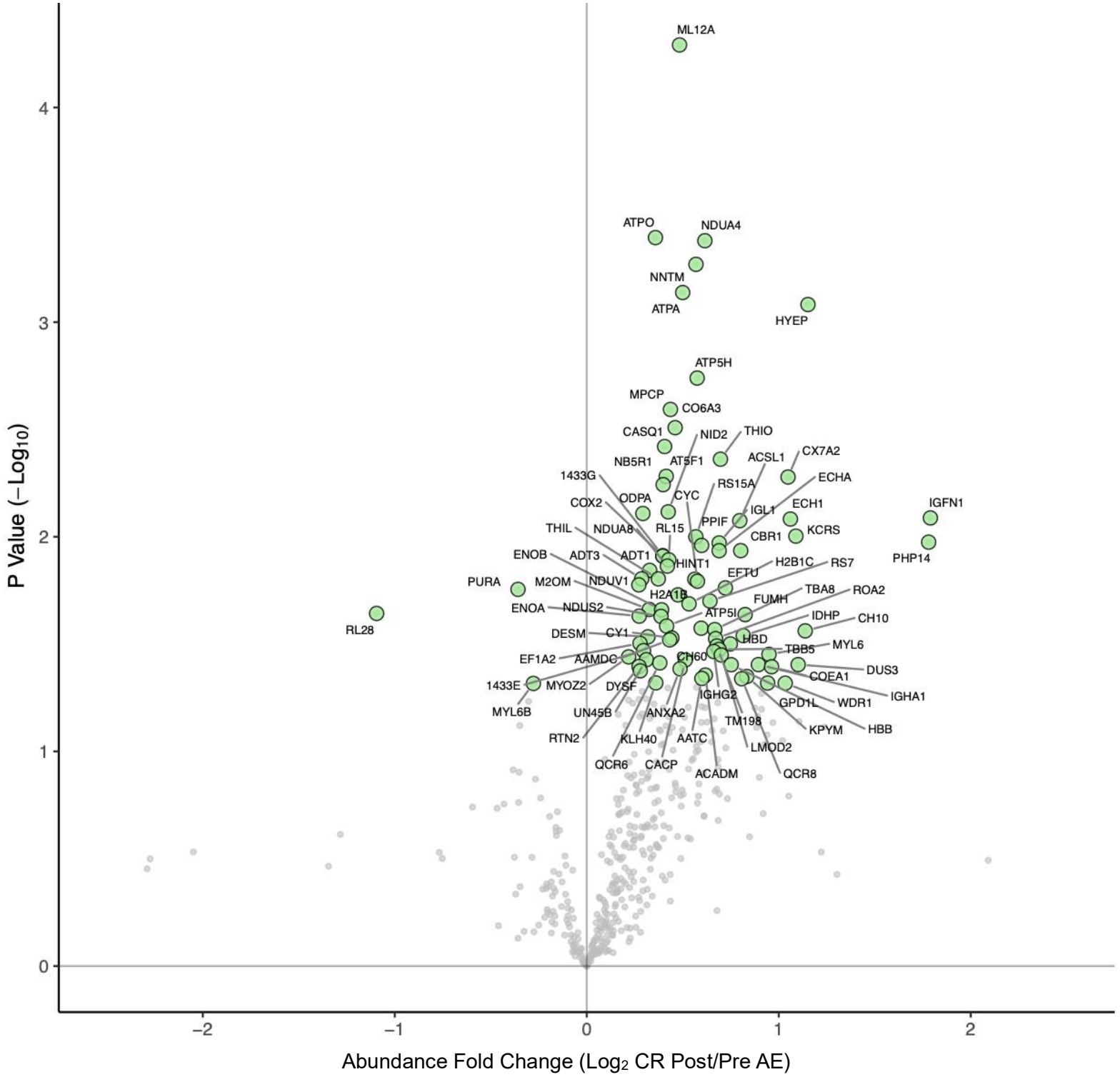


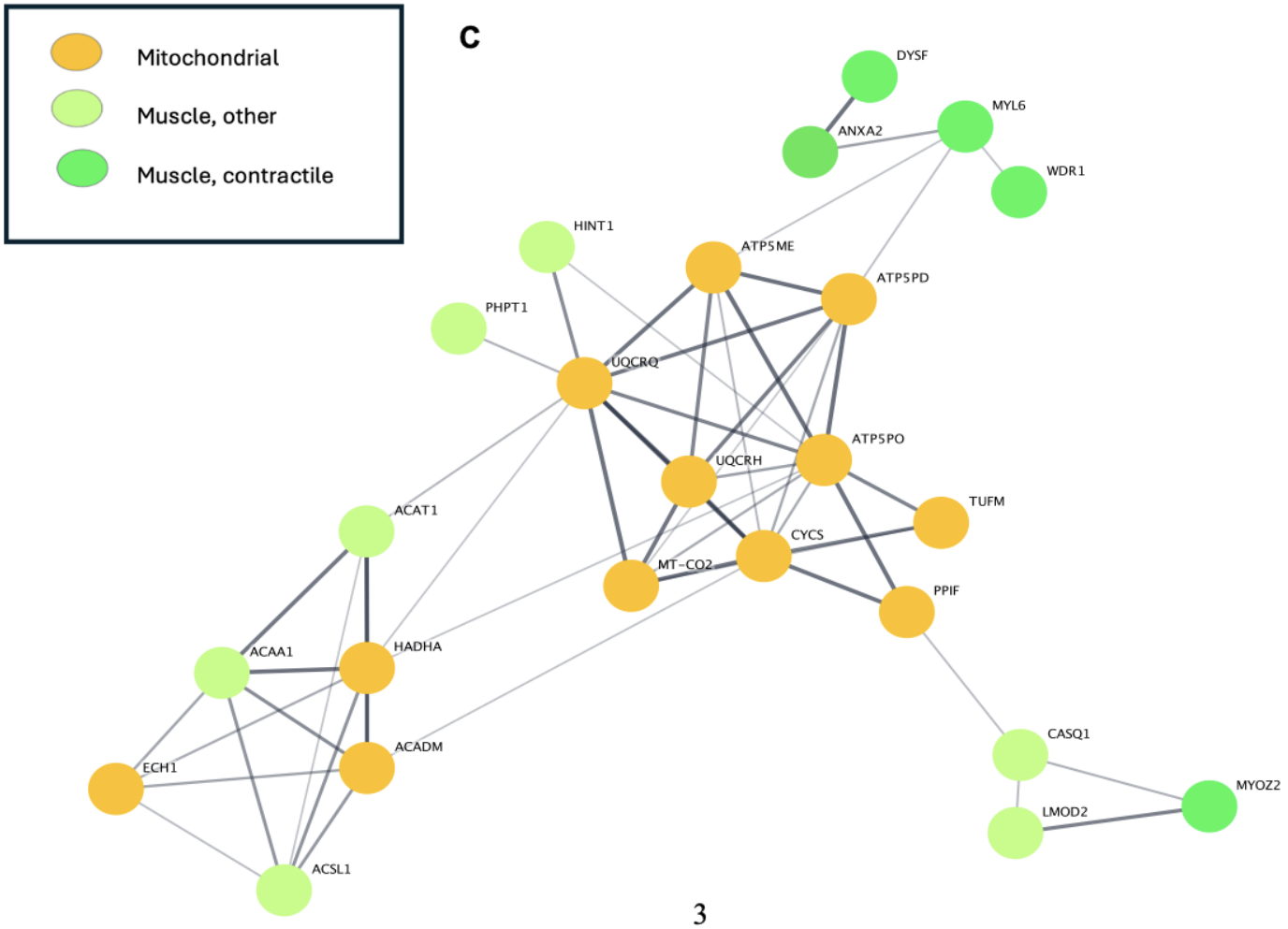
Figure 3.5. (A) Distribution plot of average abundance data (log_{10}) from 474 proteins successfully quantified in human *vastus lateralis* across all time points measured by bottom-up proteomics using liquid-chromatography tandem mass-spectrometry (LC-MS/MS) and quantified by label free quantification. Values represent 474 protein abundances quantified across all time points in all muscle samples (n = 14 participants). (B) Participant protein abundance for MYH1, MYH2 and MYH7 (Type 2x, 2a and 1, respectively) assessed as the average of the baseline and AC_Pre biopsies demonstrated as a relative distribution of MYH protein abundance. (C) Distribution plot of fractional synthesis rates (log_{10} %/d) of 41 proteins FSR successfully quantified in n = 7-14 human *vastus lateralis* in the baseline biopsy by dynamic proteome profiling. (D) Correlation matrix of protein abundances in the baseline biopsy (BL) and pre biopsy taken from the AE limb (AC_END_Pre). Average within subject correlation R=0.991 (range 0.96 -1.0).

A


DESM
KLH40
RL7A
H2B1C
RL13
H33
LAMC1
PDLI7
TPM2
PRDX1
TPM1
CMBL
H10
2.5
2.0
1.5
1.0
0.5
0.0
P Value (−Log10)
−2
−1
0
1
2
Abundance Fold Change (Log2 AC Post/Pre AE)

B


ML12A
ATPO
NDUA4
NNTM
ATPA
HYEP
ATP5H
MPCP
CO6A3
CASQ1
NID2
THIO
NB5R1
AT5F1
ACSL1
CX7A2
1433G
RS15A
ECHA
COX2
ODPA
CYC
IGL1
ECH1
IGFN1
THIL
NDUA8
RL15
PPIF
CBR1
KCRS
PHP14
ENOB
ADT3
ADT1
HINT1
H2B1C
RS7
EFTU
PURA
M2OM
NDUV1
H2A1B
TBA8
ROA2
FUMH
ENOA
NDUS2
ATP5I
IDHP
CH10
RL28
DESM
CY1
HBD
MYL6
TBB5
EF1A2
AAMDC
CH60
DUS3
COEA1
1433E
MYOZ2
DYSF
IGHG2
GPD1L
WDR1
IGHA1
MYL6B
UN45B
ANXA2
TM198
KPYM
HBB
RTN2
KLH40
AATC
LMOD2
QCR6
CACP
ACADM
QCR8
P Value (−Log10)
Abundance Fold Change (Log2 CR Post/Pre AE)



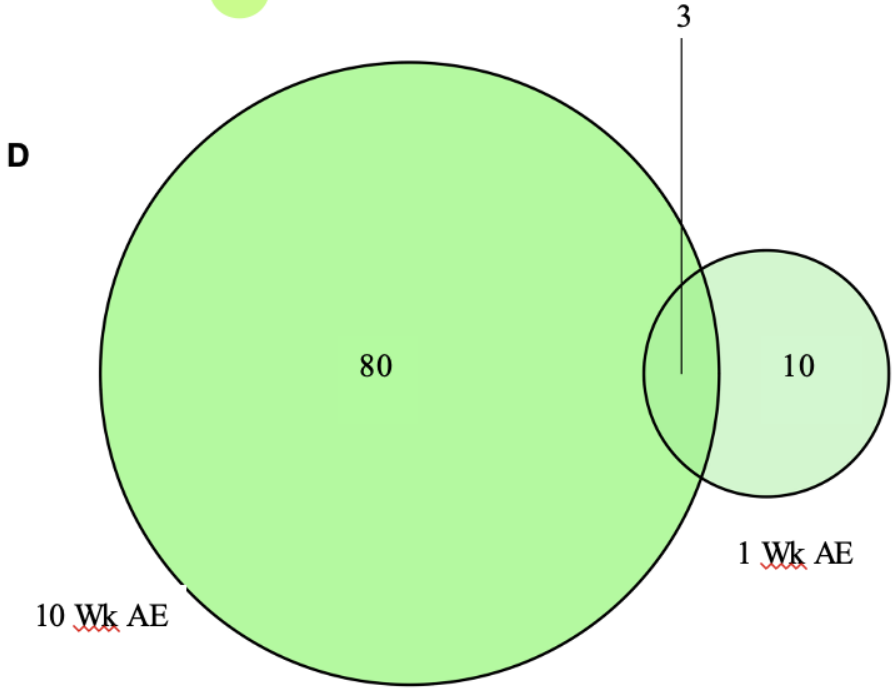
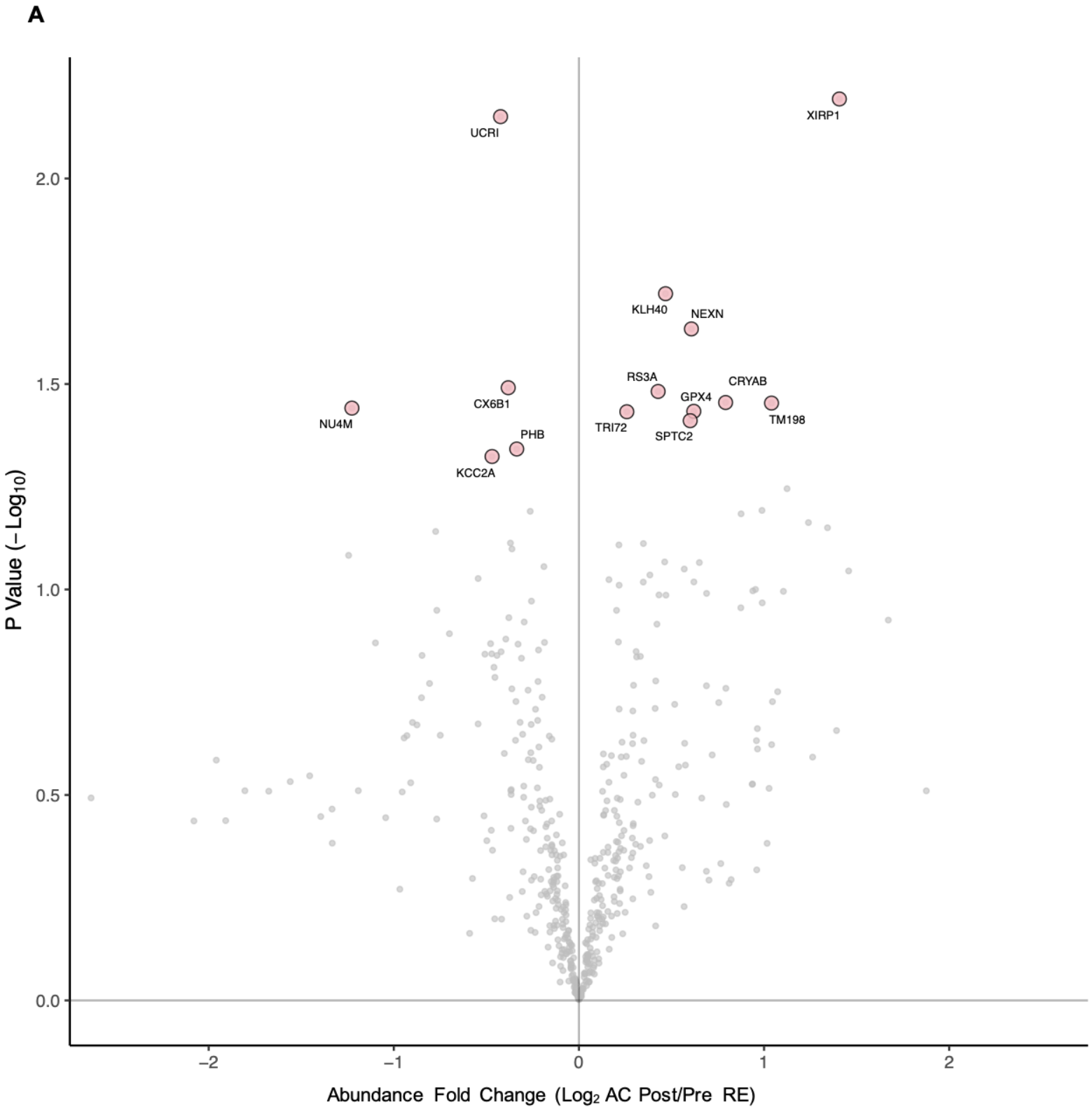
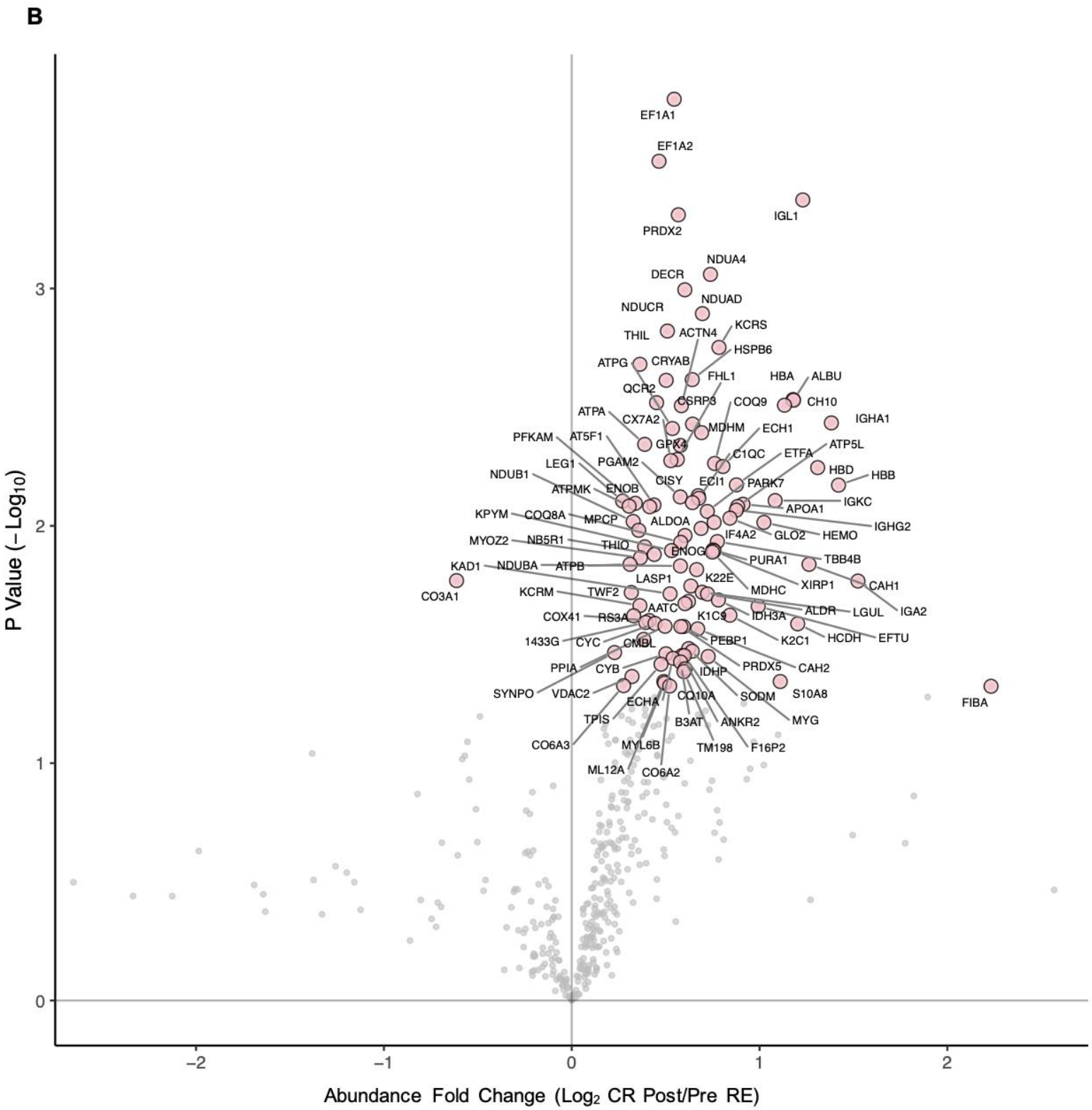
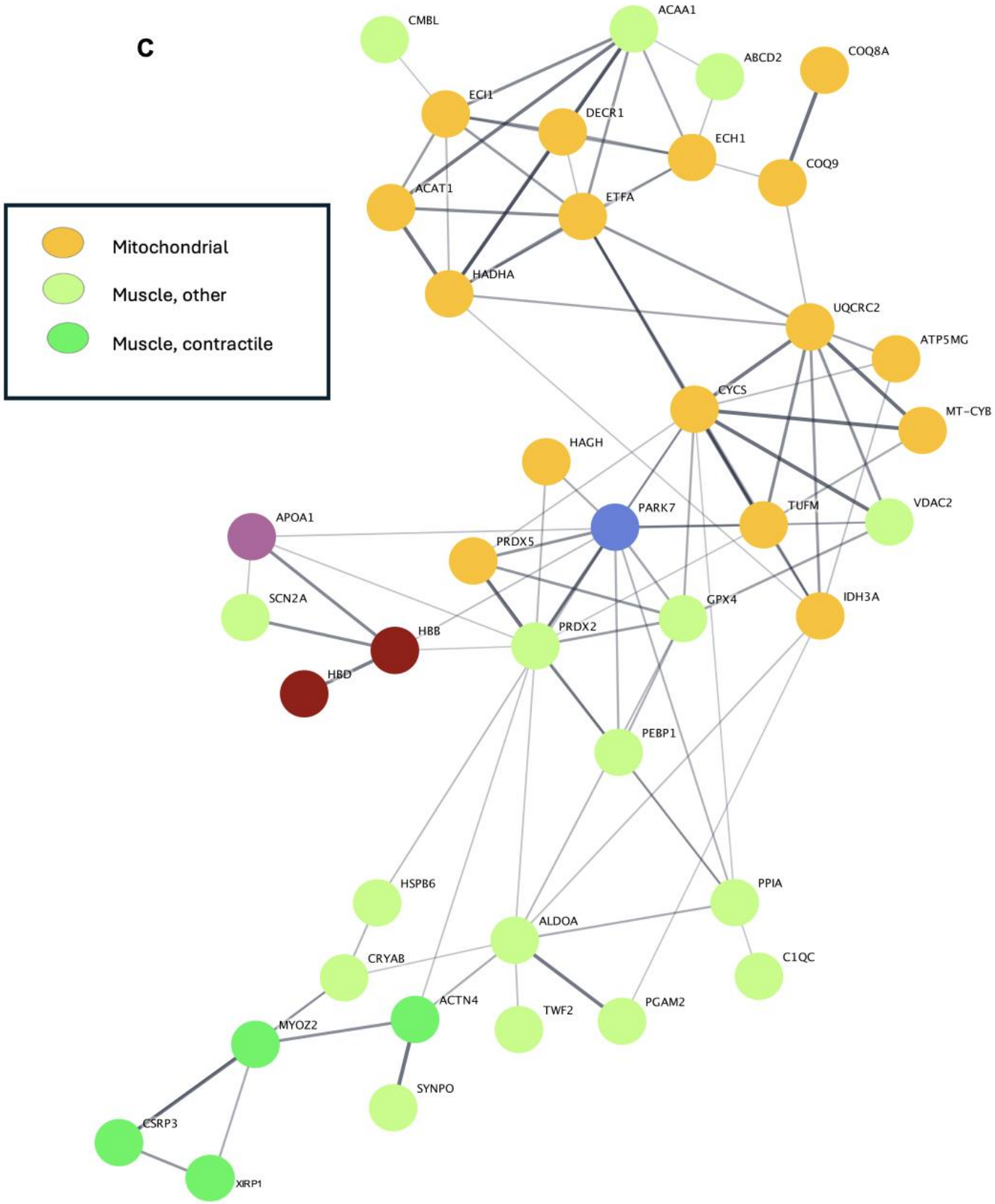


Figure 3.6. (A, B) Volcano plots of fold change (log2 AC/Pre and CR/Pre) of AE in response to 1 week (A) and 10 weeks (B) training, plotted against -log10 p value of T-test (time). (C) STRING protein interaction network of proteins that demonstrate $p<0.05$ following 10 weeks AE versus Pre. (D) Venn diagram of proteins that demonstrate a significant (time $p<0.05$) change in abundance ($fmol \cdot \mu g^{-1}$) in response to 1 week and 10 weeks training.


A
XIRP1
UCRI
KLH40
NEXN
RS3A
CRYAB
GPX4
CX6B1
NU4M
TRI72
SPTC2
TM198
PHB
KCC2A
2.0
1.5
1.0
0.5
0.0
P Value (– Log10)
–2
–1
0
1
2
Abundance Fold Change (Log2 AC Post/Pre RE)


B
P Value (– Log10)
Abundance Fold Change (Log2 CR Post/Pre RE)
EF1A1
EF1A2
PRDX2
IGL1
NDUA4
DECR
NDUCR
NDUAD
THIL
ACTN4
KCRS
HSPB6
ATPG
CRYAB
FHL1
HBA
ALBU
QCR2
CSRP3
COQ9
CH10
ATPA
CX7A2
MDHM
ECH1
IGHA1
PFKAM
AT5F1
GPX4
C1QC
ETFA
ATP5L
LEG1
PGAM2
HBD
HBB
NDUB1
CISY
ECI1
PARK7
ATPMK
ENOB
IGKC
APOA1
KPYM
COQ8A
MPCP
ALDOA
IGHG2
IF4A2
GLO2
HEMO
MYOZ2
NB5R1
THIO
ENOG
PURA1
TBB4B
KAD1
NDUBA
ATPB
LASP1
K22E
XIRP1
CAH1
CO3A1
KCRM
TWF2
MDHC
AATC
ALDR
LGUL
IGA2
COX41
RS3A
K1C9
IDH3A
1433G
CYC
CMBL
PEBP1
K2C1
HCDH
EFTU
PPIA
CYB
IDHP
PRDX5
CAH2
SYNPO
VDAC2
ECHA
CQ10A
SODM
S10A8
FIBA
TPIS
B3AT
ANKR2
MYG
CO6A3
MYL6B
TM198
F16P2
ML12A
CO6A2


C
Mitochondrial
Muscle, other
Muscle, contractile
CMBL
ACAA1
ABCD2
COQ8A
ECI1
DECR1
ECH1
COQ9
ACAT1
ETFA
HADHA
UQCRC2
ATP5MG
CYCS
MT-CYB
HAGH
PARK7
TUFM
VDAC2
APOA1
PRDX5
SCN2A
GPX4
IDH3A
HBB
PRDX2
HBD
PEBP1
HSPB6
PPIA
ALDOA
CRYAB
C1QC
ACTN4
TWF2
PGAM2
MYOZ2
SYNPO
CSRP3
XIRP1

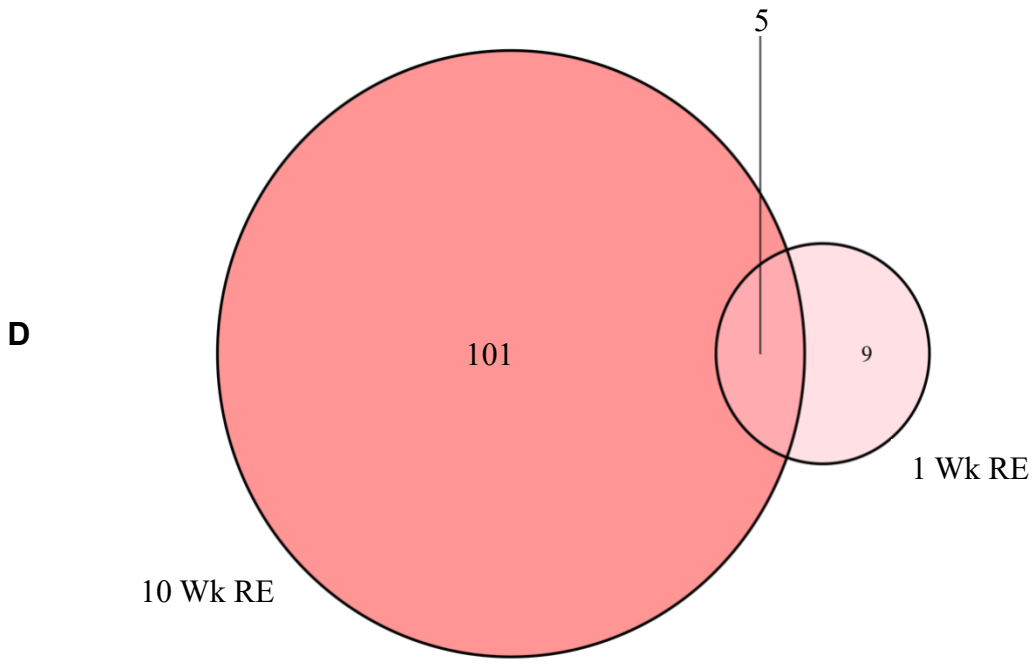
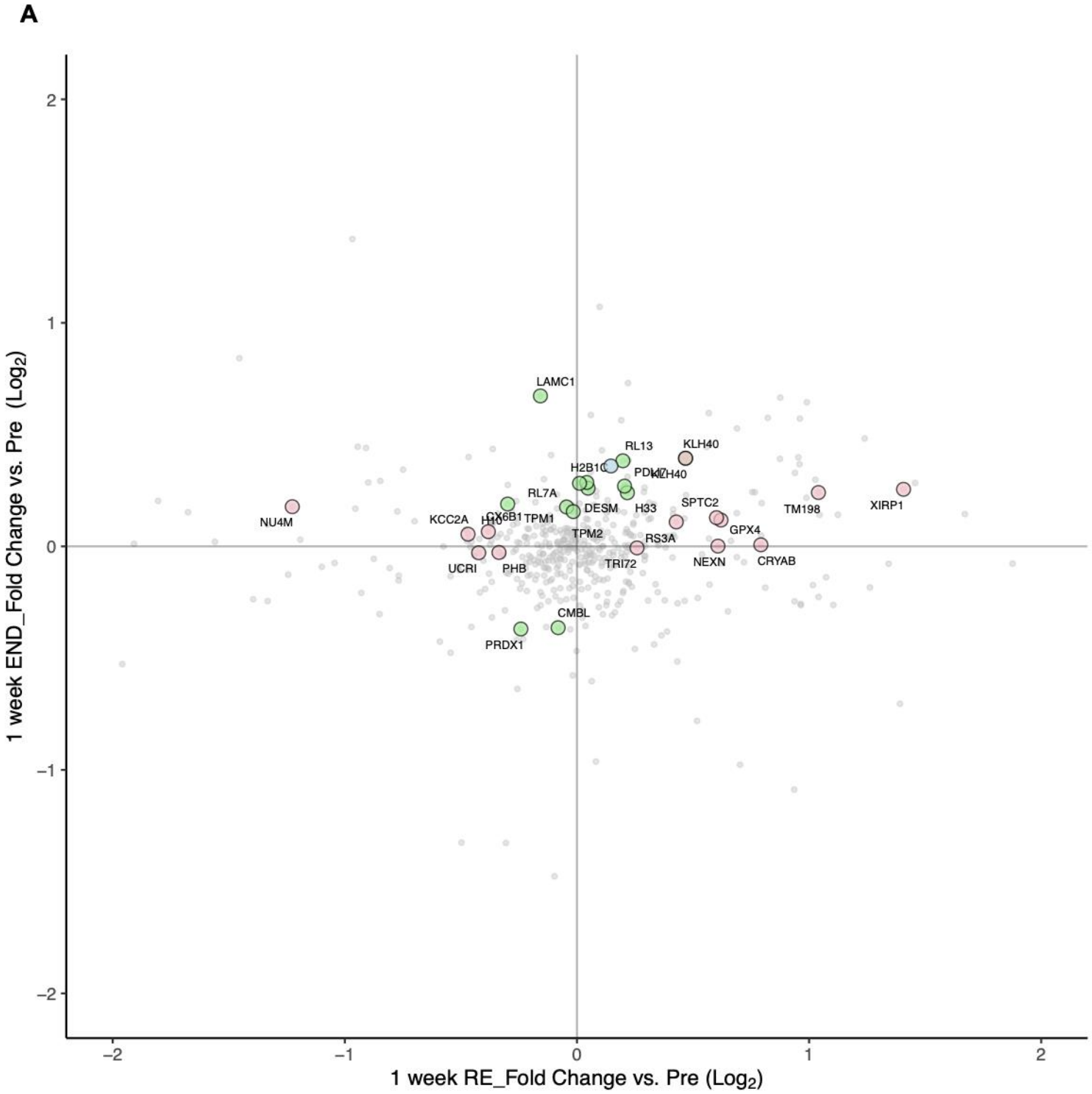
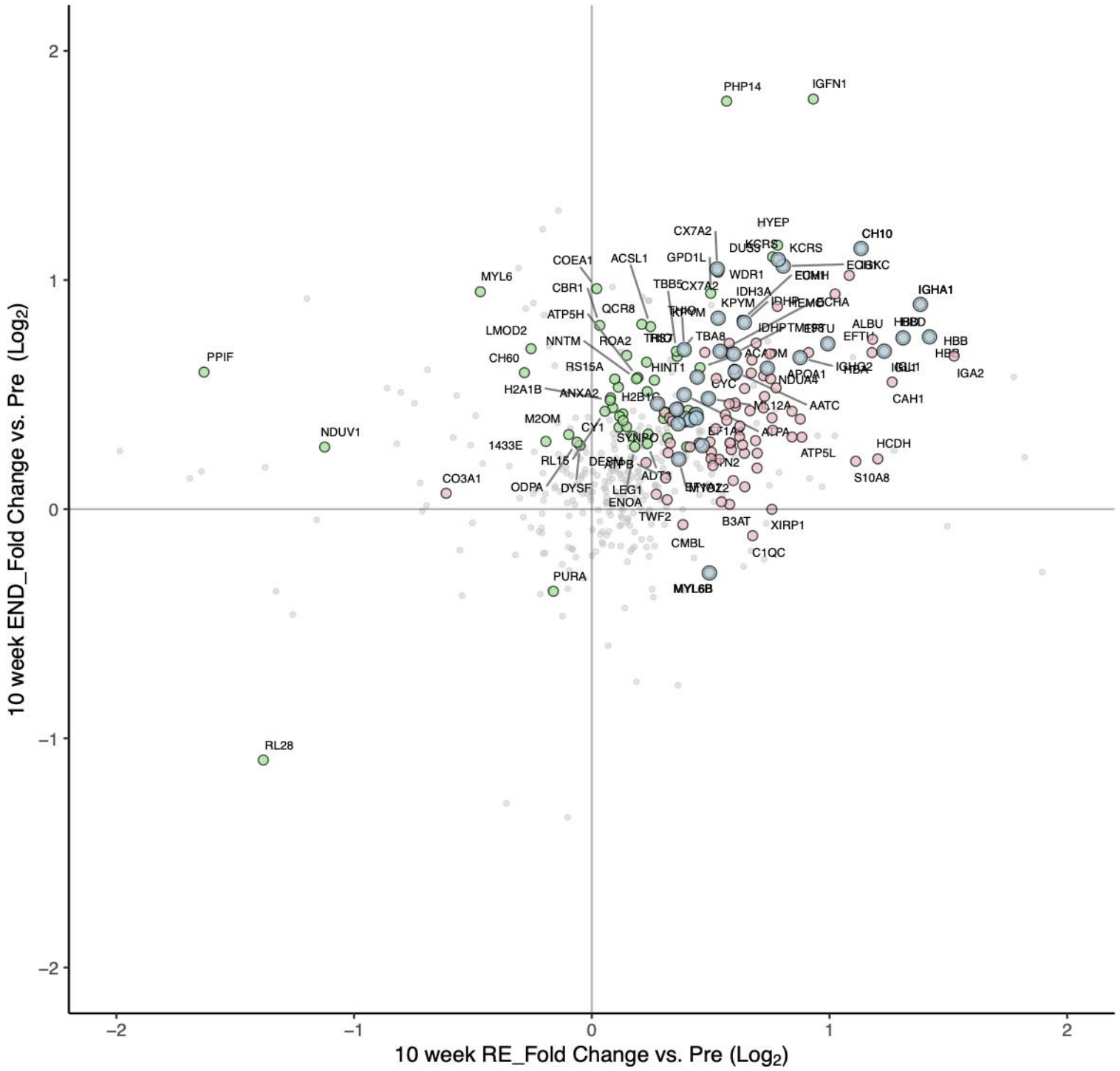


Figure 3.7. (A, B) Volcano plots of fold change (log2 AC/Pre and CR/Pre) of RE in response to 1 week (A) and 10 weeks (B) training, plotted against -log10 p value of T-test (time). (C) STRING protein interaction network of proteins that demonstrate $p<0.05$ following 10 weeks RE versus Pre. (D) Venn diagram of proteins that demonstrate a significant (time $p<0.05$) change in abundance ($fmol \cdot \mu g^{-1}$) in response to 1 week and 10 weeks training.


A
1 week END_Fold Change vs. Pre (Log2)
1 week RE_Fold Change vs. Pre (Log2)
2
1
0
−1
−2
−2
−1
0
1
2
LAMC1
RL13
KLH40
H2B1C
PDLI7
KLH40
RL7A
SPTC2
TM198
XIRP1
NU4M
KCC2A
CX6B1
TPM1
DESM
H33
TPM2
GPX4
RS3A
UCRI
PHB
TRI72
NEXN
CRYAB
CMBL
PRDX1

B


10 week END_Fold Change vs. Pre (Log2)
10 week RE_Fold Change vs. Pre (Log2)
2
1
0
−1
−2
PHP14
IGFN1
CX7A2
HYEP
CH10
KCRS
DUSS
COEA1
ACSL1
GPD1L
MYL6
WDR1
CBR1
TBB5
CX7A2
IDH3A
IGHA1
KPYM
ATP5H
QCR8
LMOD2
TBA8
ALBU
HBB
HBB
PPIF
NNTM
ROA2
CH60
HINT1
HBB
IGA2
RS15A
NDUA4
H2A1B
ANXA2
CAH1
AATC
M2OM
NDUV1
CY1
ACPA
1433E
HCDH
ATP5L
RL15
CO3A1
S10A8
ODPA
DYSF
LEG1
ENOA
TWF2
B3AT
XIRP1
CMBL
C1QC
PURA
MYL6B
RL28

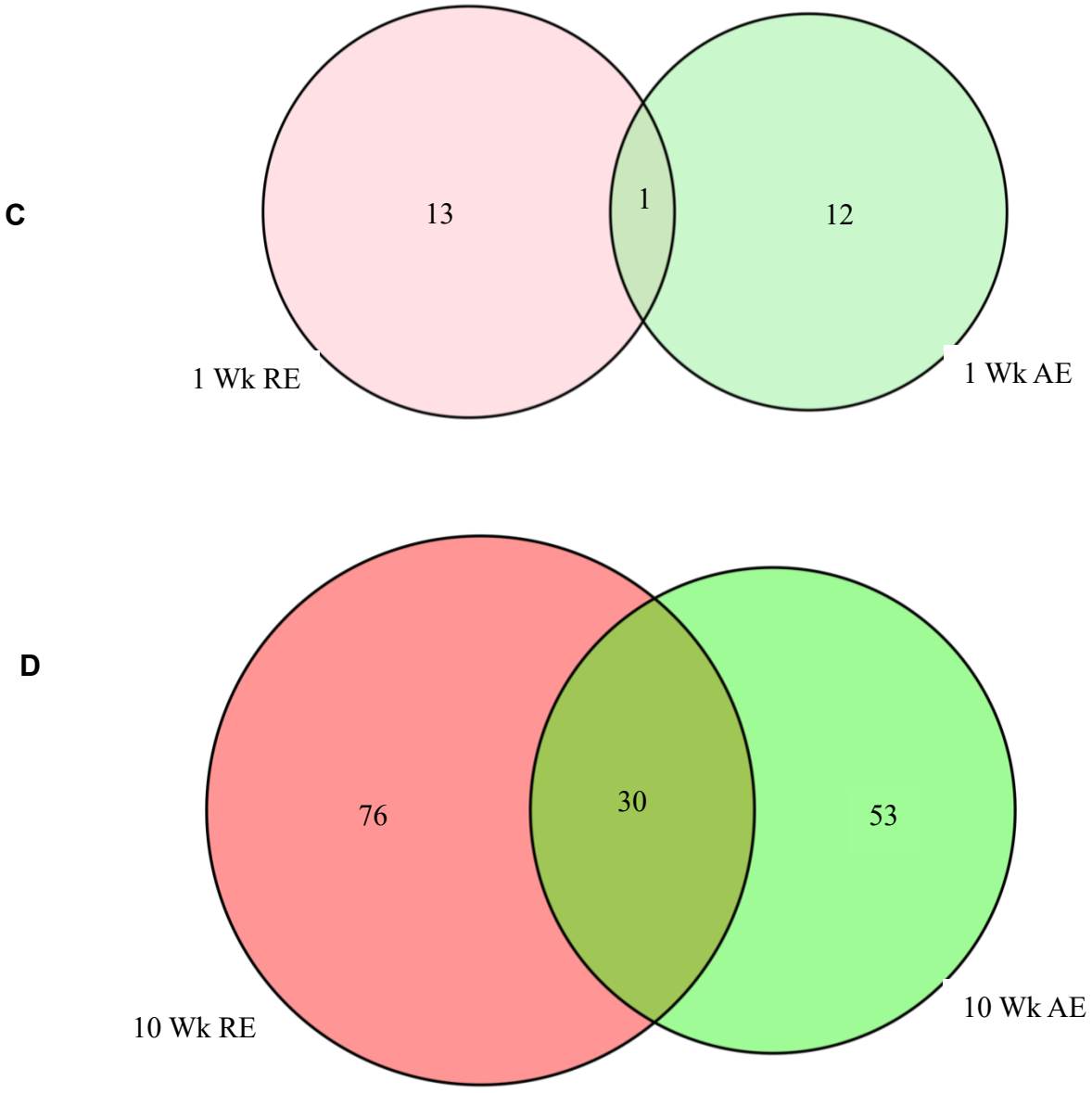


Figure 3.8. (A, B) Quadrant plots of proteins that demonstrate a significant (time $p<0.05$) change in abundance ($fmol \cdot \mu g^{-1}$) in response to 1 week and 10 weeks training. Blue points represent proteins that are common to AE and RE. (C, D) Venn diagram of proteins that demonstrate a significant (time $p<0.05$) change in abundance ($fmol \cdot \mu g^{-1}$) in response to 1 week (C) and 10 weeks (D) training.

Protein turnover rates. Calculation of mixed-protein FSR was derived from 39 individual protein FSRs (measured on n = 7-14 individuals), which incorporated the synthesis rates of the top 25 % of the most abundant proteins within our analysis. No significant differences in mixed-protein FSR ($p > 0.05$) were observed across AE and RE conditions. On average, the % change in individual participant mixed-protein FSR was ~13.6 % greater after 1 week of RE (1.49 ± 0.49 %/d) compared to baseline (1.37 ± 0.38 %/d). Ten weeks of training resulted in a 25 and 19% change increase for individual participant mixed-protein FSR in both AE (1.58 ± 0.54 %/d) and RE (1.53 ± 0.54 %/d) trained muscle compared to baseline, respectively (Figure 9)

Given the exploratory nature of this work, we elected to use paired t-tests were used to compare individual protein synthesis rates at baseline to the acute and chronic response to RE and AE. From a total of 41 proteins measured across the acute (1-week) period, 1 and 3 proteins exhibited significant ($p < 0.05$) changes in synthesis rate in response to AE (1 up) and RE (2 up, 1 down), respectively (Figure 10). In the acute period, one protein (MYOM: myomesin) showed ($p=0.10$ - 0.20) an increase in FSR vs. BL, common between AE and RE (Figure 11A). One protein (MYPC: myosin bind protein c) indicated a contrasting change in FSR between AE (increase, $p<0.10$) and RE (decrease, $p<0.05$).

From a total of 53 proteins measured across the chronic (10 week) period, 3 and 4 proteins exhibited significant ($p < 0.05$) changes in synthesis rate in response to AE (2 up, 1 down) and RE (3 up, 1 down), respectively (Figure 11B). In the chronic period, two proteins, RYR1 (ryanodine receptor 1) and FLNC (filamin C), demonstrated a significant increase ($p<0.05$) in

FSR compared to baseline. Two proteins, PDLI3 (PDZ, LIM domain protein 3) and DESM (desmin), also tended ($p<0.20$) to increase FSR relative to baseline following 10 weeks of exercise (AE and RE). One protein, MYL3 (myosin light chain 3), appeared to decrease in FSR relative to BL common between AE and RE following 10 weeks of exercise.

Inspection of the relationship of the change (vs. BL) between individual proteins' FSR (log2 %/d) between week 1 (AC) and week 10 (CR) for AE (Figure 10A) and RE (Figure 10B) indicated that proteins with greater relative FSR in week 1, also tended to have greater FSR at week 10. Similarly, proteins with increased FSR following 1 week AE, appeared to also respond analogously following 1 week RE (Figure 11A). Lastly, a comparable protein FSR relationship to 1-week EX is demonstrated again at week 10, where proteins with increased FSR following AE appeared to also respond to RE (Figure 11B).

Figures 12 and 13 summarize data from 1- and 10-week exercise that compares within-participant changes in protein abundance with the change in protein FSR, illustrating the variety of unique relationships between protein synthesis and abundance following exercise. Typical of what may be anticipated, proteins positioned in the upper right quadrant were more abundant after exercise training and exhibited a greater relative synthesis rate (compared to BL). Similarly, proteins in the lower left quadrant were less abundant after training and exhibited a lower relative synthesis rate (compared to BL). After exercise training, some proteins demonstrated a discordant relationship between synthesis and abundance. For example, proteins in the upper left quadrant exhibited a decrease in protein synthesis despite an increase in FSR (compared to BL). Lastly, proteins in the lower right quadrant exhibited an increase in abundance despite a relative

decrease in synthesis. The discordance between abundance and synthesis suggests a dynamic contributing and significant role of protein breakdown in the determination of net protein abundance and protein turnover. Table 3 (end of document) demonstrates the proteome responses of individual proteins to 10 weeks AE and RE.

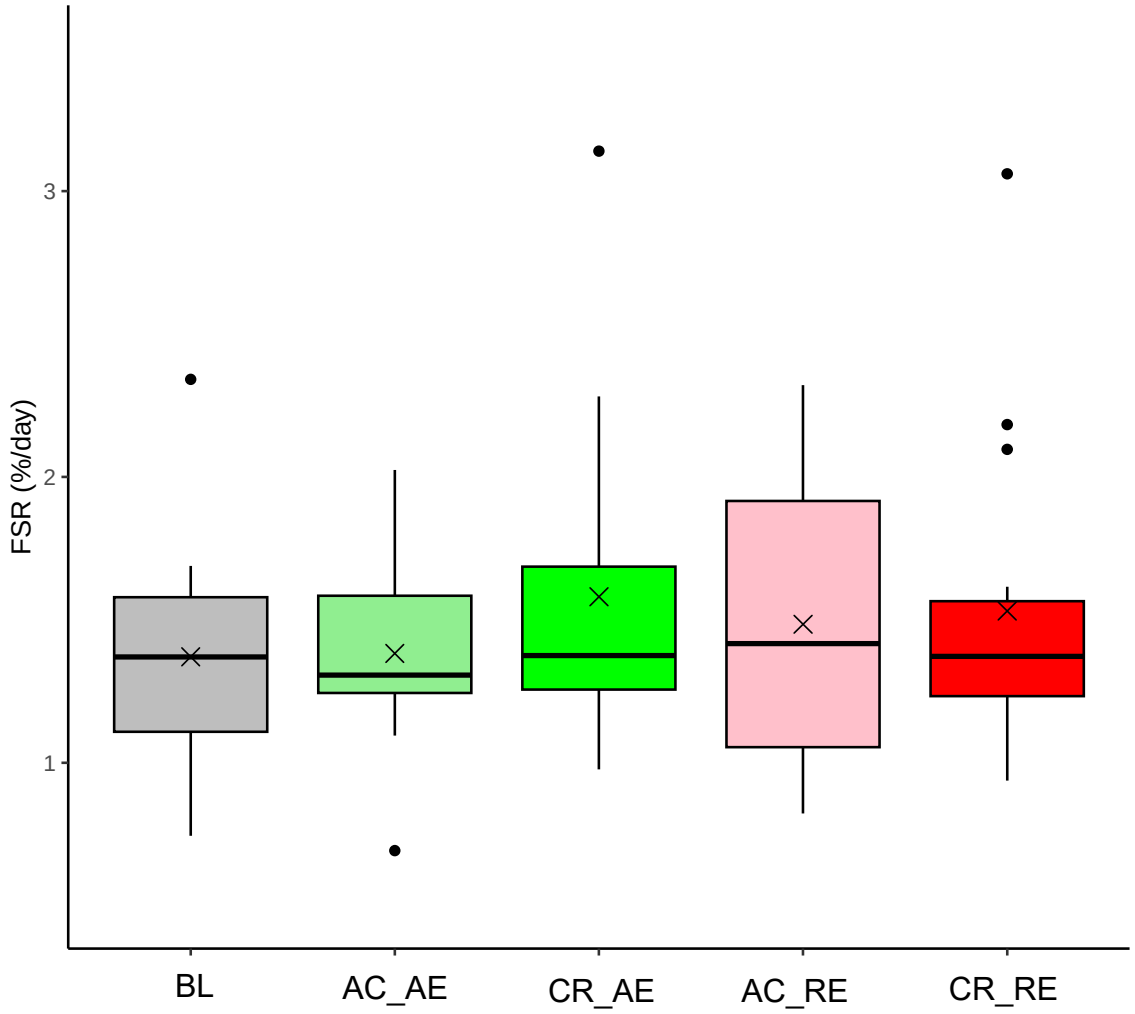
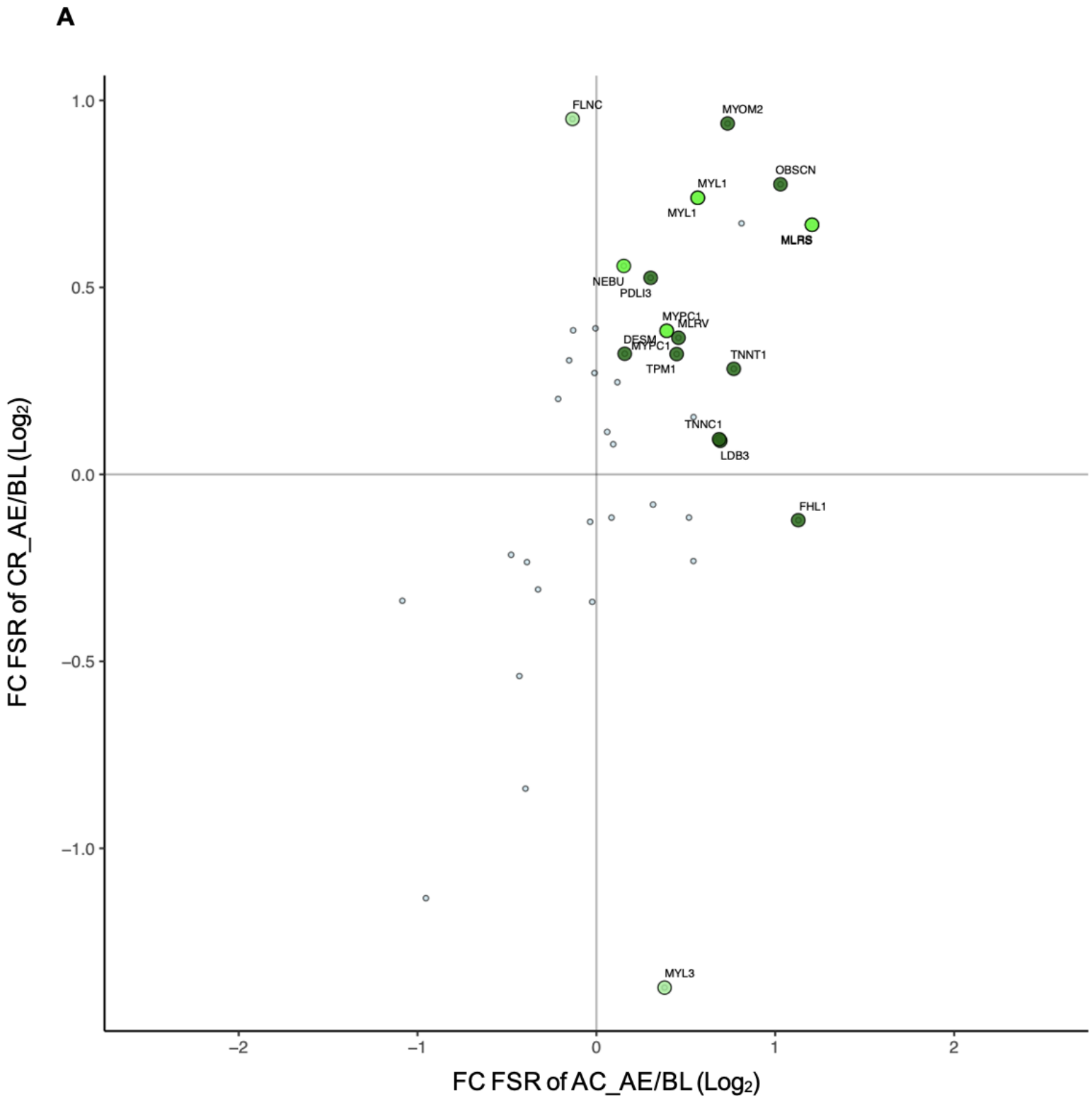


Figure 3.9. Mixed protein myofibrillar FSR, expressed as %/d, for the baseline (BL), 1st week aerobic (AC_AE), 1st week resistance (AC_RE), 9-10th week aerobic (CR_AE) and 9-10th week resistance (CR_RE) time periods. Red line separates the acute (pre-training) and chronic (post-training) periods.


A
FLNC
MYOM2
OBSCN
MYL1
MYL1
MLRS
NEBU
PDLI3
MYPC1
MLRV
DESM
MYPC1
TNNT1
TPM1
TNNC1
LDB3
FHL1
MYL3
1.0
0.5
0.0
−0.5
−1.0
−2
−1
0
1
2
FC FSR of CR_AE/BL (Log2)
FC FSR of AC_AE/BL (Log2)

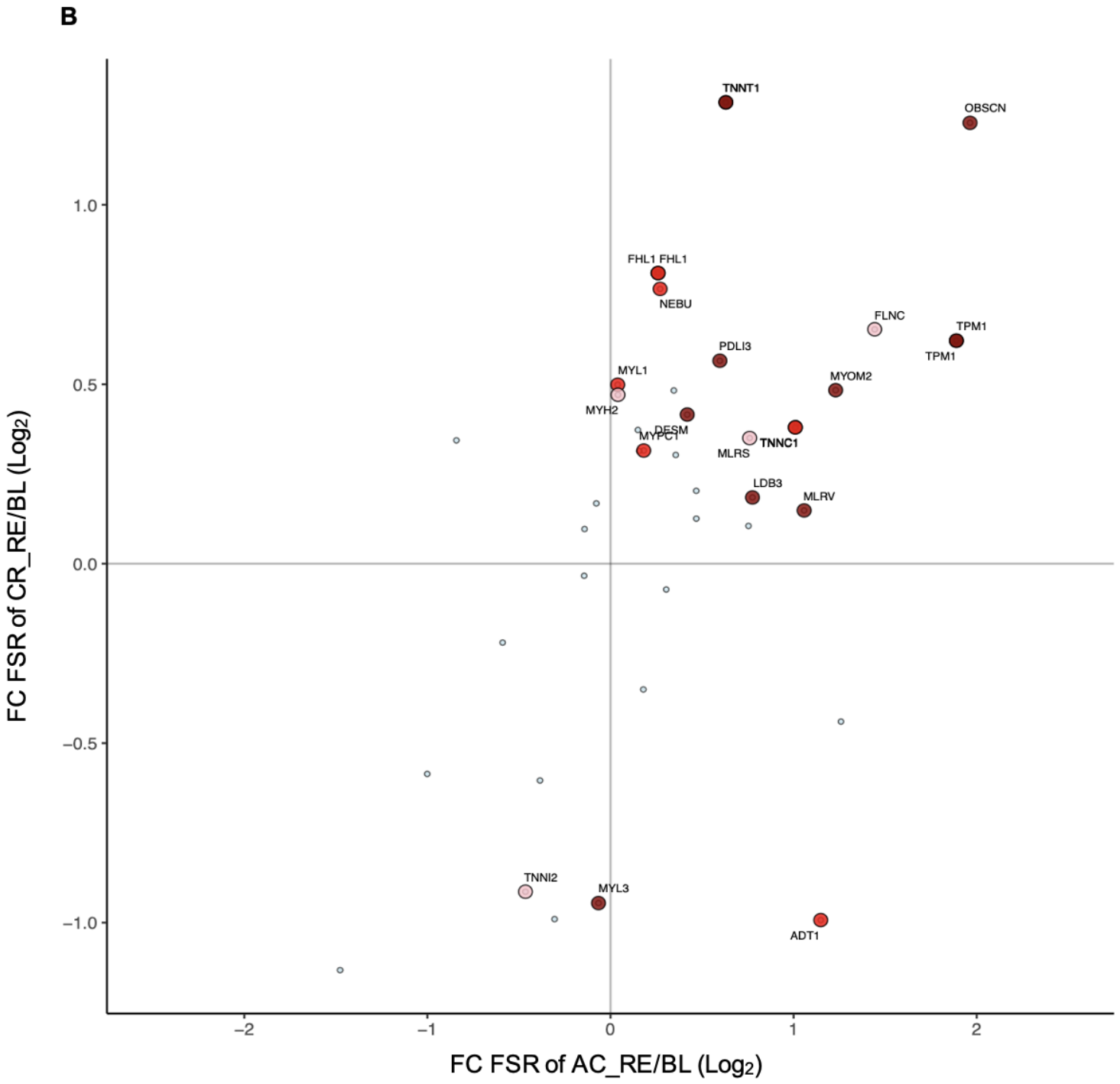
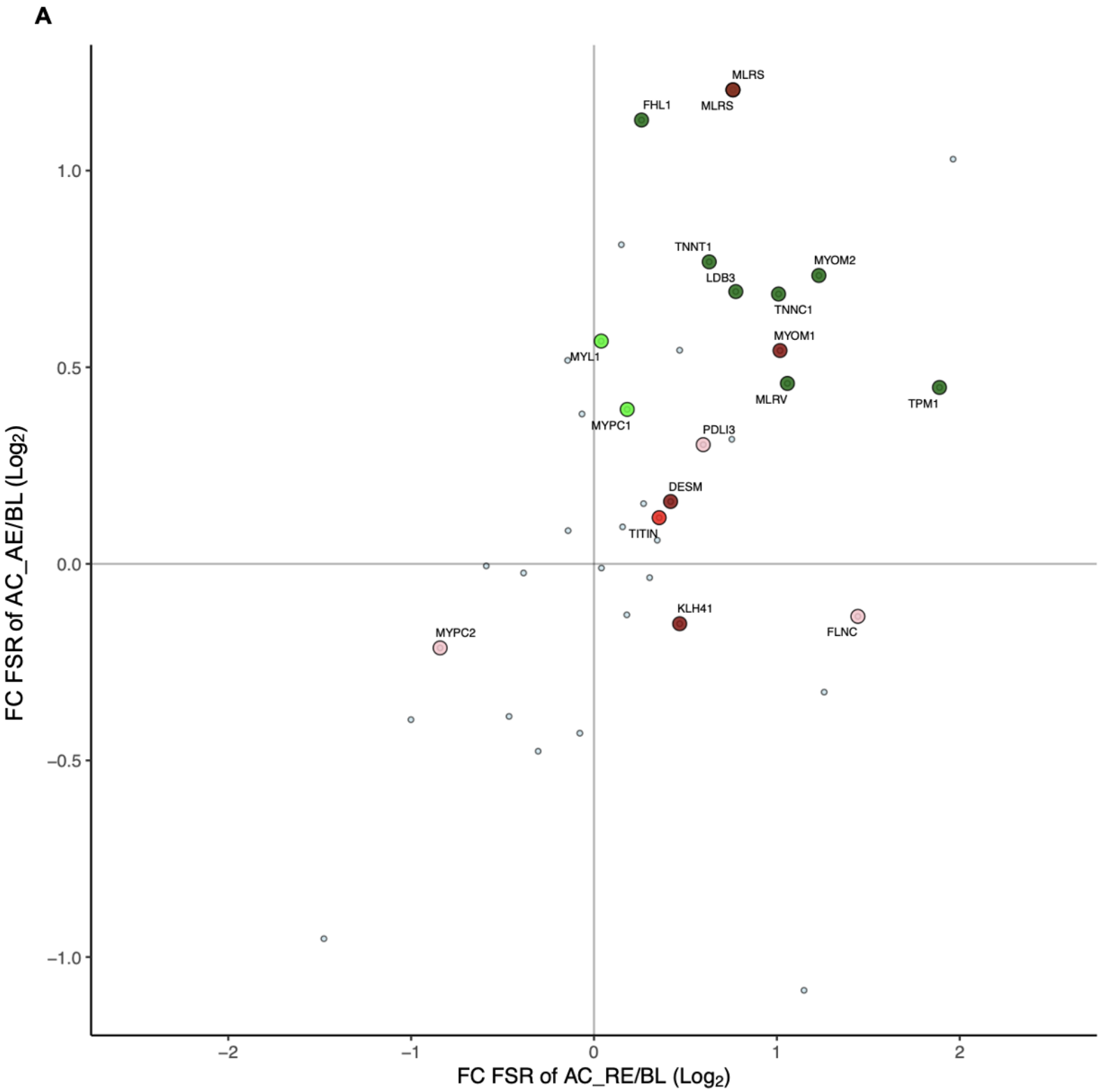


Figure 3.10. (A) Fractional synthetic responses to AE (A) and RE (B) following 1 week (AC) and 10 weeks (CR) of exercise training. Summary data presented are from n=41 (AC) and n=53 (CR) proteins' FSR in n=7-14 participants. Data presented as individual protein synthesis expressed as Log_2 (FC vs. BL). Greater relative synthesis as compared to BL in week 1 (AC) and week 10 (CR) (upper right quadrant). Lower relative synthesis in week 1 (AC) and week 10 (CR) (lower left quadrant).


A
MLRS
FHL1
MLRS
TNNT1
MYOM2
LDB3
TNNC1
MYOM1
MYL1
MLRV
TPM1
MYPC1
PDLI3
DESM
TITIN
KLH41
MYPC2
FLNC
1.0
0.5
0.0
−0.5
−1.0
−2
−1
0
1
2
FC FSR of AC_AE/BL (Log2)
FC FSR of AC_RE/BL (Log2)

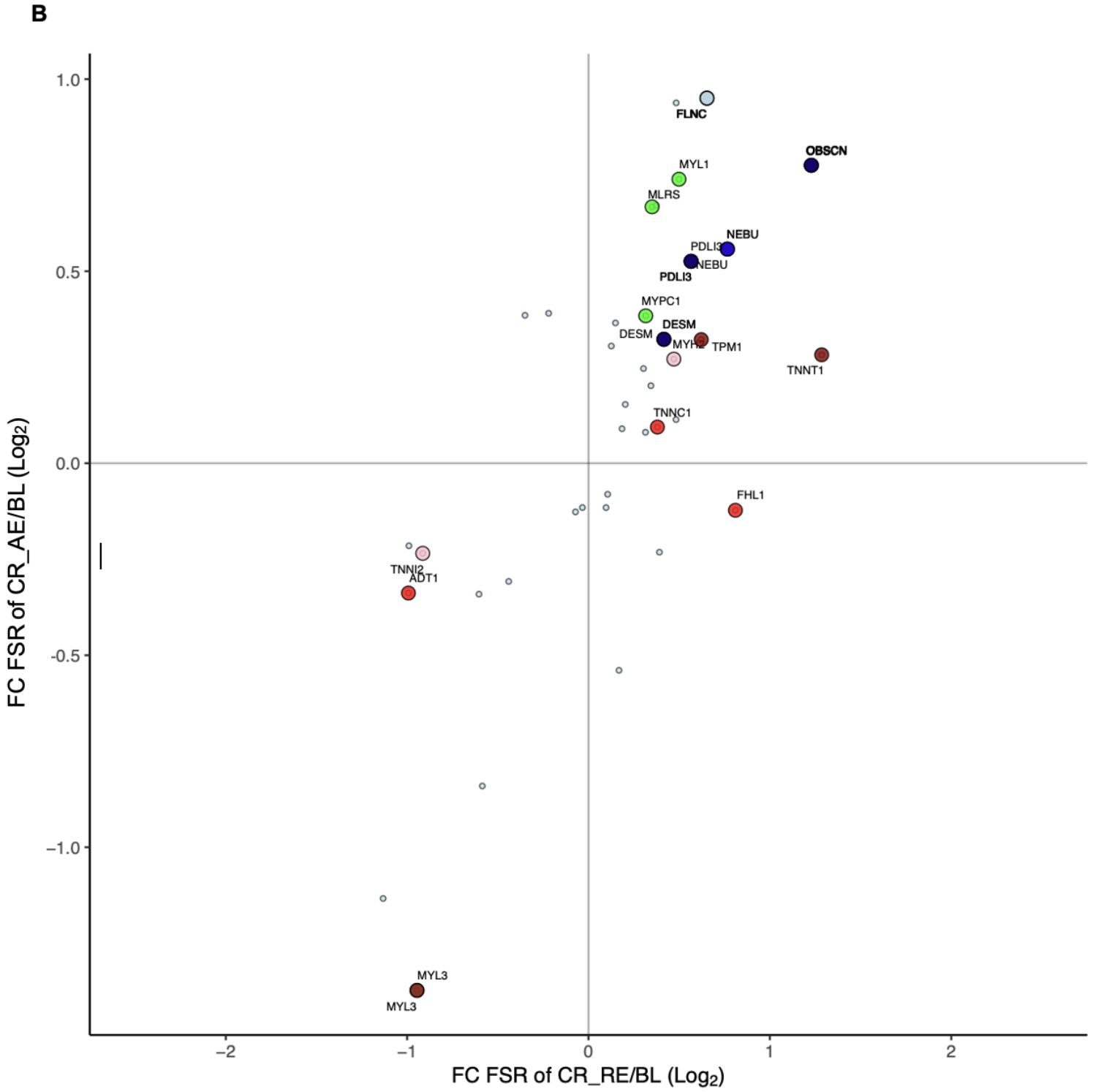
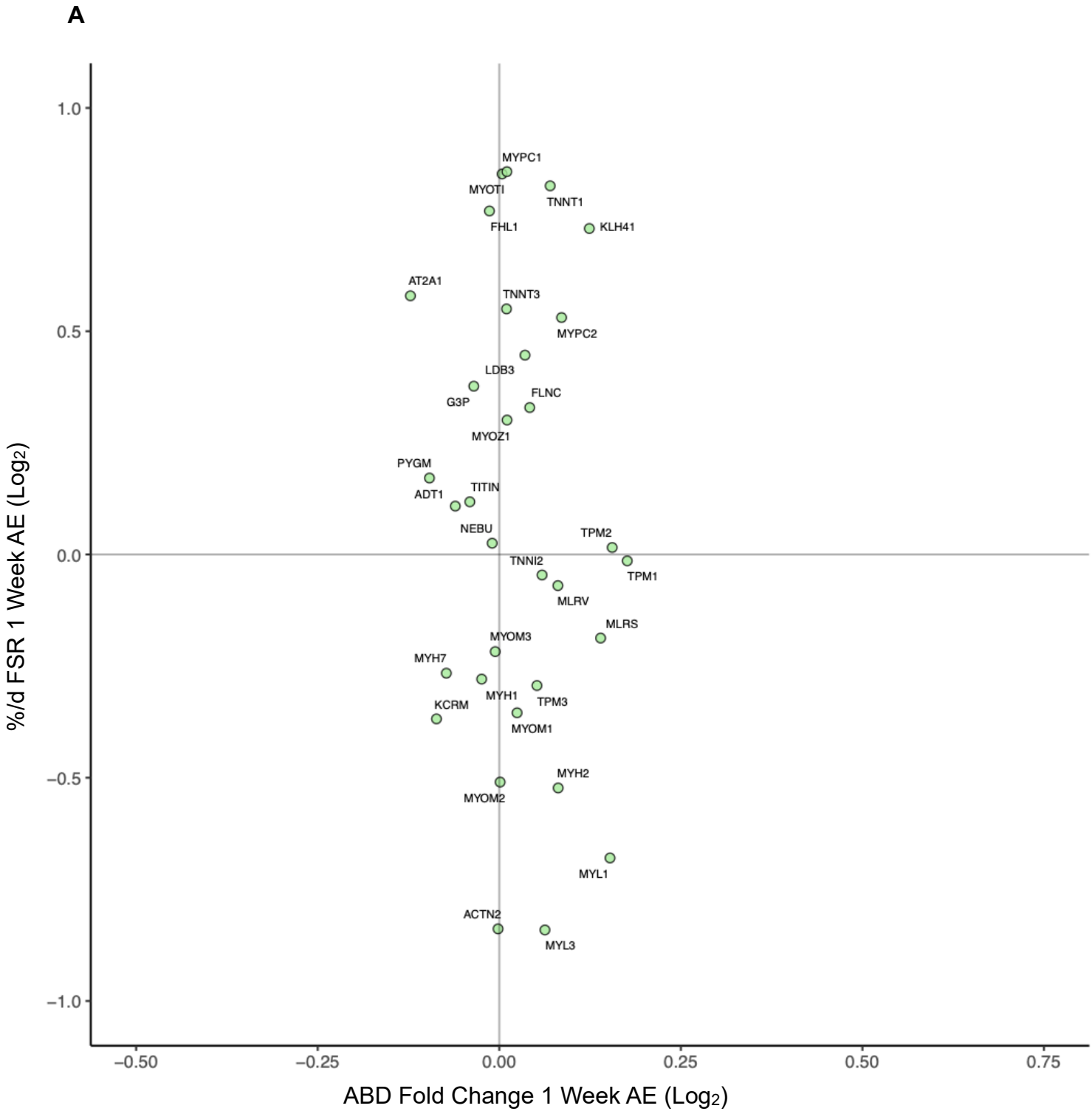


Figure 3.11. Fractional synthetic responses to 1 week (A) and 10 weeks (B) of exercise training. Greater relative synthesis in both AE and RE (upper right quadrant). Lower relative synthesis in both AE and RE (lower left quadrant). Proteins FSR significance vs. BL is denoted by color with p-values; darker (p=0.05 - 0.20) to lighter (p<0.05).


A
1.0
0.5
0.0
−0.5
−1.0
−0.50
−0.25
0.00
0.25
0.50
0.75
%/d FSR 1 Week AE (Log2)
ABD Fold Change 1 Week AE (Log2)
MYPC1
MYOT1
TNNT1
FHL1
KLH41
AT2A1
TNNT3
MYPC2
LDB3
G3P
FLNC
MYOZ1
PYGM
ADT1
TITIN
NEBU
TPM2
TNNI2
TPM1
MLRV
MLRS
MYOM3
MYH7
MYH1
TPM3
KCRM
MYOM1
MYH2
MYOM2
MYL1
ACTN2
MYL3

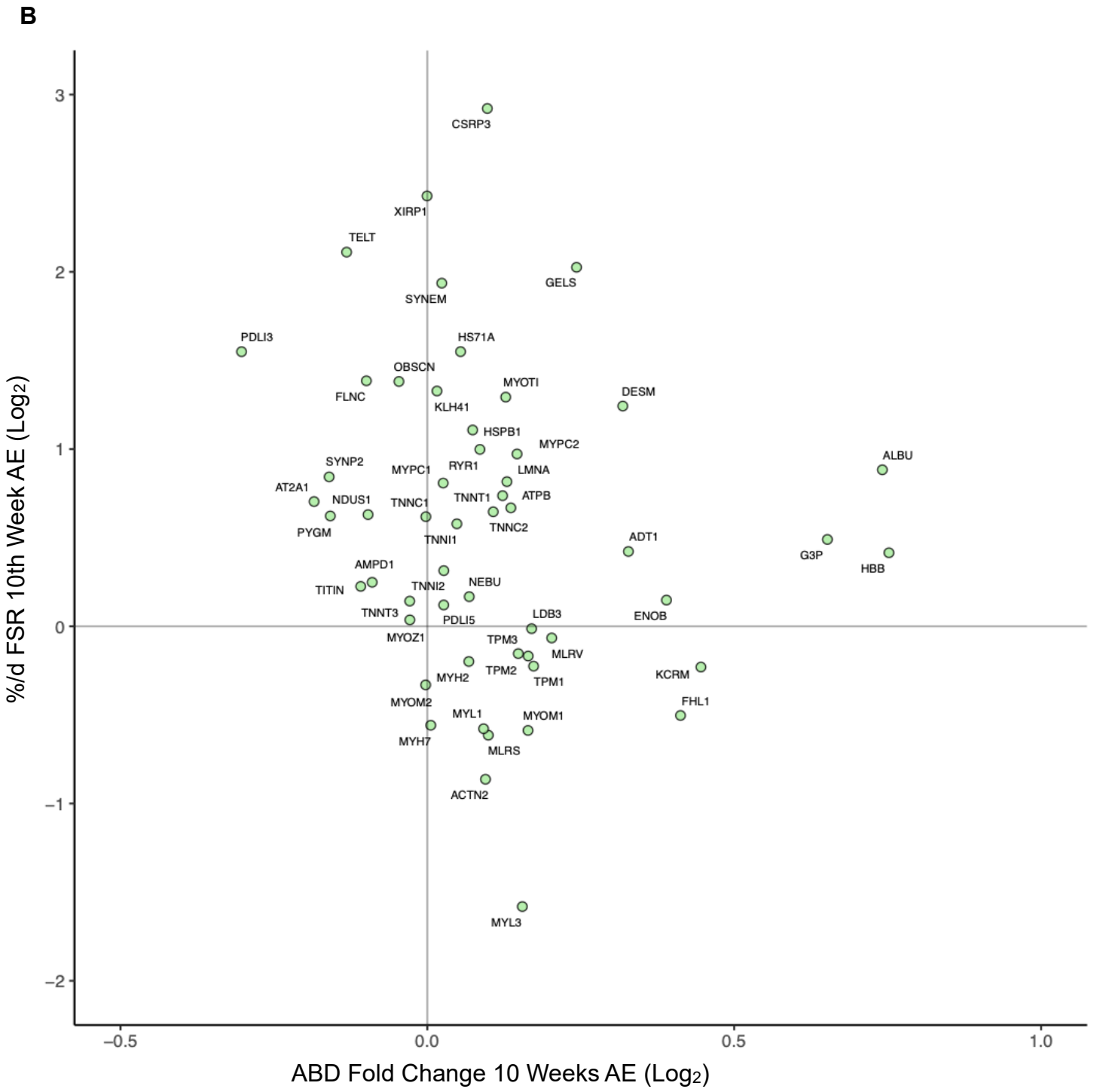
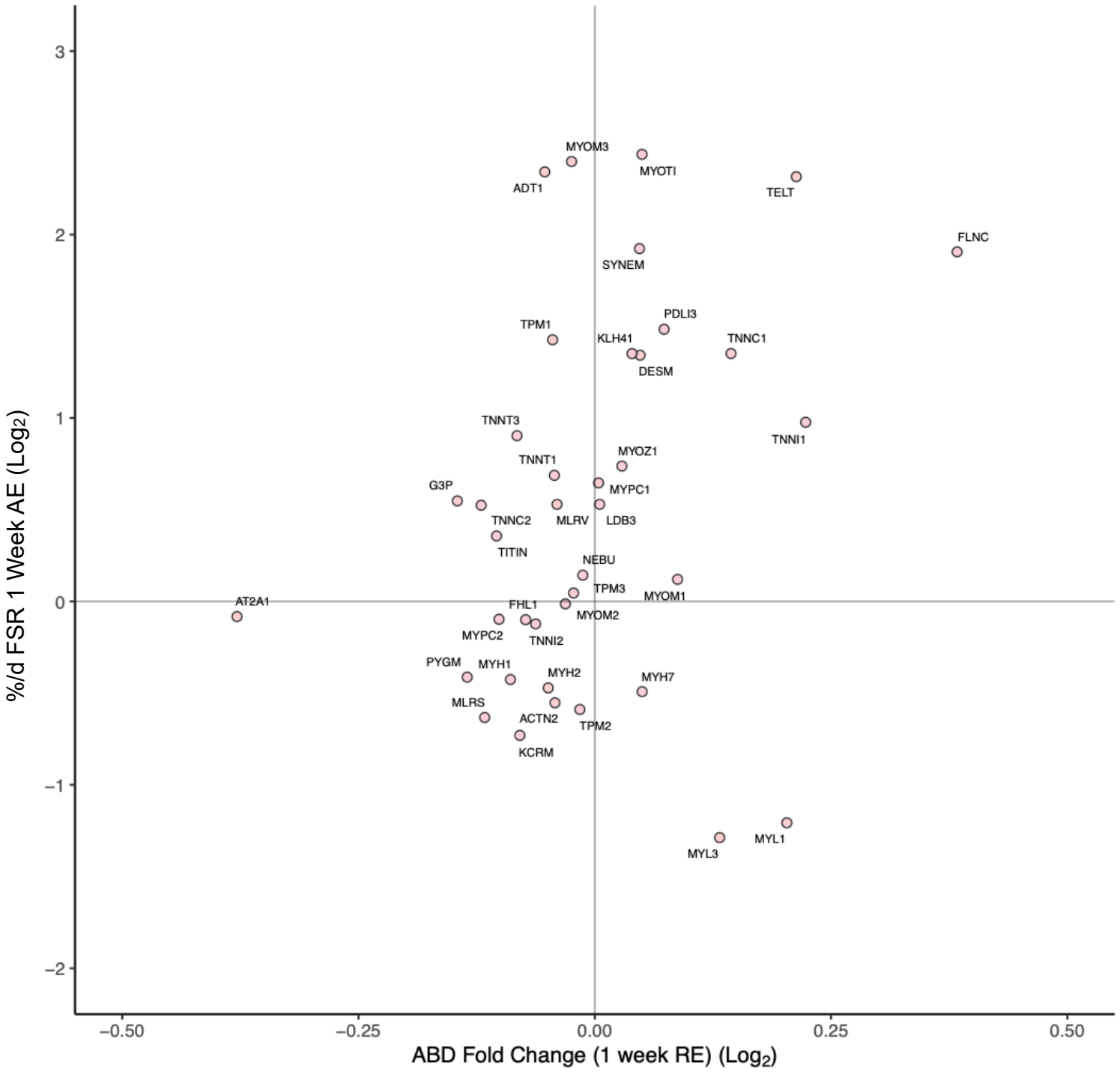


Figure 3.12. Dynamic proteome responses to AE following 1 week (A) and 10 weeks (B) of exercise training. Summary data presented are from n=41 (acute) and n=53 (chronic) proteins' FSR in n=7-14 participants. Data presented as fold change relative to BL. Increase in synthesis, increase in abundance (upper right quadrant). Increase in synthesis, decrease in abundance (upper left quadrant). Decrease in synthesis, increase in abundance (lower right quadrant). Decrease in synthesis, decrease in abundance (lower left quadrant).

A


%/d FSR 1 Week AE (Log2)
ABD Fold Change (1 week RE) (Log2)
3
2
1
0
−1
−2
−0.50
−0.25
0.00
0.25
0.50
MYOM3
MYOTI
ADT1
TELT
FLNC
SYNEM
PDLI3
TPM1
KLH41
TNNC1
DESM
TNNT3
TNNI1
MYOZ1
TNNT1
G3P
MYPC1
TNNC2
MLRV
LDB3
TITIN
NEBU
TPM3
MYOM1
AT2A1
FHL1
MYOM2
MYPC2
TNNI2
PYGM
MYH1
MYH2
MYH7
MLRS
ACTN2
TPM2
KCRM
MYL1
MYL3

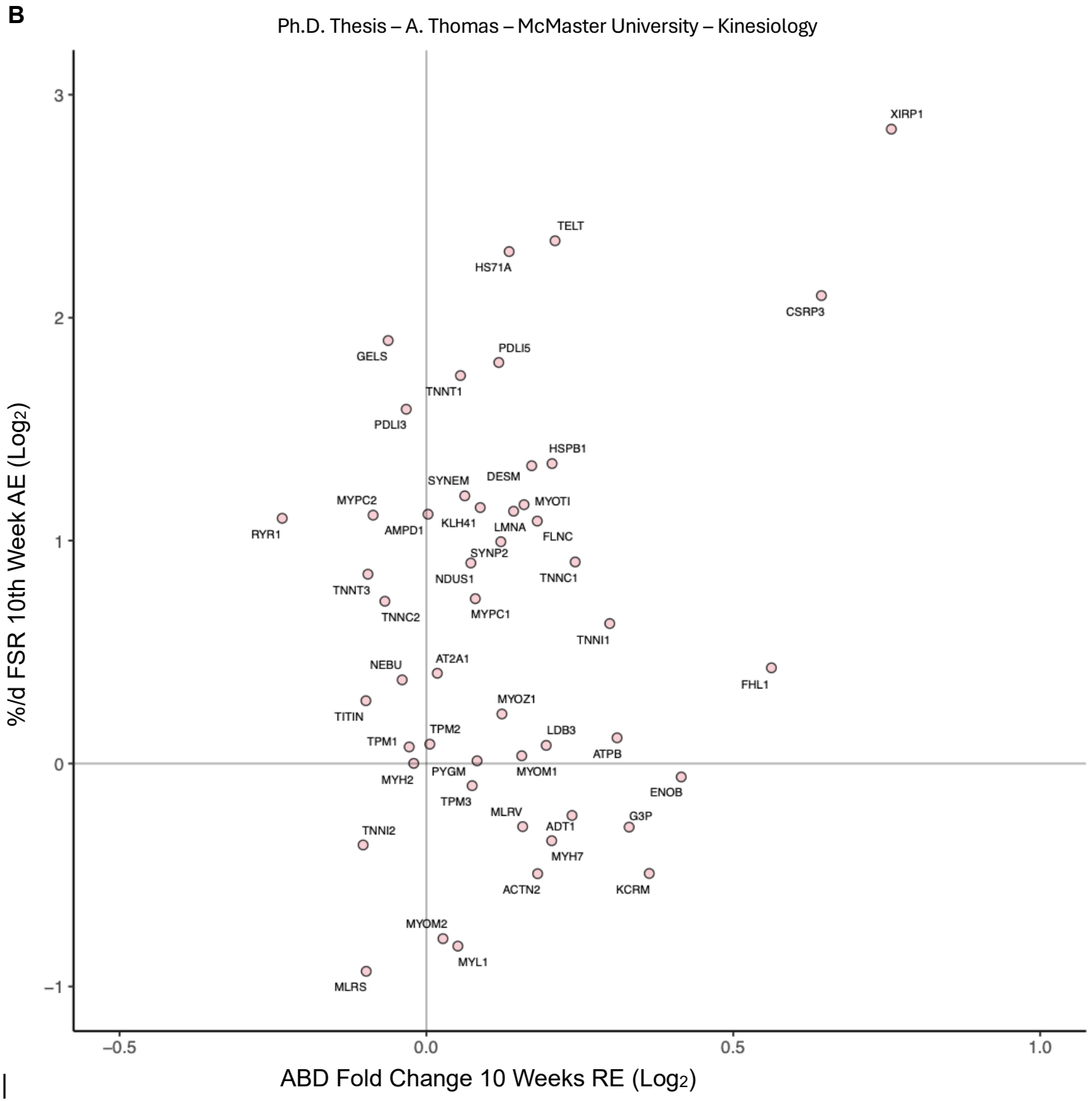


Figure 3.13. Dynamic proteome responses to RE following 1 week (A) and 10 weeks (B) of exercise training. Summary data presented are from n=41 (acute) and n=53 (chronic) proteins' FSR in n=7-14 participants. Data presented as fold change relative to BL. Increase in synthesis, increase in abundance (upper right quadrant). Increase in synthesis, decrease in abundance (upper left quadrant). Decrease in synthesis, increase in abundance (lower right quadrant). Decrease in synthesis, decrease in abundance (lower left quadrant).

Discussion

There is a paucity of information regarding the common and unique changes that occur in the muscle proteome following aerobic (AE) and resistance (RE) training. Our proteome-wide analysis, which yielded a small fraction (474 out of thousands of proteins), highlighted increases in the abundance of mitochondrial proteins alongside an increase in the abundance of proteins associated with contractile and structural elements (e.g. titin, myosin heavy chain isoforms and actin), as well as protein subunits involved with the regulation of protein synthesis (e.g. eukaryotic elongation initiation factors and ribosomal subunits). These proteome changes characterized the conserved adaptive response of muscle to each exercise mode. Interestingly, the current analyses suggest the integration of individual abundance and synthesis rate changes reveal protein breakdown also to be a principal component of differential proteome remodelling between aerobic- and resistance-trained muscle.

Overall, changes in protein abundance predominantly occurred following chronic (10 weeks; 159 proteins $p<0.05$) as opposed to acute (1 week; 29 proteins $p<0.05$) exercise training (AE and RE). Acutely, only 1 protein's (KLH40) abundance was commonly regulated ($p<0.05$) by AE and RE. The exact function of KLH40 is not currently known; however, it has been suggested to play a role as a substrate-specific adapter of a BCR (BTB-CUL3-RBX1) E3 ubiquitin ligase complex, acting as a key regulator of skeletal muscle development (Ravenscroft, Miyatake et al. 2013). In contrast, 30 proteins were regulated in common, based on abundance data, following 10 weeks of AE and RE training ($p<0.05$). GO analysis did not reveal a clear compartment or function for proteins commonly regulated following 10 weeks EX (AE and RE). However, 43% (13/30) of proteins commonly upregulated included mitochondrial subunits (ATPA, AT5F1, CX7A2,

ECHA, NDUA4) and proteins (CH10, CYC, ECH1, EFTU, IDHP, KCRS, MPCP3, NB5R1). These data highlight the relevance of regulating mitochondrial protein content alongside changes in contractile activity, regardless of modality, to support elevated metabolic stress, ultimately improving muscle function.

Eukaryotic translation initiation and elongation factors represent a crucial step in post-transcriptional processes relating to the initiation of protein synthesis. The increases in muscle protein synthesis following resistance training have been previously associated with enhanced translation initiation associated with phosphorylation of eukaryotic initiation factor (eIF4E-binding protein 1) and subsequent binding to the 5' end of mRNA to form the active translation initiation complex (Saxton and Sabatini 2017). Similarly, increased activity of eIF4F has been previously demonstrated to correlate well with myofibrillar protein synthesis in response to both aerobic and resistance exercise (Wilkinson, Phillips et al. 2008). Our dynamic proteome profiling identified a significant increase ($p<0.05$) in EF1A2 (elongation factor 1-alpha) and IF4A2 (eukaryotic initiation factor 4A) protein abundance following 10 weeks of AE and RE. EF1A2 expressed specifically in neurons and muscle, is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome (Doig, Griffiths et al. 2013). Whereas IF4A2, highly expressed in skeletal muscle, is an RNA-dependent ATPase and ATP-dependent RNA helicase that functions to melt the 5′ proximal secondary structure of eukaryotic mRNAs in order to facilitate binding of the 40S ribosomal subunit (Li, Imataka et al. 1999). While we quantify the abundance of several other proteins involved in translation elongation (e.g. EFA1A, EF1G, EF2, EFTU) and initiation (e.g. IF5A1), our analyses identified no significant differences in protein abundance or synthetic rate following 1- and 10- weeks exercise training. Unfortunately, several

known key translation initiation and elongation factors (e.g. eIF4E, eIF4F, eIF4B, eIF5A1, etc.) were not included in our current proteome profiling. Additionally, the phosphorylation status of these proteins often dictates their activity and function; this method of analysis was not included, and therefore only changes in relative abundance and synthetic rate can be reported.

In general, there was a moderate positive correlation between the changes in synthetic rate (FSR) between AE and RE training muscle after 1 week (R= 0.37) and 10 weeks (R= 0.63) of training (Figure 11). Similarly, there was a moderate positive correlation between the changes in synthetic rate after 1- and 10 weeks RE (R=0.49) (Figure 10B). However, there was no clear relationship with AE between 1- and 10 weeks (R= 0.16) (Figure 10A) individual protein FSR changes versus BL. Despite the moderate agreement (R=0.37 and 0.63) between the FSR of AE and RE, we highlight 53 and 76 proteins with differential change in abundance across 10 weeks of exercise (Figure 8D). Given that the general pattern of a linear relationship was maintained within proteins FSR following 1 and 10 weeks of exercise and 1 and 10 weeks of RE. We propose that changes in abundance would also follow a similar expression pattern. Our data indicated that while specific protein abundances were differentially regulated in response to AE and RE, that training mode-dependant changes in synthesis that we could detect did not readily dictate the changes in abundance. Therefore, we hypothesize that the selective targeting of proteins for degradation may represent another key process involved in the adaptive remodelling of some proteins of the muscle proteome. Figures 12 and 13 show changes in protein abundance across training plotted against the synthetic rate in AC and CR training and highlights the dynamic and potentially significant contribution of protein breakdown in altering the muscle proteome and, ultimately, muscle phenotype. Following 1 week of AE and RE, 44% (18/41) and

34% (14/41) of proteins' FSR fold change relative to baseline did not align (up/down or down/up) with the individual fold change relative to baseline in protein abundance, respectively. Following 10 weeks of AE and RE, ~51% (27/53) and ~40% (21/53) of proteins' FSR fold change relative to baseline did not align (up/down or down/up) with the individual fold change relative to baseline in protein abundance, respectively. These data suggest there may be an influence of protein breakdown on the determination of protein content.

The phenotypic differences between resistance and aerobic training on muscle are well established. However, there is currently limited data identifying which components of exercise-induced proteome remodelling are linked to these differences. Our data reveal a moderate overlap in protein-specific abundance changes between AE and RE training when examined across the detectable proteome. Additionally, we observed a moderate positive correlation between the protein-specific synthetic responses to both training types. Previously, measurements of protein turnover that are largely restricted to bulk/mixed-protein fractional synthesis rate have typically shown that aerobic training increases mitochondrial protein synthesis, while resistance training enhances myofibrillar protein synthesis (Wilkinson et al., 2008). However, these methods fail to provide details on protein-specific turnover, especially for lower abundance proteins, whose turnover rates may not significantly affect mixed-protein FSR measurements due to the vast dynamic range of protein synthesis of the skeletal muscle proteome. In the present analyses, we observed that commonly regulated proteins following exercise expanded from 1 to 30 following 10 weeks of training. These 30 proteins made up ~36% (30/83) and ~29% (30/103) of the total significant proteins that changed in abundance following 10 weeks of training and may be key proteins responsible for supporting the

phenotypic adaptation of each form of exercise. Additionally, our data indicated that increases in individual protein turnover are similarly influenced by aerobic and resistance training. Contrarily, 61 and 83 proteins were exclusively regulated following AE and RE, respectively. GO analyses of the AE modality-specific terms indicated an enrichment in the biological process of 'oxidative phosphorylation' (FDR = 0.00067), 'energy derivation from oxidation of organic compounds' (FDR = 0.0013) and 'sarcomere organization' (FDR= 0.0021). Additionally, the top-ranked (FDR) enriched GO terms for cellular components included 'inner mitochondrial membrane protein complex' (FDR =0.00038) and 'mitochondrial protein-containing complex' (FDR=0.00038). Contrarily, the top-ranked (FDR) GO biological process associated with proteins that changed in abundance following RE only were 'monocarboxylic acid metabolic process' (FDR = 0.0102) and 'cellular respiration' (FDR = 0.0128). Interestingly, the top-ranked (FDR) GO cellular component of these proteins was annotated as 'mitochondrion' (FDR = 1.90e-5). However, the next highest-ranked GO cellular components are 'I band' (FDR = 0.00055) and 'myofibril' (FDR = 0.00055). Overall, our exploratory analysis indicates mitochondrial proteome remodelling within the myofibrillar fraction to be a key feature of exercise-induced adaptation.

Based on the current data, protein degradation appears dynamic and a component of adaptation that requires further investigation due to the potential role in modulating the selectivity of proteome adaptation. It should be acknowledged that measuring protein breakdown has proven technically challenging (Tipton, Hamilton et al. 2018), and thus, the influence of exercise on MPS is far better characterized than MPB. However, it is generally accepted that exercise mediates MPB, such that RE has been demonstrated to increase MPB (Biolo, Maggi et al. 1995, Phillips, Tipton et al. 1997, Kumar, Atherton et al. 2009). Currently, there is minimal evidence

for the selective or 'targeted' breakdown of proteins, yet this has been demonstrated in the remodelling of the ubiquitinated proteome of *C. elegans* with aging (Koyuncu, Loureiro et al. 2021). Furthermore, post-translational modifications are key to protein function, particularly in the context of translation initiation and degradative activity. Therefore, phosphoproteomics analysis would complement the current investigation well and be able to advance our understanding of exercise-induced differences in proteome adaptations between AE and RE.

Our study focussed on, and draws inferences based on, the differences in relative protein abundances, an effective approach to highlighting qualitative changes in the muscle proteome that are associated with changes in muscle phenotype rather than absolute changes in muscle mass, i.e. protein accretion. In contrast to previous proteomic profiling studies in human muscle (Deane, Phillips et al. 2022, Roberts, Ruple et al. 2023) that found very few changes in relative protein abundance in response to resistance training, we identified 105 proteins ($p<0.05$) that increased in abundance compared to baseline after 10 weeks resistance training. Interestingly, ~71% (75/105) of these proteins were exclusive to RE and not associated with any change in abundance following AE training. Specifically, our analysis did not show substantial increases in myofibrillar protein abundance, which is typically associated with mechanical loading adaptations leading to hypertrophy (Hornberger and Farrar, 2004). These data suggest that while proteome adaptation is not absent in response to resistance training, the absolute quantification of proteomics data may be necessary to thoroughly investigate adaptations related to protein accretion and increases in muscle mass. This outcome would require precise measurement of muscle volume, and volume changes to calculate total protein content accurately. Although we did not observe clear differences in muscle mass between aerobic and resistance training groups

(Figure 2D), previous studies using similar resistance training protocols have shown significant increases (~8%) in vastus lateralis cross-sectional area (CSA) after prolonged, repeated training [3 sets of 8-12 repetitions for leg press and extension at 80% 1RM, 3 times per week for 10 weeks; (Stokes, Timmons et al. 2020)]. Additionally, we report a significant increase in vastus lateralis CSA, following 10 weeks RE, assessed by ultrasound (Figure 3). Therefore, it can be inferred that resistance training may lead to an expansion of the total proteome pool rather than a reorganization of relative protein abundance, likely contributing to improvements in strength and force output. However, our data, along with previous supplementary proteomic analyses by (Robinson, Dasari et al. 2017), suggest qualitative changes in the mitochondrial proteome of resistance-trained muscles that may partly explain the resistance training-induced enhancements in skeletal muscle phenotype.

Conclusion

In conclusion, we report the first data to integrate the profiling of abundance and synthesis rates on a protein-by-protein basis after both resistance and aerobic training, in parallel, in men and women. To date, this work likely represents the most in-depth analysis of protein-specific fractional synthesis rates in human muscle in vivo in response to differential forms of exercise training. Our data allowed for the quantification and comparison of 41 and 53 protein synthesis rates after 1 and 10 weeks of training, respectively, to complement the analysis of 474 protein abundances. Our data illustrate the common and unique networks of protein regulation following AE and RE. Specifically we highlight the influence both AE and RE have on mitochondrial proteins, likely aimed at improving the metabolic function and possibly underpinning an increase in aerobic capacity.

Furthermore, in both AE and RE, we identified changes in protein abundance that do not align with individual protein synthetic rates, suggesting that targeted degradation is an adaptive feature of the shared response to aerobic and resistance training. Indeed, the analysis of changes in abundance and synthesis rates suggests degradation is regulated and may underpin the selective turnover of mitochondrial proteins with AE and RE training. Lastly, the analyses planned for the current tissue and data are only partially complete. Future analyses to be done include an identical proteomic investigation into the soluble muscle fraction (in progress) in addition to a phosphoproteomic investigation to tie together with our current data on changes in protein abundance and synthesis rates.

10 WEEKS AE (GREEN)
vs. RE (RED)

UniProt Identifier	Protein name	ABD change (%/d)	FSR (%/d)	FSB (%/d)	FSR p.val vs. BL	ABD change (%/d)	FSR (%/d)	FSB (%/d)	FSR p.val vs. BL
ACTN2	Alpha-actinin-2	-0.008	0.674	0.683	0.507	0.039	0.829	0.036	0.076
ADT1	ADP/ATP translocase 1	-0.008	1.423	1.431	0.014*	0.003	0.992	0.003	0.106
ALBU	Albumin	-0.059	1.582	1.649	0.128	0.007	1.554	0.007	0.003*
AMPD1	AMP deaminase 1	-0.003	1.286	1.289	0.656	0.014	2.180	0.014	0.988
AT2A1	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	-0.010	1.683	1.693	0.406	0.029	1.418	0.027	0.940
ATPB	ATP synthase subunit beta, mitochondrial	0.000	1.654	1.653	0.412	0.010	1.214	0.010	0.015*
CSRP3	Cysteine and glycine-rich protein 3	-0.021	6.901	6.923	0.626	0.045	4.031	0.041	0.004*
DESM	Desmin	0.038	2.131	2.096	0.029*	0.006	2.613	0.006	0.071
ENOB	Beta-enolase	-0.009	1.240	1.250	0.022*	0.002	1.122	0.002	0.008*
FHL1	Four and a half LIM domains protein 1	-0.028	0.839	0.869	0.076	0.042	1.430	0.039	0.005*
FLNC	Filamin-C	-0.011	2.405	2.416	0.502	0.020	2.074	0.019	0.218
G3P	Glyceraldehyde-3-phosphate dehydrogenase	-0.017	1.416	1.434	0.080	0.007	1.048	0.007	0.102
GELS	Gelsolin	-0.046	3.834	3.884	0.261	-0.020	3.542	-0.020	0.805
HBB	Hemoglobin subunit beta	-0.039	1.438	1.481	0.039*	-0.033	2.057	-0.036	0.007
HS71A	Heat shock 70 kDa protein 1A	0.006	2.833	2.827	0.574	0.007	4.581	0.007	0.245
HSPB1	Heat shock protein beta-1	-0.031	2.157	2.189	0.666	-0.005	2.506	-0.005	0.381
KCRM	Creatine kinase M-type	-0.029	0.958	0.989	0.077	0.004	0.916	0.004	0.022*
KLH41	Kelch-like protein 41	0.006	2.482	2.476	0.903	0.025	2.220	0.024	0.389
LDB3	LIM domain-binding protein 3	0.008	1.081	1.074	0.234	0.026	1.151	0.025	0.083
LMNA	Prelamin-A/C	0.008	1.812	1.804	0.278	-0.004	2.200	-0.004	0.234
MLRS	Myosin regulatory light chain 2, skeletal muscle isoform	0.028	0.770	0.744	0.527	-0.011	0.658	-0.011	0.540
MLRV	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform	0.004	1.077	1.073	0.076	0.015	0.966	0.014	0.320
MYH2	Myosin-2	0.015	1.004	0.989	0.662	0.019	1.117	0.018	0.877
MYH7	Myosin-7	-0.025	0.786	0.812	0.960	0.027	0.892	0.026	0.216
MYL1	Myosin light chain 1/3, skeletal muscle isoform	0.007	0.806	0.799	0.547	-0.019	0.721	-0.020	0.594
MYL3	Myosin light chain 3	0.010	0.518	0.508	0.166	0.011	0.630	0.011	0.505
MYOM1	Myomesin-1	0.009	0.816	0.807	0.067	0.022	1.148	0.022	0.118
MYOM2	Myomesin-2	0.012	0.836	0.824	0.982	0.009	0.800	0.009	0.788
MYOTI	Myotilin	-0.008	2.425	2.434	0.237	0.017	2.239	0.016	0.228
MYOZ1	Myozenin-1	0.010	1.162	1.152	0.837	0.019	1.299	0.018	0.279
MYPC1	Myosin-binding protein C, slow-type	-0.002	1.754	1.756	0.821	0.015	1.775	0.015	0.393
MYPC2	Myosin-binding protein C, fast-type	0.021	1.961	1.941	0.381	-0.005	2.303	-0.005	0.379
NDUS1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	-0.023	1.610	1.634	0.633	0.019	1.905	0.018	0.577
NEBU	Nebulin	-0.002	1.135	1.138	0.599	-0.003	1.469	-0.003	0.758
OBSCN	Obscurin	-0.005	2.565	2.571	0.699	-0.027	3.447	-0.029	0.317
PDLI3	PDZ and LIM domain protein 3	-0.021	2.691	2.712	0.058	0.002	2.963	0.002	0.844

PDLI5	PDZ and LIM domain protein 5	0.004	1.180	1.177	0.804	0.017	3.642	0.016	0.214
PYGM	Glycogen phosphorylase, muscle form	-0.004	1.601	1.606	0.247	0.032	1.185	0.030	0.544
RYR1	Ryanodine receptor 1	-0.024	2.019	2.044	0.492	-0.020	2.158	-0.021	0.236
SYNEM	Synemin	-0.019	3.830	3.849	0.867	0.020	2.572	0.019	0.705
SYNP2	Synaptopodin-2	-0.012	1.835	1.848	0.235	-0.020	2.023	-0.021	0.369
TELT	Telethonin	0.020	4.160	4.140	0.528	0.063	4.144	0.056	0.152
TITIN	Titin	-0.004	1.222	1.226	0.340	-0.011	1.353	-0.011	0.125
TNNC1	Troponin C, slow skeletal and cardiac muscles	-0.021	1.674	1.697	0.979	0.013	1.974	0.013	0.268
TNNC2	Troponin C, skeletal muscle	0.038	1.673	1.638	0.323	-0.005	1.688	-0.005	0.677
TNNI1	Troponin I, slow skeletal muscle	-0.004	1.608	1.613	0.629	0.019	1.622	0.018	0.146
TNNI2	Troponin I, fast skeletal muscle	0.030	1.234	1.206	0.883	0.010	0.923	0.010	0.457
TNNT1	Troponin T, slow skeletal muscle	0.001	1.840	1.838	0.330	0.032	3.202	0.030	0.703
TNNT3	Troponin T, fast skeletal muscle	0.016	1.192	1.177	0.825	0.004	1.840	0.004	0.445
TPM1	Tropomyosin alpha-1 chain	0.014	0.987	0.973	0.194	-0.002	1.174	-0.002	0.810
TPM2	Tropomyosin beta chain	-0.001	1.050	1.051	0.177	0.001	1.212	0.001	0.945
TPM3	Tropomyosin alpha-3 chain	-0.013	1.023	1.036	0.117	0.008	1.084	0.008	0.473
XIRP1	Xin actin-binding repeat-containing protein 1	0.021	4.980	4.960	0.999	0.084	6.572	0.073	0.013*

Table 3. For comparison/calculation, changes in abundance have been converted to a rate of change in abundance.

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CHAPTER 4

GENERAL DISCUSSION

Introduction

Skeletal muscle, comprising approximately 40% of total body mass, is critical for the promotion and maintenance of metabolic health. In addition to providing the force generation necessary for activities of daily living, skeletal muscle, due to its various metabolic functions, also reduces the risk for non-communicable diseases, such as cardiovascular disease and type 2 diabetes (Saqib, Dai et al. 2020). Exercise is a critical factor in maintaining muscle mass, especially in aging populations, and is associated with reduced risks of premature mortality and cancer-related deaths (Ruiz, Sui et al. 2008). Despite these benefits, the molecular mechanisms driving muscle adaptation to different exercise types remain incompletely understood. To that end, the experiments detailed in the present thesis were designed to characterize the common and differential skeletal muscle signalling and protein abundance adaptations that occur in response to aerobic (AE) and resistance (RE) exercise. In study 1 (Chapter 2), we characterized the phosphoproteomic response to a single bout of AE and RE by assessing skeletal muscle immediately following (0 hr) and in recovery (3 hr) from exercise. We discovered widespread phosphorylation events, identifying nearly 13000 individual phosphosites on 3102 proteins and unique clusters specifically associated with AE and RE. This hypothesis-generating analysis prompted a targeted investigation into a RE-specific pathway that was found to correlate well with the post-exercise rise in myofibrillar protein synthesis. Given the novel identification of a RE-specific exercise pathway for regulation of translation initiation, this led to more exploratory analyses in study 2 (Chapter 3) of the individual proteins' synthetic and abundance responses to exercise to AE and RE. In study 2, we characterized the proteomic and protein synthetic response to 1- and 10-weeks AE and RE. We discovered a moderate positive correlation between the changes in individual protein synthetic rate (FSR) between AE and RE training muscle after 1-

week (R= 0.37) and 10 weeks (R= 0.63) of training. Additionally, study 2 results suggest that changes in targeted protein degradation is a component of exercise adaptation that requires further investigation due to the potential role in modulating proteome remodelling. In the discussion that follows, the primary findings of each study will be elaborated on in the context of prior research, and the strengths, weaknesses, and avenues for future research are explored.

Muscle Structure and Function

The functional attributes of skeletal muscle are underpinned by its protein constituents (i.e. the proteome) and their respective functions. The proteins comprising the proteome exist in a dynamic equilibrium of protein turnover, their content is a function of the balance of protein synthesis and degradation. Skeletal muscle is composed of a hierarchy of contractile bundles, from the level of whole muscle down to the individual myofibril and sarcomere. Studying the structure and composition of skeletal muscle can help us understand the influence of exercise on muscle plasticity and how transient signalling induces chronic proteome adaptation.

In Study 1, we investigate the skeletal muscle adaptive mechanisms by looking at signalling events that occurred immediately following and three hours post-exercise to compare with the differences in rates of protein synthesis between AE and RE. In Study 2, we replicated the exercise stimulus of Study 1 in hopes of achieving a similar initial pattern of post-exercise signalling in order to correlate signalling changes with their individual protein synthetic rates and change in abundance early (1 week) and later (10 weeks) following exercise mode-specific training within a person, which we hypothesized would be the most efficient way to establish phenotype changes against the same genetic backgrounds. Together, these studies contribute to

the current literature by expanding on previous phosphoproteomic (Hoffman, Parker et al. 2015, Blazev, Carl et al. 2022) and proteomic (Robinson, Dasari et al. 2017, Deshmukh, Steenberg et al. 2021) exercise studies performed in human subjects.

Muscle adaptation to exercise is a highly plastic process modulated by the type and intensity of the exercise performed. Exercise is typically dichotomized into an aerobic- or resistance-type stimulus, with distinct and unique muscle adaptation that occurs in response to each training modality. This dichotomization is, largely in part, due to the differential perturbance (mechanical and metabolic) that induces the signalling of distinct protein networks (i.e. kinases) for stimulation of specific protein synthesis and adaptations following aerobic and resistance exercise in skeletal muscle. Aerobic exercise, characterized by prolonged low-load contractions that create an energetic stress, typically leads to increased expression of mitochondrial genes and proteins, enhancing mitochondrial content and promoting an oxidative muscle fibre phenotype with improved fatigue resistance. In contrast, resistance exercise involving high-load contractions stimulates translation initiation primarily in the myofibrillar protein fraction, resulting in the accretion of muscle protein, hypertrophy and increased force-generating capacity. However, analysis of transient post-exercise phosphorylation signalling, a relatively well-characterized link between perturbance and protein turnover, reveals a high degree of similarity between exercise types (Wilkinson, Phillips et al. 2008, Blazev, Carl et al. 2022), indicating training mode-independent signalling events generic to more than one training type. Leading to the inquiry of how (induced by mechanical, metabolic or other stimuli), when (days, weeks or months) and where (protein pool or compartment) post-exercise signalling in skeletal muscle is able to generate a unique protein synthetic response between exercise types. To date, the vast

majority of literature ties acute phosphorylation events converging through a select series of signalling networks following exercise as the focal points of activity-stimulated protein synthesis and turnover. Within these signalling networks are a plethora of 'key' exercise-regulated proteins and phosphosites responsible for the propagation of signal transduction (e.g. kinases and phosphatases) that are upstream of the changes in protein turnover and typically associated with aerobic or resistance training, alluding to exercise specific mechanisms of adaptation. Yet, the chronic adaptations of muscle to exercise, which unfold over weeks to months, cannot be accurately predicted based on the temporary molecular responses observed immediately after each exercise session. A deeper understanding of the processes that connect the immediate muscle responses during or shortly after exercise to the long-term changes in hypertrophy and metabolic characteristics is essential for developing effective training programs or lifestyle recommendations that consistently enhance performance and health. Identifying molecular markers that can predict favourable or unfavourable changes in muscle phenotype could greatly improve exercise prescription. However, several challenges remain before this goal can be fully realized.

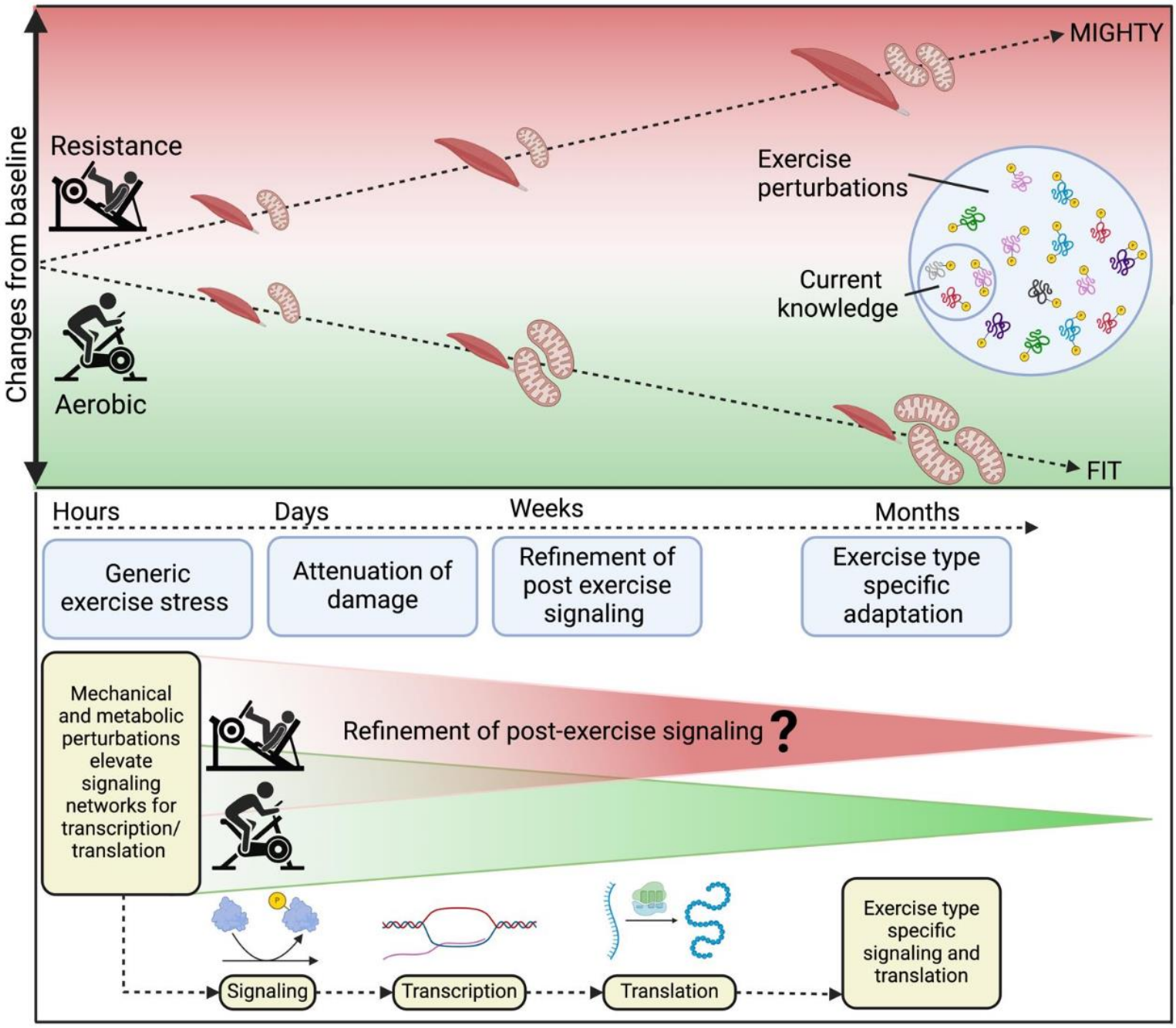

Figure 4.1. Graphic of a hypothesized overview of exercise-specific adaptations in Human Skeletal Muscle

Stable isotope tracer methods and muscle protein turnover

Skeletal muscle is unique in composition, with proteins that enable it to both generate, perceive and integrate mechanical forces (Sun, Guo et al. 2016). Mechanical loading of skeletal muscle is a potent regulator of protein turnover and eventual protein accretion leading to mass gain. When loading occurs, skeletal muscle undergoes an elevation of anabolic signals that stimulate muscle protein synthesis and, when performed chronically, increases muscle size and strength (Hornberger and Esser 2004, Currier, McLeod et al. 2023). Contrarily, the absence of mechanical stimuli – i.e., unloading – elicits a rapid loss of muscle mass (Booth 1982, Nunes, Stokes et al. 2022) . Changes in skeletal muscle mass are mediated, in part, by the acute balance in muscle protein synthesis (MPS) and muscle protein breakdown (MPB). The metabolic process of incorporating amino acids into functional muscle protein is regulated by MPS, whereas MPB is the antagonistic function, and proteins are broken down into amino acids. Rates of MPS and MPB are dynamic and subject to change following exercise and protein ingestion both of which typically induce a robust rise in MPS, whereas periods of disuse and hypo-aminoacidemia lessen MPS and spike the rate of MPB (Wolfe 2006, Nunes, Stokes et al. 2022). Calculating the balance in MPS and MPB yields net protein balance (NPB), also known as muscle protein turnover. When the rate of MPS exceeds MPB, the value of NPB is positive and accretion of muscle protein occurs (Wolfe 2006, McGlory, van Vliet et al. 2019). Following exercise, the transient increase in NPB is less negative despite a substantial rise in MPS by a concomitant rise in MPB (Phillips, Tipton et al. 1997). For example, resistance exercise has been shown to elevate MPS at 3 (112%), 24h (65%), and 48h (34%), but MPB is greater than MPS and it is also increased, albeit to lesser extent than MPS, above resting values at 3 (31%) and 24 hours (18%) post-exercise (Phillips, Tipton et al. 1997). For optimal skeletal muscle adaptation to occur, both MPS

and MPB are indispensable and tightly regulated and MPB appears to be an essential component of skeletal muscle remodelling (Tipton, Hamilton et al. 2018). Overall exercise generally elicits a greater difference in rates of MPS and MPB compared to rest (Wolfe 2006). However, while at rest and in the post-absorptive state, MPB rates exceed MPS, driving the loss of skeletal muscle mass and contractile tissue (Breen and Phillips 2013, McGlory, van Vliet et al. 2019). It is not until protein is consumed, hyperaminoacidemia ensues and MPS is stimulated that MPS exceeds MPB, and net protein accretion occurs (Joanisse, Lim et al. 2020). MPS is typically assessed as whole muscle (bulk) fraction or fractioned into myofibrillar and sarcoplasmic, and occasionally mitochondrial, fractional components. Although bulk fraction MPS is highly informative in determining the influence of a stimuli on muscle proteins, fractionated tissue provides a deeper perspective as to which proteins are being synthesized and alludes to the associated signalling mechanism underpinning the regulation of translation initiation.

Wilkinson et al. (2008) hypothesized that the divergent phenotypes observed with chronic AE and RE are associated (derived by) with differential repeated stimulation of myofibrillar and sarcoplasmic FSR (Wilkinson, Phillips et al. 2008). In their seminal study, acute myofibrillar and mitochondrial FSR were measured prior to and following 10 weeks of exercise training. In the untrained state, acute resistance exercise stimulated myofibrillar (67%) and mitochondrial (69%) FSR above resting values. Whereas acute aerobic exercise robustly stimulated mitochondrial FSR in the untrained (154%) and trained (104%) state but failed to significantly alter myofibrillar FSR (Wilkinson, Phillips et al. 2008). Additionally, following the training period resting myofibrillar FSR was greater in the resistance-trained leg in comparison to aerobic and the same relative acute resistance exercise stimulus elevated myofibrillar FSR a further 37%

above resting values (Wilkinson, Phillips et al. 2008). With this approach, Wilkinson et al. (2008) were able to observe what fraction of protein synthesis each modality stimulated, which is a direct result of the contraction type, metabolic perturbance and acute signalling. The repeated stimulation of distinct protein synthesis is almost certainly responsible for the modality-specific adaptations observed with chronic aerobic and resistance training. Skeletal muscle is, therefore, able to detect the type of contraction and modality of exercise, initiating specific downstream mechanisms that signal for adaptation. These data offer some of the first insights into training-mode specificity in the regulation of protein synthetic response in the hours following a single bout of exercise in the untrained and trained states.

Studies that use amino acid-specific labels (i.e., stable isotope labeling by amino acids in cell culture SILAC) to measure protein turnover offer an acute (typically < 12 hours) insight into bulk or fraction-specific muscle protein synthesis in vitro. However, particularly in response to exercise, this is a relatively small window to understand and interpret what is going on in the larger picture of adaptation. Longer-term biosynthetic labelling techniques allow for a more integrative approach to measuring protein synthetic responses (i.e. deuterium ingestion as deuterated water can be given for days/ weeks), and by doing so, outcomes seem to correlate better with changes in tissue mass (Brook, Wilkinson et al. 2015). Additionally, the use of longer-term tracers encompasses the sleep-wake cycles, multiple feedings, and any other day-to-day activities and their influence on the variation in muscle protein synthesis. Longer windows of protein synthesis may allow for better insight into the dynamic nature of the adaptive response to exercise training. For instance, Damas et al. (2016) demonstrated that MPS only correlated with muscle growth once an initial period of ‘damage’ was attenuated (Damas, Phillips et al. 2016).

Additionally, deuterium oxide (D_2O) tracer methods can provide novel insight as to how proteins are differentially regulated early versus later during training (Stokes, Timmons et al. 2020), and, if desired, in distinct loading states as changes in individual protein content are also mediated by protein stability and may explain why in rare instances exercise training studies have observed no significant elevation of bulk protein synthesis over periods of training (Shad, Thompson et al. 2021).

Advancements in the use of D_2O as a biosynthetic label, combined with proteomic techniques, offer enhanced resolution in understanding muscle responses such as changes in protein synthesis, degradation, and turnover rates (Hesketh, Srisawat et al. 2016, Stead, Hesketh et al. 2020) and has highlighted that proteins with different functions have different synthesis rates in human muscle (Stansfield, Barrett et al. 2024). Traditionally, mixed muscle and fractionated measurements of protein synthesis provide an average response of thousands of proteins, each of which may react uniquely to a given exercise intervention. The use of D_2O labelling alongside proteomic analysis of muscle samples can isolate the synthesis of individual proteins within the muscle. This method has demonstrated that proteins with different functions have varying synthesis rates in human muscle (Hesketh, Srisawat et al. 2016, Murphy, Shankaran et al. 2018, Stead, Hesketh et al. 2020). Indeed, profiling of individual protein synthetic rates has reported a significant increase in the FSR of 20 proteins (from 139 measured) comprising processes associated with glucose metabolism, muscle contraction, and cellular respiration in aerobic-trained muscle (Shankaran, King et al. 2016). Further, individual FSR response to resistance training in overweight males has indicated increases in FSR in 175/190 proteins, comprised of mitochondrial and sarcoplasmic proteins (Murphy, Shankaran et al. 2018). Overall, these data

indicate that individual proteins in human muscle exhibit a range of diverse responses, occurring in response to exercise training. However, both these reports mentioned (Shankaran, King et al. 2016, Murphy, Shankaran et al. 2018) lack data on protein abundance, which, in the context of exercise-induced proteome remodelling, leaves questions as to whether the reported changes in synthesis rate reflect changes in protein abundance (i.e. static protein breakdown) or underpin changes in protein turnover (i.e. concomitant dynamic protein breakdown).

The pioneering dynamic proteomic analysis of human skeletal muscle in response to RE documented both protein-specific synthesis and abundance data for nearly 100 muscle proteins (Camera, Burniston et al. 2017). In contrast to RE, profiling individual protein synthesis rates in response to AE revealed a significant increase in turnover of 22 proteins (out of 409 measured), particularly those associated with energy metabolism, proteasome activity, and cellular stress responses in aerobic-trained muscle (Srisawat, Stead et al. 2023). This research (Camera, Burniston et al. 2017, Srisawat, Stead et al. 2023) led to previously uncovered distinct patterns in protein turnover, such as proteins that aligned in synthesis and abundance (i.e. increased/decreased synthesis and corresponding change in abundance, proteins with discordant synthesis and abundance (i.e. increased/ decreased synthesis and an opposite change in abundance). Training-induced increases in the turnover rate of individual proteins have also been reproduced in dynamic proteomic analysis of human muscle samples collected before and after a 10-week HIIT intervention (Srisawat, Stead et al. 2023). Therefore, these data offer new insights of training induced adaptation that may not be readily explained using traditional mixed-protein measurements of protein turnover.

Undoubtedly, the application of dynamic methods to quantify the rates of protein synthesis (and degradation) is required to understand how skeletal muscle adapts to exercise stimuli on a protein-specific level. The phenotypic consequences of repeated exercise bouts are entirely dependent on changes in the skeletal muscle proteome, encompassing changes in protein abundance and/or activity. However, currently little is known of the specificity of training and the subsequent changes in selective synthesis and degradation that underpin skeletal muscle remodelling. Similarly, while changes in dynamic processes explain how changes in muscle phenotype are produced, changes in protein abundance represent the molecular consequence or 'output' from training stimuli and the cumulative effect of repeated transient signalling events. Therefore, training mode-specific differences in signal transduction across multiple molecular pathways likely contribute to the differential regulation of protein synthesis and degradation that result in divergent phenotypic adaptation of skeletal muscle.

Post-translational modifications (PTM) and Phosphoproteomics

Following exercise, highly coordinated mechanisms begin orchestrating an adaptive response to the metabolic and contractile (mechanical) perturbation in skeletal muscle. However, regardless of the clear differential phenotypic outcomes of chronic training in either aerobic or resistance, relatively little is known about the exercise type-specific signalling mechanisms, regulation of protein turnover or changes in abundance that are proposed to elicit an aerobic or resistance-trained muscle phenotype. Exercise, whether aerobic or resistance, results in profound transcriptional responses, with some common genes being transcribed and some that are unique (Pillon, Gabriel et al. 2020). As an (over)simplified mechanism, the current understanding is that with each exercise session, transcribed genes are subsequently translated, and the resultant

proteins accumulate to change, over time, the muscle phenotype (Egan and Zierath 2013, Egan and Sharples 2023).

A critical aspect of exercise-induced muscle adaptation is the regulation of protein activity/function through PTM, particularly phosphorylation. Phosphorylation (e.g. of S, T, or Y residues) involves the addition of a phosphate group to proteins, which can alter their activity, binding properties, or cellular localization. This modification is essential for regulating cellular processes in response to exercise, with over 500 kinases in the human 'kinome' potentially involved. Recent advancements in mass spectrometry have significantly enhanced our understanding of the phosphorylation landscape in response to exercise. For instance, liquid chromatography-mass spectrometry has revealed over 1,000 exercise-regulated phosphorylation sites in human skeletal muscle following high-intensity aerobic exercise (Hoffman, Parker et al. 2015). However, linking these molecular changes to phenotypic outcomes, such as changes in protein synthesis and turnover, has been challenging.

Phosphoproteomic analyses have the potential to uncover distinct signaling networks triggered by different exercise modalities that are potentially responsible for the divergent adaptive responses to exercise training. Non-targeted analyses are particularly valuable for discovering new mechanisms and have highlighted the limited understanding of signalling networks, including which kinases regulate specific phosphorylation sites. Despite their thoroughness, non-targeted PTM studies have often generated more questions than answers, leaving uncertainty about the key signalling events that may drive phenotypic muscle adaptations. For instance, Potts et al. (Potts, McNally et al. 2017) conducted phosphoproteomic profiling of mouse tibialis

anterior muscle collected one hour after maximal intensity contractions (MIC) induced by electrical stimulation. These data showed significant changes in phosphorylation at 621 sites on 313 proteins, with most (531/621 sites) showing increased phosphorylation post-exercise (Potts, McNally et al. 2017). Similarly, Blazev et al. (2022) (Blazev, Carl et al. 2022) identified 5,486 phosphosites on 1,573 proteins significantly regulated at one and three hours following either aerobic (90 min at 60% VO2 max), sprint (3 x 30 s all-out cycling), or resistance exercise (6 sets of 10-rep max knee extensions) in eight healthy, untrained men. They found that 420 phosphorylation sites were common across the different exercise types, including the novel gene product C18ORF25, which was confirmed as an AMPK substrate. In study 1, we utilized phosphoproteomics and measured protein synthetic responses to investigate the signalling events following AE and RE. We hypothesized that specific clusters of phosphopeptides would be uniquely regulated by RE and specifically associated with the post-exercise increase in MPS. We demonstrated similar widespread phosphorylation in the immediate (0 hr) and recovery (3 hr) period post-exercise as detected by Blazev et al. (2022) (Blazev, Carl et al. 2022) (see Chapter 2, supplementary Figures 2 and 4). Together these data support that exercise tightly regulates post-exercise phosphorylation signaling, likely to direct subsequent protein turnover responses to the stress of the exercise stimulus and provide a response to minimize homeostatic disturbance due to subsequent exercise stresses.

Study 1 employed a unilateral exercise design, allowing for within-subject comparisons; we identified 12,899 phosphosites on 3,102 proteins across 3 different time points and 2 exercise conditions, representing the most extensive compendium of exercise-regulated phosphorylation sites to date. Specifically, we observed 85 phosphopeptides that were significantly regulated

following RE, with 12 unique to RE alone, suggesting a role in RE-specific signalling mechanisms. Our findings highlighted several potential key phosphopeptides and associated kinases potentially involved in RE-induced muscle adaptations. Notably, phosphopeptides such as CRYAB_(S19), BAG3_(T62), EIF4B_(S500), IGFN1_(S787), WNK1_(S1978), and HSPB1_(S83) demonstrated RE-specific responses. The regulation of these phosphopeptides could function as signalling mechanisms that lead to hypertrophy and muscle growth in response to chronic RE.

In addition to RE-specific responses, we observed shared phosphorylation events following both AE and RE, suggesting common regulatory pathways. For instance, proteins such as VDAC_(S127) and BOLA1_(S81), which are involved in mitochondrial function and oxidative stress protection, were regulated by both exercise modalities. Furthermore, canonical phosphopeptides like p70, AKT, mTOR, AMPK, and p38 MAPK were implicated in exercise adaptation signalling, although their precise roles in distinguishing between AE and RE phenotypes remain unclear.

Lastly, we used a targeted approach in Chapter 2 and performed Western blot analyses, correlating phosphorylated:total protein ratios with changes in MPS post-exercise. Phosphopeptides like MKK3_(S189), a target protein identified by our analyses and rodent tissue (data not shown) showed significant associations with MPS increases following RE, suggesting its potential role in muscle hypertrophy-specific signalling. Chapter 2 can be summarized by the valuable insights our research provided in the phosphoproteomic landscape of skeletal muscle adaptation to AE and RE. We identified novel exercise-induced signalling networks and proteins,

particularly those associated with the post-exercise rise in MPS following RE. These findings contribute to our understanding of the molecular mechanisms underlying exercise-induced muscle adaptations and highlight potential targets for optimizing training programs and health interventions. To directly follow up the research in Chapter 2, Chapter 3 continued exploring these pathways, particularly of protein expression outcomes, measuring the turnover rates of individual proteins over extended periods to better determine AE and RE training outcomes and phenotypic adaptation. Together, the data contained in Chapters 2 and 3 mark a significant step forward in elucidating the complex signalling networks involved in muscle adaptation to different exercise modalities, offering new avenues for research and practical applications in sports science and clinical scenarios.

Proteomic investigations and mitochondrial proteins

Our laboratory has previously demonstrated that a single session of RE can significantly increase mitochondrial protein synthesis in the untrained state (Wilkinson, Phillips et al. 2008). However, results of mitochondrial content have demonstrated equivocal results, showing either a decrease (Kon, Ohiwa et al. 2014), no change (Wilkinson, Phillips et al. 2008, Porter, Reidy et al. 2015), or an increase following RE (Tang, Hartman et al. 2006). Proteomic investigations have the potential to further resolve the intricacies of mitochondrial adaptations of human muscle to exercise, extending knowledge of mitochondrial proteome remodelling beyond the archetypal targets of training adaptations. For example, Holloway et al. (2009) utilized proteomic analysis of human muscle to catalogue the abundance of 256 protein spots separated via 2-dimensional Gel Electrophoresis (2-DiGE) before and after 6-wks of HIIT (6 x 1 min bouts at VO2max completed 3x / week) (Holloway, O'Gorman et al. 2009). Their analyses (Holloway, O'Gorman et

al. 2009) highlighted gains in the abundance of individual enzymes associated with ATP resynthesis, including ATP synthase-a and -b as well as succinate dehydrogenase subunits (Holloway, O'Gorman et al. 2009). Additionally, time-series profiling using high-resolution gel maps of the mitochondrial proteome during the first 2 weeks of an aerobic training programme identified ~1.3- to 2-fold increases in abundance of mitochondrial respiratory chain I and V subunits and tricarboxylic acid cycle enzymes, despite no changes in mitochondrial: nuclear DNA ratio (Egan, O'Connor et al. 2013). These data indicate important characteristics and aspects of proteome remodelling beyond the typical myofibril and bulk mitochondrial protein accretion, in line with previous work identifying training induced increases in mitochondrial respiration without parallel increases in markers of total mitochondrial content (Granata, Oliveira et al. 2016). These previous findings align with our study 2 investigation into the myofibrillar protein fraction following 1- and 10- weeks of exercise training. In study 2 we found that a good proportion of the proteins significantly elevated in abundance following 10 weeks AE and RE were annotated as mitochondrial proteins. Indicating a significant role for mitochondrial proteome remodelling with exercise training. However, unfortunately, study 2 did not have an assessment of bulk mitochondrial content or mitochondrial function (i.e., respirometry or enzyme activity) to pair with our findings of protein abundance and FSR. Therefore, we are left to speculate whether the increase in mitochondrial protein abundance observed in study 2 represents an increase in total mitochondrial content or remodelling of specific complexes/ subunits within the mitochondria and the functional significance of the findings.

Liquid chromatography-tandem mass-spectrometry analyses of exercised muscle have enabled the quantification of thousands of proteins, providing even more comprehensive insight into

muscle responses to exercise training. Granata et al., (2021) quantified the abundance of 726 mitochondrially annotated proteins in human biopsy samples, identifying 185 mitochondrial proteins that change significantly in abundance in response to differing aerobic training volumes (Granata, Caruana et al. 2021). However, similar to the aforementioned proteomic analyses of exercised muscle, the changes in abundance of > 150 mitochondrial proteins were not parallelled to increases in bulk mitochondrial content. Overall, these works highlight that individual protein remodelling of mitochondria may underly, at least part, of the improvements in mitochondrial function induced by exercise training. Protein-specific remodelling of the mitochondrial proteome may represent a previously underexplored aspect of training adaptations. While bulk increases in protein content may predominantly be associated with aerobic training, our proteomic analysis of exercised muscle highlights that mitochondrial remodelling is not exclusive to aerobic stimuli. Other previous work reports an increase in the abundance of 141 mitochondrial proteins after 3 months of regular resistance training (4 sets of 8–12 repetitions, 4x / week) (Robinson, Dasari et al. 2017). Furthermore, enrichment analysis of proteomic profiles from chronically (> 15 years) resistance- or aerobic-trained muscle reported a greater abundance of proteins associated with oxidative phosphorylation to be a prominent feature of aerobic-trained muscle and, despite to a lesser extent, significantly enriched in resistance muscle (Emanuelsson, Arif et al. 2024). Altogether, exercise appears to induce a variety of changes to the muscle mitochondrial proteome. Ultimately, untargeted analyses of skeletal muscle responses to training suggest that while adaptation is much more prominent in aerobically trained muscle, mitochondrial adaptations involve much more complex remodelling rather than a bulk proportional increase in muscle mitochondrial protein content and function. These data, in combination with our findings from Study 2, indicate an exercise training-induced improvement

in mitochondrial bioenergetics and quality. The improvement in mitochondrial bioenergetics likely underpins an extensive proportion of the increased metabolic capacity elicited by regular exercise training. A limitation of the current thesis, particularly Study 2 (Chapter 3), is the lack of a soluble muscle fraction. The identification of sarcoplasmic and mitochondrial proteins in the myofibrillar isolated fraction can be a result of the imperfect separation of protein fractions. However, for the present analyses we postulated the changes in mitochondrial proteins detected in the myofibrillar fraction (i.e., intermyofibrillar mitochondria) might reflect (albeit in much lower abundance) any alterations in the soluble fraction-contained mitochondrial protein.

Due to their vastly unique content in highly trained aerobic and resistance athletes, mitochondria are potentially a key differentiating factor for the performance and phenotypic divergence observed with aerobic vs. resistance training. However, mitochondria may also have an important role in skeletal muscle adaptation to chronic resistance training. Mitochondrial content appears essential for the maintenance of muscle mass, with evidence from rodent models suggesting that muscle mass is highly dependent on sufficient mitochondrial content (Roberts, McCarthy et al. 2023). Additionally, impairment of mitochondrial biogenesis is linked with muscle atrophy (Joseph, Adhihetty et al. 2013, Ross, Coppotelli et al. 2019). Furthermore, PGC-1a knocked out in rodent models demonstrates a modest reduction in hypertrophy of plantaris muscle after 14 days of synergist ablation (Perez-Schindler, Summermatter et al. 2013). Results in human skeletal muscle and resistance exercise-induced mitochondrial biogenesis are discordant; however, some improvements in maximal coupled respiration have been observed despite little to no change in gene expression or protein content (Porter, Reidy et al. 2015, Groennebaek and Vissing 2017, Memme, Erlich et al. 2021). Therefore, a degree of resistance

exercise signalling (i.e. AMPK, MAPKs) is likely to stimulate mitochondrial adaptation in support of greater metabolic and energetic demands associated with resistance training, albeit to a lesser extent than aerobic exercise.

Exercise specific changes in protein content

Previous work from our lab (Damas, Phillips et al. 2016) demonstrated that the acute exercise-induced increase in protein synthesis only correlated with hypertrophy after an initial attenuation of damage had occurred. From this, it is proposed that chronic exercise refines the skeletal muscle transcriptional and translational programs to initiate selective signalling pathways that drive phenotypic changes (Wilkinson, Phillips et al. 2008, Kumar, Atherton et al. 2009). Additionally, in an untrained state, we initially proposed that a significant overlap of exercise-induced signalling patterns existed in response to the initial exposures to exercise, regardless of modality, therefore inferring that acute signalling adapts as an individual becomes more accustomed to the stress of exercise training. For example, in untrained individuals, the acute signalling response of canonical signalling pathways, including AMPK, ERK1/2 and p38, is attenuated after only 10 days of intensified aerobic exercise, such that acute exercise no longer elicits a significant elevation in phosphorylation (Benziane, Burton et al. 2008). We hypothesized that the molecular responses to acute exercise in an untrained state may largely consist of a 'generic stress' response to the unaccustomed perturbation. Over time, with repeated mode-specific exercise bouts, muscle adapts to the stimuli, refining the perturbation and targeting it towards efficient adaptation for improved muscle function and substrate utilization specific to the exercise stimulus; this thesis would be consistent with the data from Wilkinson et al. (2008) that we have previously generated based on fraction-specific data (Wilkinson, Phillips et al. 2008).

Interestingly, the proteins we could detect in our analysis from Chapter 3 demonstrate a shift toward greater overlap (i.e. more shared significantly upregulated proteins following 10 weeks of exercise training in comparison to 1 week). We initially proposed the acute exercise-induced regulation of protein transcription/ translation in unaccustomed skeletal muscle would stimulate a greater proportion of pathways common to AE and RE in early (i.e. 1 week) training as opposed to later (i.e. 10 weeks). We hypothesized that the proteins commonly regulated by AE and RE early in training would be associated with energetic stress and the synthesis of proteins associated with muscle ultrastructural remodelling. Unfortunately, in Chapter 3, we did not have any measure of acute exercise-induced phosphoproteomic signalling in the trained state to compare with our unaccustomed signalling data in Chapter 2. Assessment of the phosphoproteomic alterations in muscle in response to exercise following a period of training would advance our understanding of how 'global' signalling adapts in response to AE and RE. Additionally, it would be interesting to characterize the relationship between widespread phosphorylation signalling post-exercise and individual protein synthetic rates in exercise-trained muscle.

In study 1 (Chapter 2), we identified that a sustained exercise-stimulated phosphorylation response (seen immediately post-exercise and persisting for 3h) may be a differentiating factor between AE and RE. Specifically, we observed a large cluster of proteins that are robustly phosphorylated immediately following RE, with a substantial relative proportion of these proteins still demonstrating increased phosphorylation 3 hours following exercise, compared to AE (see Chapter 2, Figure 2). In line with this, Blazev et al. (2022) also identify a stark contrast in the number of phosphosites differentially regulated in recovery from AE and RE (Blazev, Carl

et al. 2022). Specifically, Blazev et al. (2022) demonstrate 1 versus 687 phosphosites significantly regulated 3 hours following AE and RE, respectively (Blazev, Carl et al. 2022). From these data, in combination with study 1, several individual phosphoproteins (i.e. MAPKAPK2, MAPKAPK3 and MAPAPK5) were hypothesized to play a key role in post-exercise signalling and appeared to be associated with the subsequent rise in myofibrillar protein synthesis (see Chapter 2, Supplementary Figure 4). Altogether, these data (Blazev et al. 2022, Hoffman et al. 2015, as well as our data) demonstrate that the phosphoproteomic response to exercise type can be characterized in a meaningful and reproducible manner, leading to the identification of novel exercise-regulated signaling networks and proteins. These methods (i.e. phosphoproteomics and other PTM omic techniques) will advance our understanding of the widespread and complex post-exercise signalling and be a source for hypothesis generation for new areas of targeted research when networks or proteins of interest are discovered.

Targeted studies on canonical exercise PTM have demonstrated that the signalling response is malleable with training. Acute signalling responses can change as individuals adapt to the stress of exercise training, potentially explaining why many transient molecular pathways are not highly activated after a period of chronic training (Benziane, Burton et al. 2008). Benziane et al. (2008) demonstrated that in untrained individuals, the acute signalling response of several canonical pathways (e.g., AMPK, ERK1/2, and p38) is adaptable after just 10 days of intensified aerobic exercise (Benziane, Burton et al. 2008). Moreover, exercise training can reduce acute VEGF expression following exercise, with only an 18% difference in capillaries per muscle fibre (Richardson, Wagner et al. 2000). Similarly, in resistance and aerobic trained athletes, an acute bout of exercise did not activate AMPK phosphorylation in their respective disciplines; however,

a significant increase was observed when engaging in unfamiliar (i.e., opposite to the athletes' regular exercise stimulus) exercise (Coffey, Zhong et al. 2006). Unfortunately, Study 1 (Chapter 2) did not include a chronically trained timepoint and study 2 has yet to perform phosphoproteomic analysis on the muscle tissue, so we are unable to directly assess whether signalling altered in response to exercise in either study. However, given our assessment of FSR in study 2, it can be inferred that the level of post-exercise signalling responsible for translation initiation would have been comparable in week 1 and week 10, as individual proteins, both within and between exercise modality demonstrated a moderate correlation in FSR.

Strengths and Weakness of the Research

Study 1 is part of a collaborative project aimed at characterizing the mechanisms underpinning myofibrillar protein synthesis and skeletal muscle hypertrophy in response to muscle loading. Study 1 had a significant strength in its pairing of both widespread and targeted analyses. We also expanded on previous global phosphoproteomic studies in humans (Hoffman, Parker et al. 2015, Blazev, Carl et al. 2022) by contrasting outcomes directly with protein synthesis across the recovery period. However, study 1 is not without limitations. Due to the chosen method of tracer analysis ($^{13}C_6$ Phenylalanine), the period in which protein synthesis could be assessed was limited to 3 hours post-exercise. As discussed in Chapter 1, the protein synthetic response is upregulated rapidly post-exercise and can remain elevated for ~24 hours (Di Donato, West et al. 2014), as such, we are only capturing a relatively brief (yet important) window of synthesis and signaling. Another limitation of Study 1 is a reduced sample size (n=4) for the pilot Cohort and also that the cohorts were not specifically recruited to have similar baseline characteristics (i.e. sex, BMI, training history, etc.). Therefore, although participants in each of the different cohorts

completed an identical intervention, the phosphoproteomic data from Cohort 1 is biologically distinct with targeted analyses of Cohort 2. Lastly, to keep individual limbs free from any 'concurrent' exercise during the study period, cross-assessment of physiological measures, i.e. 1RM on the aerobic limb, and vice versa, were not performed. Therefore, we are limited in our analyses and cannot directly contrast the limbs for physiological measures (i.e. 1RM, Peak Watts) at baseline or following training.

Study 2 is part of an ongoing collaborative effort aimed at characterizing the proteomic adaptations to aerobic and resistance training, in both the early (1 week) and later (10 weeks) phase. Study 2 is notable for its robust sample size (n=14), the inclusion of both males and females and the use of a unilateral design, all of which distinguish it as a peer with other proteomic investigations in exercise training. Nevertheless, Study 2 also has limitations, such as the absence of cross-assessment of physiological measures (common to Study 1) and technical issues with the metabolic cart, which limited our assessment during VO_2 ramp test to only being able to collect peak wattage (W) as a proxy indicator of aerobic fitness. Lastly, due to technical issues with the LC-MS, we are currently limited to analysis of only the myofibrillar muscle fraction. Pairing our investigation of the myofibrillar protein fraction (which often contains only a fraction of proteins in comparison to soluble (Roberts, Ruple et al. 2023)) with the soluble fraction will greatly expand our understanding of the current work. Both studies share the limitation that omic techniques may not fully capture the complete (phospho)proteome, and many (phospho)proteins are not yet annotated. Future directions could include examining the omic differences in highly trained persons to expand upon our knowledge of the proteome in 'highly adapted' tissue. Additionally, exploring other PTMs (i.e. ubiquitination and acetylation of lysine residues) and following up with targeted human analyses or mechanistic gain or loss of

function work in rodents or cell models is crucial for better understanding that the observations made in humans and leading to discovery of relevant mechanisms. Additionally, further analyses of the soluble fraction and phosphoproteomics are planned to provide insights into resting and recovery post-exercise.

Summary and main contributions of the thesis

In Study 1, we identified a novel signalling network predominantly regulated by RE and robustly associated with the post-exercise increase in myofibrillar protein synthesis. This study provides an overview of a newly proposed pathway likely contributing to the RE-induced rise in protein synthesis. Study 2 presents the first data integrating protein abundance profiling and synthesis rates on a protein-by-protein basis after both resistance and aerobic training, simultaneously in males and females. This research likely represents the most comprehensive analysis to date of protein-specific fractional synthesis rates in human muscle in vivo in response to different forms of exercise training. Our data allowed the quantification and comparison of 41 and 53 protein synthesis rates after 1 and 10 weeks of training, respectively, alongside the analysis of 474 protein abundances. The findings illustrate the shared and unique networks of protein regulation following AE and RE. Specifically, we highlight the influence of both AE and RE on mitochondrial proteins, likely aimed at enhancing metabolic function and potentially increasing aerobic capacity. Furthermore, we observed changes in protein abundance that do not align with individual protein synthetic rates, suggesting targeted degradation as an adaptive response shared between aerobic and resistance training. This analysis suggests that degradation may be selectively regulated, potentially driving the turnover of mitochondrial proteins with AE and RE training.

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