nature portfolio

Peer Review File

Aerobic and Resistance Exercise-Regulated Phosphoproteome and Acetylproteome Modifications in Human Skeletal Muscle

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications. Previous journal names have been redacted.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript from Pataky et al. maps the divergence in proteomic, phosphoproteomic, acetylproteomic, transcriptomic and metabolomic responses to acute aerobic versus resistance exercise in human skeletal muscle. These acute exercise analyses are complemented by additional omics-based analyses of human muscle biopsy responses to chronic aerobic versus resistance exercise training collected as part of this group's previous 2017 study published in Cell Metabolism. Overall the clarity and comprehensiveness of the revised manuscript has improved relative to the authors' original submission to [journal name redacted], and the revised manuscript with an overall lack of follow-up and/or mechanistic target validation now falls more within the scope, standard and relative level of significance to their specialised research field (and some other related fields from the wide readership) that would be expected for potential publication in Nature Communications.

In the revised manuscript the authors have made efforts to sufficiently address most of the major and minor comments from myself and Reviewer #2. These revisions include a more critical discussion of their specific findings and overall study's novelty relative to other related studies from the field, as well as highlighting some study limitations and addressing most other methodological considerations identified by reviewers that warranted further clarification and/or discussion in the manuscript. While overall this revised manuscript has sound methodology with most of the authors' conclusions appropriately supported by their datasets, there are still some remaining major and minor limitations and other aspects of the manuscript that warrant further revision in the manuscript as detailed in my major and minor comments below.

Major Comments:

1. Abstract, Lines 36-37 and associated Discussion/Conclusions (e.g., Lines 627-629): To ensure all of the authors' conclusions are fully supported by their datasets, some remaining statements in the manuscript need to be removed and/or further toned down since the authors have not performed any functional and/or mechanistic validation experiments demonstrating that top phosphoproteomic and/or acetylproteomic targets identified in their datasets indeed mechanistically regulate physiological adaptations to different modes of acute and chronic exercise. For example, to term 'likely' in the last sentence of the Abstract should be toned down to another word such as 'may' or 'potentially' to better align with the conclusions that can be drawn from the datasets, as well as other terms such as 'critical' insight. In line with this comment, please ensure this toned-down wording is used consistently throughout the manuscript so associated discussion points and conclusions are not overstated and are fully backed by the datasets, given the overall lack of follow-up validation and/or mechanistic experiments included in this manuscript (e.g., the authors' concluding statement 'These skeletal muscle post-translational signatures that likely dictate the different physiological adaptations to different exercise modes based on our results.' in Lines 627-629).

2. Figures 2A and 5A: In their responses to Reviewer #1, the authors state they have now specified the number of male and female participants and mean age in the diagrams for both the chronic exercise training study and acute exercise study

design shown in Figures 2A and 5A, respectively. Apologies if I have missed these additions to these respective figure panels, but it appears these details are still not shown. Please ensure these revised figure panels are included with your revised manuscript to help improve overall clarity and transparency of the study design to this journal's wide readership.

3. Results related to Reviewer #2's Comment #8: While this reviewer acknowledges the authors' comments related to the different scope and level of experimental validation of 'omics datasets that is typically expected for potential publications in Nature Communications relative to their previous target journal [journal name redacted] before this manuscript was transferred, the authors have primarily focused their rebuttal on the extensive time, funding and effort required to mechanistically validate the ribosomal chaperone NACA without acknowledging and/or attempting other relatively straightforward validation experiments (i.e., not mechanistic validation experiments) of known exercise-regulated phosphoprotein targets. However, Reviewer #2's specific comment about taking a better look and 'benchmarking' known acute aerobic and resistance exercise-regulated kinases such as 'the AMPK, mTOR, CaMK/CnA or other signaling pathways' is still valid and typically the current standard for exercise phosphoproteomic/acetylproteomic studies published from this field.

While some known surrogate upstream/downstream regulators and substrates of these known/proposed exercise-regulated kinases have been identified within the large phosphoproteomic dataset, key kinase regulatory phosphosites and/or substrate targets identified are not specifically discussed or further highlighted/interrogated in the manuscript outside of performing kinase activation predictions. Furthermore, this types of basic benchmarking and validation experiments in studies involving exercise physiology and omics-based analysis of skeletal muscle are feasible with a small amount of sample available and not costly or time-consuming with well-validated and specific antibodies available (e.g., immunoblot analysis of AMPK, mTOR and at least one respective well-established substrate). Therefore, some additional benchmarking validation of key kinases known to be regulated by acute aerobic versus resistance exercise, or at least some further discussion of the authors' quantification of key acute aerobic and resistance exercise-regulated kinases/substrates within their phosphoproteomic datasets is warranted to more effectively benchmark their exercise interventions and muscle signaling responses observed in their study's participant cohort relative to other relevant targeted and global studies published in this field to date. This is especially important considering that most other previously-published exercise studies in this field typically involve more homogenous human cohorts with less range of age, exercise capacity and other participant characteristics such as BMI (i.e., despite the authors' paired analysis used, the range of 18-55 years recruited for their acute study is much more heterogenous in terms of whole-body physiology and specifically muscle physiology and associated responses molecular to exercise, compared to the more typical age range of 18-30 years recruited for their exercise training study that is more in line with norms in the field).

Considering other important limitations raised by Reviewer #2's Comment #2 related to the overall lack of data in this study relating to exercise training descriptors between the aerobic and resistance exercise training programs, this muscle biopsy acute exercise response benchmarking strategy or at least acknowledgement of these previous points raised by Reviewer #2 in the authors' responses and some further discussion of these key targets in the manuscript is warranted. Performing some basic validation experiments outside of just focusing the authors' manuscript revisions on other points such as potential differential kinase localization and sample-to-sample muscle biopsy variability will help increase the field and readership's abilities to compare the targeted, divergent responses in key acute exercise-regulated kinases in this study across other studies from the field. This will in turn improve the overall comprehensiveness and value of this study to this specialised research field, without "delaying for publication while waiting for basic experiments related to NACA" as the authors repeatedly state in their rebuttal to multiple reviewers' related comments.

Minor Comments:

1. Introduction, Lines 56-58: The authors are commended for raising this important background about the lack of convergence between transcriptomic and proteomic responses to exercise training in the revised manuscript (which importantly is also applicable to acute exercise). However, this statement should be further expanded and/or clarified to more effectively highlight the complex layers of both pre/post-transcriptional control of gene expression as well as pre/post-translational control of protein expression (i.e., it is not as simple as just stating 'a balance between (protein) synthesis and degradation'). The associated statement 'can now be explained' as currently written should also be toned down, as it implies that the molecular basis for this lack of convergence has now been fully unravelled. These complex mechanisms and regulatory layers underlying the molecular control of acute and chronic exercise-induced gene and protein expression and their functional consequences in physiological adaptations are still mostly unknown and only beginning to be discovered by the exercise field.

2. Results, Lines 385-392: The authors are commended for highlighting the potential role of secreted factors such as "exerkines" in their acute one-legged exercise model, which may induce off-target effects on the non-exercised leg muscle and other tissues throughout the body after being released by the acutely exercised leg muscle. However, the authors should consider further revising how these potential off-target effects are described in the Results and/or Discussion, as these potential effects do not just involve the metabolome, but also may involve crosstalk involving other types of biological cargo (e.g., proteins, DNA, RNA, lipids and cytokines) which for example can be stored inside extracellular vesicles and have potential effects on skeletal muscle that are not detected by skeletal muscle metabolomic analyses. More detailed description of these 'other important molecular responses', outside of just discussing the metabolomic responses in acutely exercised muscle, is therefore warranted in this section of the Results and/or the Discussion to improve clarity/transparency to readers and better capture the current state of the field.

Reviewer #3

(Remarks to the Author)
The authors have thoughtfully considered my comments and suggestions. They have diligently addressed open questions in the manuscript and clarified methods that were missing from the initial submission. I would like to thank them for clarifying statistical analysis and making the raw data available. Looking forward to seeing the article in press.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have now sufficiently addressed all remaining major and minor comments. The authors are commended for taking the time and effort to carefully consider and complete the suggested revisions, which have helped improve the overall manuscript text and figures.

Collectively, this manuscript represents a significant hypothesis-generating resource for the exercise physiology research field and beyond, stemming future mechanistic investigations into phosphoproteomic and acetylproteomic regulation of skeletal muscle molecular targets involved in the divergent physiological responses to aerobic versus resistance exercise.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript from Pataky et al. maps the divergence in proteomic, phosphoproteomic, acetylproteomic, transcriptomic and metabolomic responses to acute aerobic versus resistance exercise in human skeletal muscle. These acute exercise analyses are complemented by additional omics-based analyses of human muscle biopsy responses to chronic aerobic versus resistance exercise training collected as part of this group's previous 2017 study published in Cell Metabolism. Overall the clarity and comprehensiveness of the revised manuscript has improved relative to the authors' original submission to [**journal name redacted**], and the revised manuscript with an overall lack of follow-up and/or mechanistic target validation now falls more within the scope, standard and relative level of significance to their specialised research field (and some other related fields from the wide readership) that would be expected for potential publication in Nature Communications.

In the revised manuscript the authors have made efforts to sufficiently address most of the major and minor comments from myself and Reviewer #2. These revisions include a more critical discussion of their specific findings and overall study's novelty relative to other related studies from the field, as well as highlighting some study limitations and addressing most other methodological considerations identified by reviewers that warranted further clarification and/or discussion in the manuscript. While overall this revised manuscript has sound methodology with most of the authors' conclusions appropriately supported by their datasets, there are still some remaining major and minor limitations and other aspects of the manuscript that warrant further revision in the manuscript as detailed in my major and minor comments below.

We thank the reviewer for the insightful and thoughtful comments on this revised manuscript. We appreciate the reviewer for recognizing the sound methodology, novelty of the study, and the potential for publication in Nature Communications. As advised by the reviewer we have addressed the reviewer's remaining major and minor comments, especially performing additional immunoblotting experiments and careful interpretation of the results, as described below.

Major Comments:

1. Abstract, Lines 36-37 and associated Discussion/Conclusions (e.g., Lines 627-629): To ensure all of the authors' conclusions are fully supported by their datasets, some remaining statements in the manuscript need to be removed and/or further toned down since the authors have not performed any functional and/or mechanistic validation experiments demonstrating that top phosphoproteomic and/or acetylproteomic targets identified in their datasets indeed mechanistically regulate physiological adaptations to different modes of acute and chronic exercise. For example, to term 'likely' in the last sentence of the Abstract should be toned down to another word such as 'may' or 'potentially' to better align with the conclusions that can be drawn from the datasets, as well as other terms such as 'critical' insight. In line with this comment, please ensure this toned-down wording is used consistently throughout the manuscript so associated discussion points and conclusions are not overstated and are fully backed by the datasets, given the overall lack of follow-up validation and/or mechanistic experiments included in this manuscript (e.g., the authors' concluding statement 'These skeletal muscle

post-translational signatures that likely dictate the different physiological adaptations to different exercise modes based on our results.' in Lines 627-629).
We have now made the following changes to are more balanced interpretation of the results:

- Line 36: changed "critical" to "new"
- Line 37: changed "likely" to "potentially"
- Line 417: inserted "many"
- Line 531: changed "caused" twice to "resulted in" and "induced"
- Line 535: changed "critical" to "new"
- Line 651: removed the word "clearly"
- Line 659: changed "likely" to "may"

2. Figures 2A and 5A: In their responses to Reviewer #1, the authors state they have now specified the number of male and female participants and mean age in the diagrams for both the chronic exercise training study and acute exercise study design shown in Figures 2A and 5A, respectively. Apologies if I have missed these additions to these respective figure panels, but it appears these details are still not shown. Please ensure these revised figure panels are included with your revised manuscript to help improve overall clarity and transparency of the study design to this journal's wide readership.

The reviewer is correct, and we have now addressed these omissions. Please find the new figures with panels that show the number of males and females in each group.

3. Results related to Reviewer #2's Comment #8: While this reviewer acknowledges the authors' comments related to the different scope and level of experimental validation of 'omics datasets that is typically expected for potential publications in Nature Communications relative to their previous target journal [**journal name redacted**] before this manuscript was transferred, the authors have primarily focused their rebuttal on the extensive time, funding and effort required to mechanistically validate the ribosomal chaperone NACA without acknowledging and/or attempting other relatively straightforward validation experiments (i.e., not mechanistic validation experiments) of known exercise-regulated phosphoprotein targets. However, Reviewer #2's specific comment about taking a better look and 'benchmarking' known acute aerobic and resistance exercise-regulated kinases such as 'the AMPK, mTOR, CaMK/CnA or other signaling pathways' is still valid and typically the current standard for exercise phosphoproteomic/acetylproteomic studies published from this field.

While some known surrogate upstream/downstream regulators and substrates of these known/proposed exercise-regulated kinases have been identified within the large phosphoproteomic dataset, key kinase regulatory phosphosites and/or substrate targets identified are not specifically discussed or further highlighted/interrogated in the manuscript outside of performing kinase activation predictions. Furthermore, this types of basic benchmarking and validation experiments in studies involving exercise physiology and omics-based analysis of skeletal muscle are feasible with a small amount of sample available and not costly or time-consuming with well-validated and specific antibodies

available (e.g., immunoblot analysis of AMPK, mTOR and at least one respective well-established substrate). Therefore, some additional benchmarking validation of key kinases known to be regulated by acute aerobic versus resistance exercise, or at least some further discussion of the authors' quantification of key acute aerobic and resistance exercise-regulated kinases/substrates within their phosphoproteomic datasets is warranted to more effectively benchmark their exercise interventions and muscle signaling responses observed in their study's participant cohort relative to other relevant targeted and global studies published in this field to date. This is especially important considering that most other previously-published exercise studies in this field typically involve more homogenous human cohorts with less range of age, exercise capacity and other participant characteristics such as BMI (i.e., despite the authors' paired analysis used, the range of 18-55 years recruited for their acute study is much more heterogenous in terms of whole-body physiology and specifically muscle physiology and associated responses molecular to exercise, compared to the more typical age range of 18-30 years recruited for their exercise training study that is more in line with norms in the field).

Considering other important limitations raised by Reviewer #2's Comment #2 related to the overall lack of data in this study relating to exercise training descriptors between the aerobic and resistance exercise training programs, this muscle biopsy acute exercise response benchmarking strategy or at least acknowledgement of these previous points raised by Reviewer #2 in the authors' responses and some further discussion of these key targets in the manuscript is warranted. Performing some basic validation experiments outside of just focusing the authors' manuscript revisions on other points such as potential differential kinase localization and sample-to-sample muscle biopsy variability will help increase the field and readership's abilities to compare the targeted, divergent responses in key acute exercise-regulated kinases in this study across other studies from the field. This will in turn improve the overall comprehensiveness and value of this study to this specialised research field, without "delaying for publication while waiting for basic experiments related to NACA" as the authors repeatedly state in their rebuttal to multiple reviewers' related comments.

The reviewer is correct in that although we have identified surrogate upstream/downstream regulators we did not provide a benchmark of the acute exercise phospho-response in muscle in our submission. We have now provided immunoblot data demonstrating increased phosphorylation of AMPK and mTOR and some of their well-documented downstream substrates such as ACC^{Ser79} pP70S6K^{Thr389} and pRPS6$^{Ser240/244}$ in response to acute aerobic and resistance exercise (Figure 5, panels I-J). Also, we have added text on lines 424-429, 574-576, 793-797, and 1127-1131.

Minor Comments:

1. Introduction, Lines 56-58: The authors are commended for raising this important background about the lack of convergence between transcriptomic and proteomic responses to exercise training in the revised manuscript (which importantly is also applicable to acute exercise). However, this statement should be further expanded and/or clarified to more effectively highlight the complex layers of both

pre/post-transcriptional control of gene expression as well as pre/post-translational control of protein expression (i.e., it is not as simple as just stating 'a balance between (protein) synthesis and degradation'). The associated statement 'can now be explained' as currently written should also be toned down, as it implies that the molecular basis for this lack of convergence has now been fully unravelled. These complex mechanisms and regulatory layers underlying the molecular control of acute and chronic exercise-induced gene and protein expression and their functional consequences in physiological adaptations are still mostly unknown and only beginning to be discovered by the exercise field.

Thank you for this recommendation. We have now expanded on this section of the introduction to provide more clarity. (see lines 56-63).

2. Results, Lines 385-392: The authors are commended for highlighting the potential role of secreted factors such as "exerkines" in their acute one-legged exercise model, which may induce off-target effects on the non-exercised leg muscle and other tissues throughout the body after being released by the acutely exercised leg muscle. However, the authors should consider further revising how these potential off-target effects are described in the Results and/or Discussion, as these potential effects do not just involve the metabolome, but also may involve crosstalk involving other types of biological cargo (e.g., proteins, DNA, RNA, lipids and cytokines) which for example can be stored inside extracellular vesicles and have potential effects on skeletal muscle that are not detected by skeletal muscle metabolomic analyses. More detailed description of these 'other important molecular responses', outside of just discussing the metabolomic responses in acutely exercised muscle, is therefore warranted in this section of the Results and/or the Discussion to improve clarity/transparency to readers and better capture the current state of the field.

We agree with the suggestion of the reviewer and have now included text further describing other possible off-target effects such as EV delivery of molecular signaling molecules (see lines 402-405). We limited the addition of these interpretations to the Results section where the topic was initially introduced (versus also adding it to the Discussion) due to word restrictions.

Reviewer #3 (Remarks to the Author):

The authors have thoughtfully considered my comments and suggestions. They have diligently addressed open questions in the manuscript and clarified methods that were missing from the initial submission. I would like to thank them for clarifying statistical analysis and making the raw data available. Looking forward to seeing the article in press.

Thank you.