nature portfolio

Peer Review File

Temporal phosphoproteomics reveals circuitry of phased propagation in insulin signaling

Corresponding Author: Professor Hadi Al-Hasani

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Subject: Temporal phosphoproteomics reveals circuitry of phased propagation in insulin signaling

Turewicz et al. report their study on the effect of insulin on global phosphorylation in primary human skeletal muscle cells derived form 5 normal weight human participants in a time-depend manner. The author has quantified >10,000 phosphorylation sites in response to the insulin treatment, and have presented extensive data interpretation and bioinformatic analysis, and for most part, enough detail are provided in the methods for the work to be reproduced. However, they are major concerns.

Major concerns:
• Lack of human participants' clinical characteristics:
o Only age, weight, heigh and BMI were provided.
o The sample size is small (total 5 participants), and 3 are Lean while 2 are overweight, who may have different levels of prediabetes and/or insulin sensitivity and insulin resistance, even though they are healthy.
o Physical activity, fasting plasma glucose, fasting plasma insulin, 2-hour OGTT, and HbA1c may affect insulin signaling, insulin sensitivity, and/or insulin resistance as well as the primary outcome of the experiments, insulin-stimulated phosphorylation, while these data were missing.
o It will also be helpful if insulin sensitivity was measured in these participants by hyperinsulinemic-euglycemic clamp.
• Since both proteome and phosphoproteome data were acquired, it may help to normalize the individual phosphorylation site abundance to the corresponding protein abundance (even when the corresponding unphosphorylated peptide was not detected). This may correct for sample loading and provide information on whether the observed increase or decrease of phosphorylation level is due to the changes in protein abundance or not.
• Page 10, "Heatmap of the top 35 overrepresented kinases (p-value < 0.15)", why not use P<0.05?
• An additional donor variability assessment is needed. There are 11,612 phosphopeptides with quantitative data for at all time points phosphorylation site. For each time point comparison to t0 (e.g., t5 min to t0), the CV for the fold change for each of the 11,612 phosphopeptides for the 5 donors needs to be calculated. Then the mean and medium for the 11,612 CVs can be obtained to indicate the variability of the 5 donors.
• RNASeq was performed on only 3 donors, and it is unclear how reliable these results will be due to the small sample size and relative large variation in primary human cell lines from different donors compared to immortalized cell lines from the same donor or animal models from similar genetic background.

Minor concerns:
• Page 7, a reference is needed for "phosphorylation of S50, a major activating site in the tyrosine phosphatase PTPN1 (Figure 3b)".
• Page 10, "PPI network conducted network propagation utilizing 10,642 proteins and 139,266", shall it be "PPI network and conducted network propagation utilizing 10,642 proteins and 139,266"
• Page 15, (RxRxxS/T, missing ")"
• Page 20, "After starvation for 4 h," while Page 2 "6h"
• Page 30, "167 167 corresponding", "125 125 corresponding phosphopeptides", "25,803 25,803 total proteome"?
• Supplemental Figure 9: the color scale on the right did not cover all the fold changes (e.g., SRSF1).

Reviewer #2

(Remarks to the Author)
Insulin signaling in muscle cells plays a pivotal role in regulating various physiological processes and is essential for maintaining glucose homeostasis in the body. Dysregulation of insulin signaling in muscle cells is implicated in the pathophysiology of several diseases, most notably type 2 diabetes mellitus. Thus, understanding the temporal dynamics and regulated pathways of insulin intracellular signaling cascade in muscle cells is crucial for developing targeted therapies to mitigate the impact of insulin resistance-related diseases.

In this manuscript, Turewicz and colleagues performed a detailed, time-resolved analysis of the skeletal muscle phosphoproteome following insulin stimulation, combining differentiated primary human myotubes from healthy donors and high-resolution mass spectrometry. The authors identify and track ~13,000 phosphopeptides at six different time points post-insulin exposure, and identify dynamic changes in the abundance of thousands of phosphopeptides. Interestingly, the authors found that a significant fraction of phosphosites is exclusively regulated at only one time point, revealing distinct clusters dependent of the duration of insulin stimulation. The authors also identified insulin targets with low donor variability, which included several phosphosites in well established insulin targets, but also in novel putative non-canonical targets. Multiple bioinformatics approaches were used to identify activated kinases, reconstruct a PPI signaling network and identify significantly enriched pathways. These analyses revealed predominantly activated kinases and structured a signaling PPI network with a core subnetwork of 18 phosphoproteins with low donor variability. Moreover, the authors found that insulin alters the activity of multiple biological pathways with a distinct temporal pattern. Remarkably, RNA processing and splicing appears among the top regulated pathways in the latter time points, including numerous spliceosomal proteins and regulators of alternative splicing that are differentially phosphorylated in response to insulin. This is in line with a previous publication (referenced by the authors) reporting that myoblast from T2D donors present dysregulated phosphorylation of proteins involved in mRNA splicing.

This is a novel and interesting work that provides a very detailed time-resolved roadmap of insulin signaling cascade in skeletal muscle. The work is well-written and contain good figures to make it easy to follow. The phosphoproteomics data (although not my area of expertise) are well-described and provides significant novelties into the dynamics of insulin-stimulated phosphorylation, its protein targets and their corresponding downstream pathways. My only big-picture comment is that the work is mainly descriptive and I was expecting to see some biological insights, for instance related to the development of insulin resistance in skeletal muscle, or a further analysis of the impact of insulin signaling in mRNA splicing.

Comments:

1. Previous GWAS studies on insulin resistance, T2D and related glycemic traits have identified hundreds of genetic variants and a number of them have been reported to exert their function in skeletal muscle. It would be interesting to explore whether targets of insulin signaling identified in the study, particularly novel putative non-canonical targets, are enriched in GWAS signals, directly or in their neighboring PPI network. If this were the case, it would be also interesting to explore whether GWAS hits are overrepresented in targets with low or high donor variability, which might provide novel insights of the inheritance of insulin resistance.

2. The present study presents an unprecedented resolution of insulin signaling cascade in skeletal muscle and would be a very useful resource to further characterize the signaling defects underlying insulin resistance in T2D. Multiple stressors such as hyperinsulinemia, glucocorticoids and inflammatory cytokines have been reported to induce insulin resistance both in vitro and in vivo. Phosphoproteomic analysis of insulin resistant-induced myotubes may shed light on the dysregulation and rewiring of insulin signaling in insulin resistant conditions, and may pinpoint to candidate kinases and signaling subnetworks as causal drivers of insulin resistance in muscle. Alternatively or in parallel, the authors could integrate previously published phosphoproteomics datasets of T2D or insulin resistant muscle cells to identify dysregulated signaling networks in pathological conditions.

3. The extensive post-transcriptional regulation of proteins involved in mRNA splicing regulation by insulin signaling is a very interesting point and in line with previous studies showing that insulin regulates alternative splicing in liver and muscle cells, and that T2D muscle cells exhibit altered phosphorylation of spliceosomal factors. However, with the present analysis is unclear to which extent insulin-signaling induce splicing changes in skeletal muscle. The authors performed an isoform-based splicing analysis using transcript quantification from RNAseq reads and kallisto, which I do not think is ideal since might be biased by other transcriptional changes not related to splicing. Event-based methods such Vast-tools, rMATS or MAJIQ that compute the PSI index are more suited to quantify splicing changes. A more complete analysis of insulin-induced splicing changes, including the different types of altered splicing events (intron retention, exon skipping, alternative 5' or 3' splice sites), the extent of PSI changes and pathway enrichment analysis among others, may provide novel insights on the regulation and functional impact of insulin-induced splicing in muscle. Additionally, feature analysis of regulated alternative splicing events such as exon length, splice site strength or motif enrichment could help to identify candidate spliceosomal components or RNA-binding proteins as key regulators of insulin-induced splicing.

Minor:
- No validations using complementary methods are shown to demonstrate the robustness of the time-resolved analysis of the insulin-induced phosphoproteome, for instance for selected cases showing time-specific phosphorylation changes. Similarly, selected events of insulin-induced alternative splicing changes should be ideally validated by RT-PCR or other methods.

Reviewer #3

(Remarks to the Author)
Turewicz and Skagen et al conducted a groundbreaking temporal phosphoproteomics analysis of insulin-regulated pathways in human myotubes, uncovering a complex network of transient phosphorylation and dephosphorylation events. The study is the first of its kind to explore the temporal phosphoproteome, addressing a critical gap in the existing literature, which primarily provides snapshots of muscle phosphoproteome at one time point.
The methodology employed is commendable, with comprehensive phosphoproteome coverage and rigorous bioinformatics analysis. The results offer valuable insights for the scientific community. However, there are notable concerns and suggestions for manuscript improvement:
Major Concerns:
1. Lack of Proper Control: The absence of adequate controls is a major concern. Comparing time series data with T0 may not be fair, especially considering the one-hour extra starvation for cells treated with insulin at the 1-hour time point. Including controls at all timepoint will be overkill but author should have at least included 2-3 controls at early, intermediate and late stage.
2. Donor Variability: Caution is advised in interpreting findings related to donor variability due to the limited sample size. Human primary cells' inherent unpredictability may contribute to observed heterogeneity. The method used to identify donor-variable/invariable phosphosites requires clarification, as correlation analysis assumes independence, which may not be valid for donor-matched samples.
3. Descriptive Nature of Manuscript: The manuscript is overly descriptive, particularly in the results section. Shortening the results section and consolidating figures is suggested to enhance readability.
4. Lack of Functional Experiments: The manuscript could benefit from incorporating functional experiments. Such experiments could be conducted in muscle cell culture models. Alternatively, authors can demonstrate the functional relevance of insulin-regulated splicing proteins in model system.
Minor Comments:
1. Phosphosite Occupancy: Clarify how phosphosite occupancy is calculated and emphasize the need for corresponding protein/peptide quantification for accurate occupancy determination.
2. Proteome and Phosphoproteome Overlap: Highlight the limitation of poor total proteome coverage leading to minimal overlap between proteome and phosphoproteome datasets. Consider re-measuring the proteome for deeper coverage.
3. Supplementary Figure 1A: Increase the text size for better readability.
4. Methods Section Typos: Address typographical errors in the methods section, particularly in the sentences discussing PCA analysis.
5. References Formatting: Ensure all references are correctly formatted throughout the manuscript, paying attention to details like in the network propagation section.
Addressing these concerns and implementing the suggested improvements will enhance the manuscript's overall quality and impact.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This is a revised manuscript, and the authors have addressed most of concerns and the manuscript is improved, however, some concerns remain and need to be addressed before suitable for publication in the journal.

Major concerns:
1) Lack of human participants' clinical characteristics:
● Physical activity, 2-hour OGTT, and HbA1c may affect insulin signaling, insulin sensitivity, and/or insulin resistance as well as the primary outcome of the experiments, insulin-stimulated phosphorylation, while these data were missing.
• Before the biopsy procedure, 1). Were participants fasted overnight? 2). Were they allowed to do exercise? If yes, what kind of exercise was allowed? If not, how many days they were not allowed to exercise?
• Were 2-h OGTT and HbA1c performed on these subjects?

2) The 'Precursor Ions Quantifier' node was used to normalize the phosphopeptide abundances based on the total phosphopeptide content. Does this approach assume the total phosphopeptide content in different samples (with or without insulin treatment) is the same? If yes, this would be a concern. It is common practice to assume the total protein content in different samples is the same, however, it is likely that total phosphorylation would be different in samples with or without insulin treatment. It is better to normalize individual phosphopeptide level derived from phosphopeptide enrichment to the total protein level derived from the global proteome, when the corresponding non-phosphorylated peptides or the corresponding protein abundances derived from the global proteome are lacking.

3) Is there a fold change cut off (e.g., 1.5-fold change as used for phosphorylation analysis) for significantly changed transcript by RNASeq? The variation of transcript abundance in the samples may be considered moderate (median CV of the Illumina RNASeq read counts from three donors was 27.4% (mean 33.4%)), however, it may not have enough power to detect small differences in 3 donors.

Minor concerns:

It is better to move the Supplemental table headers before the table instead of after the table.

(Remarks on code availability)
Can not find the code by click the link provided.

Reviewer #2

(Remarks to the Author)
I thank the authors for the considerable time and effort dedicated to address my concerns and suggestions. I appreciate the thoroughness of the revisions and the additional data you have incorporated in response to my feedback.

In my opinion, the modifications you have made to the manuscript significantly strengthen the impact and relevance of the findings. I have no additional comments. Thank you again and congratulations for the nice work.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)
I think that this very interesting and groundbreaking manuscript has been thoroughly reviewed by all three reviewers and that the authors have gone to great length to address all the concerns addressed by the reviewers including reviewer 3.

(Remarks on code availability)

REVIEWER COMMENTS

We like to thank the reviewers for their positive feedback and their highly valuable comments and suggestions.

Reviewer #1 (Remarks to the Author):

Turewicz et al. report their study on the effect of insulin on global phosphorylation in primary human skeletal muscle cells derived from 5 normal weight human participants in a time-dependent manner. The author has quantified >10,000 phosphorylation sites in response to the insulin treatment, and have presented extensive data interpretation and bioinformatic analysis, and for most part, enough details are provided in the methods for the work to be reproduced. However, they are major concerns.

Major concerns:
1) Lack of human participants' clinical characteristics:

- *Only age, weight, height and BMI were provided.*
- *The sample size is small (total 5 participants), and 3 are Lean while 2 are overweight, who may have different levels of prediabetes and/or insulin sensitivity and insulin resistance, even though they are healthy.*
- *Physical activity, fasting plasma glucose, fasting plasma insulin, 2-hour OGTT, and HbA1c may affect insulin signaling, insulin sensitivity, and/or insulin resistance as well as the primary outcome of the experiments, insulin-stimulated phosphorylation, while these data were missing.*
- *It will also be helpful if insulin sensitivity was measured in these participants by hyperinsulinemic-euglycemic clamp.*

Thank you very much for your comments. We apologize for inadvertently providing insufficient information for the donors regarding metabolic traits. We have added additional information to the donor description that now, in addition to age, body weight, height and BMI includes body composition (lean mass, fat mass), VO2max, fasting glucose levels, fasting insulin levels, fasting C-peptide levels, and HOMA-IR as a measure of insulin sensitivity of the donors (**Supplemental Table 1**). Furthermore, we have used myotubes from the donors to measure insulin-stimulated glycogen synthesis in vitro and added these functional data to the revised manuscript (**Figure 1**). While we agree that hyperinsulinemic-euglycemic clamps represent the gold standard for determining insulin sensitivity, numerous studies have demonstrated that HOMA-IR may correlate well with the clamp-derived insulin sensitivity index (ISI). We also agree that our sample size represents a limitation in terms of the phenotypic spectrum of the donors and this issue is now addressed in the discussion section (**p 26**).

2) *Since both proteome and phosphoproteome data were acquired, it may help to normalize the individual phosphorylation site abundance to the corresponding protein abundance (even when the corresponding unphosphorylated peptide was not detected). This may correct for sample loading and provide information on whether the observed increase or decrease of phosphorylation level is due to the changes in protein abundance or not.*

Thank you for this very important point. Our analysis of the global proteome showed that insulin stimulation for 60 min hardly had any effect on the abundance of proteins (now **Supplemental Figure 4, Supplemental Table 2**), strongly suggesting that the altered abundances in phosphopeptides relate to phosphorylation events. Furthermore, sample loading normalization has been applied using the respective established node in the workflow of Proteome Discoverer 3.0 as described now in methods (**p. 30**). In essence, for sample loading normalization the 'Precursor Ions Quantifier' node was used to normalize the phosphopeptide abundances based on the total phosphopeptide content.

In order to validate our approach we further investigated the relation of phosphopeptide abundance and protein abundance using two different strategies as suggested by the reviewer:

1) phosphopeptide:peptide normalization: We correlated the individual abundance ratios of phosphopeptides with the ratios normalized to the corresponding non-phosphorylated peptides quantified in our proteome measurements. For a total of 1,367 phosphopeptides, a peptide with identical amino acid sequence could be quantified in the total proteome (without phospho enrichment). Of note, normalization was not possible for 11,829 phosphopeptides (89.64%) because no matching peptide was quantified in the total proteome.
2) phosphopeptide:protein normalization: We correlated the abundance ratios of the phosphopeptides with the ratios normalized to the corresponding protein abundances derived from the analysis of the global proteome. Here 5,322 phosphopeptides could be normalized to matching protein abundances. However, normalization was not possible for 7,874 phosphopeptides (59.67%) because no matching protein was quantified in the total proteome.

The data are now shown in **Supplemental Figure 4**. As illustrated in the Figure, the abundance ratios of the phosphopeptides were highly correlated to the normalized ratios (r=0.9519 for phosphopeptide:peptide and r=0.9848 for phosphopeptide:protein normalization, respectively), indicating that changes in phosphopeptide abundance are due to phosphorylation events and not alterations in protein abundance. The correlation is even higher for differentially regulated peptides (r=0.9919 and r=0.997, respectively; plots not shown). As a result, we believe that these data demonstrate the validity of our approach.

We address this issue and discuss the limitations in the revised manuscript (**results and discussion section, pp 3, 26**).

3) *Page 10, "Heatmap of the top 35 overrepresented kinases (p-value < 0.15)", why* ***not use P<0.05?***

We have changed the Figure accordingly to include kinases with a p-value for overrepresentation < 0.05. In addition, we included six kinases with borderline significance (p < 0.075; indicated in the figure legend). **Figure 5a** now shows a total of 19 overrepresented (instead of 35) protein kinases. The phylogenetic tree of the kinases is now shown in **Supplemental Figure 10**.

4) *An additional donor variability assessment is needed. There are 11,612 phosphopeptides with quantitative data for at all time points phosphorylation site. For each time point comparison to t0 (e.g., t5 min to t0), the CV for the fold change for each of the 11,612 phosphopeptides for the 5 donors needs to be calculated. Then the mean and medium for the 11,612 CVs can be obtained to indicate the variability of the 5 donors.*

Thank you for this comment. We have, as suggested, further assessed donor variability of the phosphopeptides and calculated CVs for each donor (5 x 11,612 = 58,060 values) and each time point (7 x 11,612 = 81,284 values), see below. We found that the CV values (mean: 31.73%, median: 26.93%) were similar for the different time points across the donors (median range: 25.85% - 28.79%) as illustrated in the revised **Figure 1c**. Distribution of CVs for abundance values from all donors and all time points, and all technical replicates are now included in the supplemental information (**Supplemental Figure 2**).

Phosphoproteome (7 time points x 11,612 peptides)	**CV Mean (%)**	**CV Median (%)**
1 min	30.99	25.85
2.5 min	31.50	26.78
5 min	31.06	26.28
15 min	31.72	26.97
30 min	31.63	26.72
60 min	33.49	28.79
overall	*31.73*	*26.93*

Phosphoproteome (5 donors x 11,612 peptides)	**CV Mean (%)**	**CV Median (%)**
Donor A	29.45	24.65
Donor B	29.64	25.30
Donor C	29.52	24.86
Donor D	30.90	26.18
Donor E	26.54	21.49
overall	*29.21*	*24.51*

5) RNASeq was performed on only 3 donors, and it is unclear how reliable these results will be due to the small sample size and relative large variation in primary human cell lines from different donors compared to immortalized cell lines from the same donor or animal models from similar genetic background.

We agree that our RNASeq analysis of three donors constitutes a limitation of our study, yet the nature and magnitude of changes in gene expression was in concordance with the literature. The median CV of the Illumina RNASeq read counts from our three donors was 27.4% (mean 33.4%) in both basal and insulin stimulated states (see below), indicating moderate variation of transcript abundance in the samples. In contrast, a recently published RNASeq data set from our laboratory using skeletal muscle and liver of the inbred C57BL/6J mouse strain displayed lower variation (median CV for muscle 10.19% (mean 22.06%); 12.80% (mean 25.14%) for liver tissue) using four animals per group (B6 Sed Control; Springer *et al.* Diabetes 2024 73:1058-1071), thus confirming larger variation in primary human cell lines vs. inbred mice.
In our present study, we used our RNASeq data to analyze alternative splicing in myotubes from the three donors. Principal component analysis of all identified splice events demonstrated that splicing was mostly dependent on the donor (see new **Supplemental Figure 17a**), indicating marked variation between the individual samples. However, PCA based on the insulin-regulated splicing events (FDR<0.01; ΔPercent Spliced-In (PSI)>0.1) clearly separated basal and insulin-stimulation across all donors (**Supplemental Figure 17b**), demonstrating that in human primary skeletal muscle cells, insulin acutely affects alternative splicing in a rather donor independent manner. Thus, while donor variation is present compared to cultured model cell lines/inbred animal models, we were able to discriminate basal and insulin-regulated alternative splicing across the different donors. Nevertheless, replication is required that include larger numbers of donors, and a broader spectrum in phenotypic variation. This issue and the limitations of our study are now discussed in our revised manuscript (**discussion section, p. 26**). Moreover, the median CV values for RNASeq are now included in the methods section of the revised manuscript (**p. 30**).

RNASeq (3 donors x 26,848 transcripts)	**CV Mean (%)**	**CV Median (%)**
basal	32.84	26.93
insulin	33.89	27.86

Minor concerns:
Page 7, a reference is needed for "phosphorylation of S50, a major activating site in the tyrosine phosphatase PTPN1 (Figure 3b)".
The respective reference (Ravichandran *et al.* Mol Endocrinol. 2001; PMID: 11579209) has been added.

Page 10, "PPI network conducted network propagation utilizing 10,642 proteins and 139,266", shall it be "PPI network and conducted network propagation utilizing 10,642 proteins and 139,266"
Apologies - the sentence has been corrected accordingly.

Page 15, (RxRxxS/T, missing ")
The sentence has been corrected accordingly.

Page 20, "After starvation for 4 h," while Page 2 "6h"
Apologies - the error on p20 has been corrected to 6 h starvation.

Page 30, "167 167 corresponding", "125 125 corresponding phosphopeptides", "25,803 25,803 total proteome"?
Apologies, we have corrected the errors in the methods section and elsewhere.

Supplemental Figure 9: the color scale on the right did not cover all the fold changes (e.g., SRSF1).
We apologize for the error. We have corrected the color map and scale accordingly.

Reviewer #2 (Remarks to the Author)

Insulin signaling in muscle cells plays a pivotal role in regulating various physiological processes and is essential for maintaining glucose homeostasis in the body. Dysregulation of insulin signaling in muscle cells is implicated in the pathophysiology of several diseases, most notably type 2 diabetes mellitus. Thus, understanding the temporal dynamics and regulated pathways of insulin intracellular signaling cascade in muscle cells is crucial for developing targeted therapies to mitigate the impact of insulin resistance-related diseases.
In this manuscript, Turewicz and colleagues performed a detailed, time-resolved analysis of the skeletal muscle phosphoproteome following insulin stimulation, combining differentiated primary human myotubes from healthy donors and high-resolution mass spectrometry. The authors identify and track ~13,000 phosphopeptides at six different time points post-insulin exposure, and identify dynamic changes in the abundance of thousands of phosphopeptides. Interestingly, the authors found that a significant fraction of phosphosites is exclusively regulated at only one time point, revealing distinct clusters dependent of the duration of insulin stimulation. The authors also identified insulin targets with low donor variability,

which included several phosphosites in well established insulin targets, but also in novel putative non-canonical targets. Multiple bioinformatics approaches were used to identify activated kinases, reconstruct a PPI signaling network and identify significantly enriched pathways. These analyses revealed predominantly activated kinases and structured a signaling PPI network with a core subnetwork of 18 phosphoproteins with low donor variability. Moreover, the authors found that insulin alters the activity of multiple biological pathways with a distinct temporal pattern. Remarkably, RNA processing and splicing appears among the top regulated pathways in the latter time points, including numerous spliceosomal proteins and regulators of alternative splicing that are differentially phosphorylated in response to insulin. This is in line with a previous publication (referenced by the authors) reporting that myoblast from T2D donors present dysregulated phosphorylation of proteins involved in mRNA splicing.
This is a novel and interesting work that provides a very detailed time-resolved roadmap of insulin signaling cascade in skeletal muscle. The work is well-written and contain good figures to make it easy to follow. The phosphoproteomics data (although not my area of expertise) are well-described and provides significant novelties into the dynamics of insulin-stimulated phosphorylation, its protein targets and their corresponding downstream pathways. My only big-picture comment is that the work is mainly descriptive and I was expecting to see some biological insights, for instance related to the development of insulin resistance in skeletal muscle, or a further analysis of the impact of insulin signaling in mRNA splicing.

Comments:

1. *Previous GWAS studies on insulin resistance, T2D and related glycemic traits have identified hundreds of genetic variants and a number of them have been reported to exert their function in skeletal muscle. It would be interesting to explore whether targets of insulin signaling identified in the study, particularly novel putative non-canonical targets, are enriched in GWAS signals, directly or in their neighboring PPI network. If this were the case, it would be also interesting to explore whether GWAS hits are overrepresented in targets with low or high donor variability, which might provide novel insights of the inheritance of insulin resistance.*

Thank you for this suggestion. We have investigated, to our knowledge for the first time, the overlap of potential insulin targets in primary human myotubes with GWAS genes/proteins.
We used data from a recent very large-scale multi-ancestry meta-analyses of T2D (T2D Global Genomics Initiative; >2m/428k controls/cases; Suzuki K *et al*, Nature 2024) which has identified 1,289 independent association signals at genome-wide significance that map to 611 loci. From these, we compiled a list of 761 human protein coding candidate genes and evaluated the overlap with the proteins identified in our study that are differentially phosphorylated in response to insulin. for a more robust analysis, we used a filtered subset of 1,552 significantly insulin-regulated

phosphopeptides with continuous quantitative information for each time point (fold change versus t_0 for t_1 - t_{60}) which maps to 656 unique protein IDs. Our comparison revealed 43 proteins in the overlap. Among these, three genes DNAJC2, TCF12 and TNRC6B belong to the group of non-canonical targets having phosphosites with low donor variability. However, our approach has some limitations. GWAS may assess phenotypes that might be only indirectly linked to insulin action in skeletal muscle. Furthermore, we included only phosphopeptides with continuous quantitative information for each single time point in our analysis and we did not filter for myotube-specific expression which likely affects the number in the overlap.
Accordingly, the applied filter sets may be less suitable to confirm previous diabetes-linked diabetes signals but still provide a useful basis for future studies in this direction. We have aggregated the data in a new Table (**Supplemental Table 3**) and we address the issue in the revised manuscript (**results section, p. 10, discussion section, p. 22**).

2. *The present study presents an unprecedented resolution of insulin signaling cascade in skeletal muscle and would be a very useful resource to further characterize the signaling defects underlying insulin resistance in T2D. Multiple stressors such as hyperinsulinemia, glucocorticoids and inflammatory cytokines have been reported to induce insulin resistance both in vitro and in vivo. Phosphoproteomic analysis of insulin resistant-induced myotubes may shed light on the dysregulation and rewiring of insulin signaling in insulin resistant conditions, and may pinpoint to candidate kinases and signaling subnetworks as causal drivers of insulin resistance in muscle. Alternatively or in parallel, the authors could integrate previously published phosphoproteomics datasets of T2D or insulin resistant muscle cells to identify dysregulated signaling networks in pathological conditions.*

We have followed the suggestion and integrated previously published phosphoproteomics datasets of metabolically challenged and/or insulin resistant muscle cells. We compared our data with two recent phosphoproteomics studies, i) one of cultured rat L6 myotubes that were exposed to various cell stressors resembling aspects of exercise signaling in vivo and interfering with insulin signaling (Needham et al., 2019, Cell Reports 29, 1524–1538 // pmid 31693893) and ii) a study of insulin regulation with human myoblasts (iMyos) derived from iPS cells of T2D patients and healthy controls (Batista et al Cell Metab 2020 32:844-859. //pmid 32888406.). We indeed identified overlapping insulin regulated phosphosites in our and the aforementioned reports, supporting a possible role of these insulin target proteins in the pathology of insulin resistance and diabetes.
Notably, many of these sites display pronounced time dependence which may affect evaluation of these sites in the context of cellular stress and/or insulin resistance as different time points were investigated across the different studies. We therefore speculate that detection of dysregulated signaling networks in T2D might be improved if the temporal aspect of phosphosite occupancy is considered.

In line with our study on insulin targets, pathway overrepresentation analysis of iPSC-derived Myoblasts (iMyos) found RNA splicing among the most significantly differentially regulated processes in T2D. Interestingly, the overlap of individual phosphosites is moderate, possibly due to different kinetics in the phosphorylation of spliceosomal proteins.
The results are illustrated in **Supplemental Figure 9** and addressed in the text of the revised manuscript (**results section, p. 10**).

3. *The extensive post-transcriptional regulation of proteins involved in mRNA splicing regulation by insulin signaling is a very interesting point and in line with previous studies showing that insulin regulates alternative splicing in liver and muscle cells, and that T2D muscle cells exhibit altered phosphorylation of spliceosomal factors. However, with the present analysis is unclear to which extent insulin-signaling induce splicing changes in skeletal muscle. The authors performed an isoform-based splicing analysis using transcript quantification from RNAseq reads and kallisto, which I do not think is ideal since might be biased by other transcriptional changes not related to splicing. Event-based methods such Vast-tools, rMATS or MAJIQ that compute the PSI index are more suited to quantify splicing changes. A more complete analysis of insulin-induced splicing changes, including the different types of altered splicing events (intron retention, exon skipping, alternative 5' or 3' splice sites), the extent of PSI changes and pathway enrichment analysis among others, may provide novel insights on the regulation and functional impact of insulin-induced splicing in muscle. Additionally, feature analysis of regulated alternative splicing events such as exon length, splice site strength or motif enrichment could help to identify candidate spliceosomal components or RNA-binding proteins as key regulators of insulin-induced splicing.*

Thank you for the insight and these very helpful comments. We followed the reviewer's suggestion and reanalyzed our RNASeq data. As suggested, we applied multivariate analysis of transcript splicing (MATS) to identify transcripts that were differentially spliced in response to insulin. The new results are included in the revised **Figure 9.** Using this approach, we identified 153 transcripts for 131 coding genes and 9 long non-coding RNAs (lncRNAs) that were differentially spliced in response to insulin (ΔPSI>10%; **Figure 9c**). The majority of detected splicing events (48%) included skipped exons (SE), followed by alternative 3' splicing (A3SS), mutually exclusive exons (MXE), retained introns (RI), and 5' splicing (A5SS). Examples for the insulin-regulated splicing events and their effect size, i.e. extent of PSI changes, are now illustrated in **Figure 9d**. Interestingly, principal component analysis of the insulin-regulated splicing events (FDR<0.01; ΔPercent Spliced-In (PSI)>0.1) separated basal and insulin-stimulated conditions across all donors (**Supplemental Figure 17b**), demonstrating that in human primary skeletal muscle cells insulin acutely affects alternative splicing in a rather donor independent manner.

We now provide detailed information on insulin-regulated spliced transcripts in **Supplemental Table 10** including basal/insulin, delta PSI values, SD and corresponding FDR/p-values, the specific genomic coordinates of the splicing events, and predicted splicing leading to NMD. These data allow further analysis of exon lengths and respective motifs and may be a useful resource to further conduct mechanistic studies on candidate splicing proteins in future studies.
In addition to **Figure 9**, **Supplemental Figure 16, Supplemental Figure 16** and **Supplemental Table 10**, we have made subsequent changes in the methods section (**p. 35**), the results section (**p. 20**) and the discussion (**p. 26**) to reflect our new approach and new data.

Minor:
No validations using complementary methods are shown to demonstrate the robustness of the time-resolved analysis of the insulin-induced phosphoproteome, for instance for selected cases showing time-specific phosphorylation changes. Similarly, selected events of insulin-induced alternative splicing changes should be ideally validated by RT-PCR or other methods.

In order to validate our approach, we have performed new experiments and conducted Western blot analysis to investigate time-dependent insulin-stimulated phosphorylation of selected proteins. As illustrated in the revised **Figure 3c and Supplemental Figure 8**), and described in the results section (**p. 7**), the Western blots closely reflect the kinetics of phosphorylation changes obtained by mass spectroscopy, as exemplified for targets phosphorylated rather early (e.g. TSC2 S1462, reaching a plateau), intermediate (e.g. MAPK1/3 S202/204; ascending/descending) or late (e.g. eIF4B S422, continuously increasing) during the time course. Ideally we would have also engaged in validation of splicing events. However, in this study we have refrained from that due to the complexity of splicing variants and subsequent requirements for the assay development.

Reviewer #3 (Remarks to the Author):

Turewicz and Skagen et al conducted a groundbreaking temporal phosphoproteomics analysis of insulin-regulated pathways in human myotubes, uncovering a complex network of transient phosphorylation and dephosphorylation events. The study is the first of its kind to explore the temporal phosphoproteome, addressing a critical gap in the existing literature, which primarily provides snapshots of muscle phosphoproteome at one time point.
The methodology employed is commendable, with comprehensive phosphoproteome coverage and rigorous bioinformatics analysis. The results offer valuable insights for the scientific community. However, there are notable concerns and suggestions for manuscript improvement:

Major Concerns:

1. *Lack of Proper Control: The absence of adequate controls is a major concern. Comparing time series data with T0 may not be fair, especially considering the one-hour extra starvation for cells treated with insulin at the 1-hour time point. Including controls at all time points will be overkill but author should have at least included 2-3 controls at early, intermediate and late stage.*

Thank you for raising this important point. We have followed this excellent suggestion of the reviewer and conducted experiments where we additionally analyzed the global phosphoproteome of human primary myotubes under starving conditions over time in the absence of insulin. Differentiated myotubes from three different donors were starved for 6 h. Subsequently, the cells were harvested at 0 min, 2.5 min, 15 min and 60 min, representing additional controls for the time periods of the early (T2.5), intermediate (T15) and late (T60) stage. The samples were processed according to our workflow and the respective (TiO2/Fe-IMAC-) enriched phosphopeptides were quantified as before using mass spectrometry. The results of this experiment are now included in the revised manuscript and shown in **Supplemental Figure 5.** As illustrated in the Figure , a total of 68 phosphopeptides (61 unique peptides without duplicates between time points) exhibited significant changes versus 0 min ($p < 0.05$; $|FC| > 1.5$) at different time points in the absence of insulin: 23/16/29 phosphopeptides at 2.5/15/60 min, respectively.
We then investigated the overlap of changed phosphopeptide abundance in the absence and presence of insulin for the different time points. We identified 13 phosphopeptides that exhibited regulation at least at one time point under both conditions, without insulin and with insulin stimulation, with little overlap of the differentially regulated phosphosites between the three basal time points (**Supplemental Figure 5d**). Consistent with previous reports, S570 in TBC1D4 is among the phosphosites regulated in both basal/starving and insulin stimulated states as the MacKintosh lab identified this residue as a substrate for both, AKT and AMPK (Geraghty et al. 2007 Biochem J. 407: 231–241 PMID: 17617058). This issue is now discussed in the discussion section (**p. 21**)
Taking together, we agree that continuous starving (between hours 6 and 7) exerts a relatively small effect on the phosphoproteome, likely due to achieving a steady state. While we found 61 unique phosphopeptides changed, versus 2,741 peptides in the insulin stimulated state, the overall effect does not affect our main findings.

2. *Donor Variability: Caution is advised in interpreting findings related to donor variability due to the limited sample size. Human primary cells' inherent unpredictability may contribute to observed heterogeneity. The method used to identify donor-variable/invariable phosphosites requires clarification, as correlation analysis assumes independence, which may not be valid for donor-matched samples. >>limitations Exploratory study rather than cross-sectional clinical study..”*

We agree that our sample size represents a limitation and this issue is now addressed in the discussion section (**p 26**). In fact, our work represents an exploratory study only, and further cross-sectional clinical studies are needed to cover a broader spectrum of phenotypes.
To quantitatively assess the variability of our data, we have included the CV values for the phosphoproteomes (across both, donors and time points) in the revised manuscript (**Figure 1c, Supplemental Figure 2**).
We apologize for not describing sufficiently the method used to identify donor-variable/invariable phosphosites. We have complemented the methods section (**p. 32**) by describing our approach in more detail:

In the method used, the seven time point-specific abundance values of a particular donor were combined into a temporal profile for each phosphopeptide. Mathematically, the profile a_i for Donor A and phosphopeptide i is a seven-dimensional vector of abundance values a_{ij} where $j \in \{t_0, t_1, t_{2.5}, t_5, t_{15}, t_{30}, t_{60}\}$ represents one of the seven time points. Each of the five donor-specific temporal profiles a_i, b_i, c_i, d_i and e_i represents the realization of a variable that is independent of the other four variables, since sample-matched values by definition are only found within a profile and not between two profiles. To assess donor variability, we calculated the Pearson's correlation coefficient r for each of the 10 pairwise combinations (a_i, b_i), (a_i, c_i), (a_i, d_i), (a_i, e_i), (b_i, c_i), (b_i, d_i), (b_i, e_i), (c_i, d_i), (c_i, e_i), (d_i, e_i) of the five independent profiles, as well as the average correlation coefficient across the 10 pair combinations.

Thus, while we agree that correlation analysis of time-resolved data derived from the same donor would not be valid due to the lack of independence, we here correlate the profiles from the donors, which are in fact independent from each other.

3. *Descriptive Nature of Manuscript: The manuscript is overly descriptive, particularly in the results section. Shortening the results section and consolidating figures is suggested to enhance readability.*

We have consolidated several figures and also included new data as requested by the reviewers. Nevertheless, we tried our best to shorten the results section in the revised manuscript while maintaining the text flow. We hope that the readability has been improved.

4. Lack of *Functional* Experiments: The manuscript could benefit from incorporating functional experiments. Such experiments could be conducted in muscle cell

culture models. Alternatively, authors can demonstrate the functional relevance of insulin-regulated splicing proteins in model system.

Thank you for this comment. We followed your excellent suggestion and have used the myotubes from the donors to measure insulin-stimulated glycogen synthesis in vitro and added these functional data to the revised manuscript (**Figure 1a, Supplemental Figure 6b**). We also conducted Western Blot experiments to validate the time course of phosphorylations of selected insulin targets (**Figure 3c**, **Supplemental Figure 8**). Lastly, we conducted new experiments with human myotubes to investigate the phosphoproteome during starvation (**Supplemental Figure 5**). We agree that direct functional evidence is required to assess the functional relevance of phosphorylations, and their temporal patterns. In response to the suggestion of reviewer #2, we have investigated the overlap of potential insulin targets in primary human myotubes with GWAS genes/proteins. Among the 43 proteins we found in the overlap, several proteins are identified as possible candidates for future functional studies related to insulin resistance (**Supplemental Table 3**).

Minor Comments:

1. *Phosphosite Occupancy: Clarify how phosphosite occupancy is calculated and emphasize the need for corresponding protein/peptide quantification for accurate occupancy determination.*

Thank you for this comment. We apologize for being unclear on this issue.
Evaluation of phosphorylation at a specific site was performed by the ptmRS algorithm employed by Thermo's ProteomeDiscoverer software. However, we were unable to quantify the corresponding non-phosphopeptide for each phosphopeptide quantified as the overlap of the phosphoproteome and global proteome was limited (please see also reply to Reviewer #1, **Supplemental Figure 4** and below).
Instead we used the abundance value ratios of the phosphopeptides relative to time zero (T0) to determine relative occupancy of the respective site. While we cannot determine the absolute amount of phosphopeptide at time T0, our analysis of the relative phosphosite occupancy allows us to determine the fold increase or decrease of that site relative to the reference value. Thus, as the term "phosphosite occupancy" might be misleading, we have changed it to "relative phosphosite occupancy" in the text where applicable (**pp. 16, 17, 24, legend Figure 8**). We now explain the calculation of relative phosphosite occupancy in the methods section ('Temporal dynamics of relative phosphosite occupancy', **p. 35**).

2. *Proteome and Phosphoproteome Overlap: Highlight the limitation of poor total proteome coverage leading to minimal overlap between proteome and*

phosphoproteome datasets. Consider re-measuring the proteome for deeper coverage.

This issue has also been raised by Reviewer #1 (please see above) and we added a new Figure (**Supplemental Figure 4**) and refer to the limitations in the revised manuscript (**results and discussion section, pp. 3, 26**) to address this point.
In essence, the overlap of enriched phosphopeptides and the corresponding non-phosphorylated peptides quantified in the global proteome is about 10% (1,367 peptides). Moreover, approx. 40% of the phosphopeptides (5,322 peptides) can be related to respective protein abundances obtained in the total proteome. Nevertheless, despite the limited coverage, our correlation analysis strongly indicates that changes in phosphopeptide abundance are due to phosphorylation events and not alterations in protein abundance (**Supplemental Figure 4c,d**). It is evident that the enrichment procedure for phosphopeptide is highly effective, likely capturing peptide species with very low relative abundance in the total proteome. An increase in the coverage of the proteome will likely lead to a proportional increase in the overlap, adding to the accuracy of quantification.

3. Supplementary Figure 1A: Increase the text size for better readability.

We have increased the size of the graph and text size of the figure.

4. Methods Section Typos: Address typographical errors in the methods section, particularly in the sentences discussing PCA analysis.

Apologies, we have corrected the errors in the methods section and elsewhere.

5. References Formatting: Ensure all references are correctly formatted throughout the manuscript, paying attention to details like in the network propagation section.

We have formatted the *references accordingly.*

Addressing these concerns and implementing the suggested improvements will enhance the manuscript's overall quality and impact.

We thank the reviewers for the valuable comments and suggestions. We hope that our revised manuscript has been improved.

Reviewer #1 (Remarks to the Author):

This is a revised manuscript, and the authors have addressed most of concerns and the manuscript is improved, however, some concerns remain and need to be addressed before suitable for publication in the journal.

Major concerns:

1) Lack of human participants' clinical characteristics:

- *Physical activity, 2-hour OGTT, and HbA1c may affect insulin signaling, insulin sensitivity, and/or insulin resistance as well as the primary outcome of the experiments, insulin-stimulated phosphorylation, while these data were missing.*
- *Before the biopsy procedure, 1). Were participants fasted overnight? 2). Were they allowed to do exercise? If yes, what kind of exercise was allowed? If not, how many days they were not allowed to exercise?*
- *Were 2-h OGTT and HbA1c performed on these subjects?*

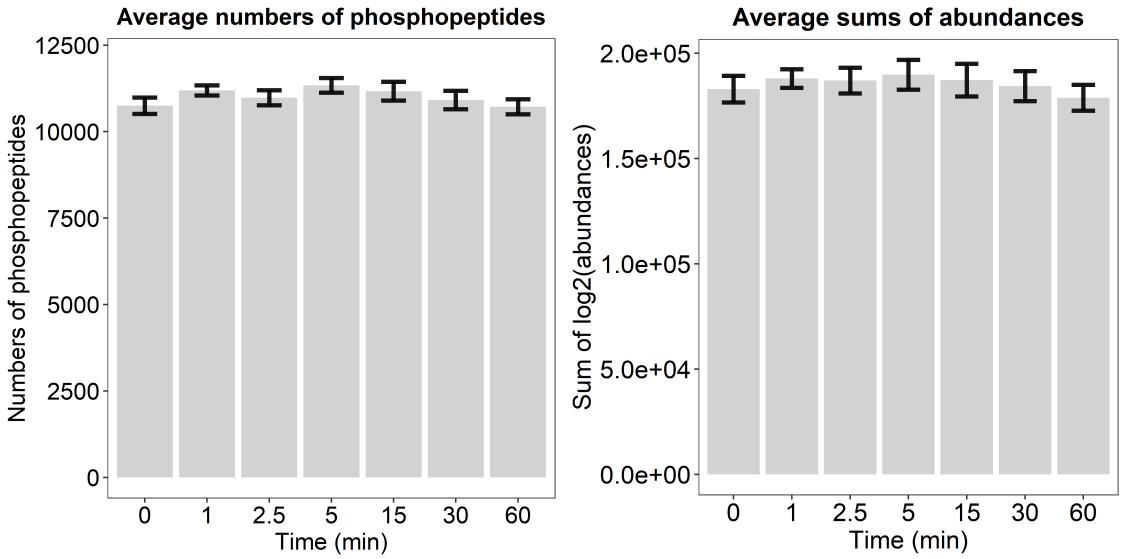
Thank you very much for this comment. The participants were fasted overnight before taking the blood samples for analysis. While we measured fasting glucose, insulin, and C-peptide levels, we did not determine HbA1c or 2-h OGTT. There were no specific restrictions regarding exercise behavior. We have included this information in the table legend (Supplemental Table 1) to clarify this issue.

2). Were they allowed to do exercise? If yes, what kind of exercise was allowed? If not, how many days they were not allowed to exercise?

There were no specific restrictions regarding exercise behavior. We have included this information in the table legend (Supplemental Table 1) to clarify this issue.

3) The 'Precursor Ions Quantifier' node was used to normalize the phosphopeptide abundances based on the total phosphopeptide content. Does this approach assume the total phosphopeptide content in different samples (with or without insulin treatment) is the same? If yes, this would be a concern. It is common practice to assume the total protein content in different samples is the same, however, it is likely that total phosphorylation would be different in samples with or without insulin treatment. It is better to normalize individual phosphopeptide level derived from phosphopeptide enrichment to the total protein level derived from the global proteome, when the corresponding non-phosphorylated peptides or the corresponding protein abundances derived from the global proteome are lacking.

Thank you very much for this thoughtful comment and for raising this important issue. Prior to normalization, we indeed considered the possibility that total phosphorylation could be different in samples with or without insulin treatment. We therefore determined the timepoint-specific numbers and abundances of quantified phosphopeptides prior to normalization. As illustrated in the Figure, both numbers and mean abundances were similar for each time point, and no statistically significant difference was observed between any of these values.



Consequently, the resulting normalization factors in the 'Precursor Ions Quantifier' node used for normalization of phosphopeptides for each timepoint were not statistically different between any of these values including t0.

Norm.Factor	**0 min**	**1 min**	**2.5 min**	**5 min**	**15 min**	**30 min**	**60 min**
Mean	1.88	2.01	1.82	2.18	2.31	2.02	2.34
Standard error	0.258	0.262	0.231	0.359	0.356	0.268	0.268

In fact, the p-value of the corresponding two-way ANOVA (basal vs inulin) is p = 0.8068,.and the adjusted p-values of the posthoc test (Tukey's HSD test) for the individual time point comparisons (0 min vs each time point) are also not significant (lowest padj t0 vs t60 >.0.91).

We conclude that our approach for normalization is valid because the total phosphorylation is not different in samples with or without insulin treatment.

3) Is there a fold change cut off (e.g., 1.5-fold change as used for phosphorylation analysis) for significantly changed transcript by RNASeq? The variation of transcript abundance in the samples may be considered moderate (median CV of the Illumina RNASeq read counts from three donors was 27.4% (mean 33.4%)), however, it may not have enough power to detect small differences in 3 donors.

Thank you for this comment. We did not apply a cut off value for RNA Seq fold changes (FC) and listed all 21 differentially expressed transcripts in Supplemental Table 9 with padj<0.05). However, we have now added a column for the FC values to Supplemental Table 9. Fourteen of the 21 differentially expressed genes exhibit FC > 1.5.

Minor concerns:
It is better to move the Supplemental table headers before the table instead of after the table.

As suggested, we have moved the Supplemental table headers.

Reviewer #1 (Remarks on code availability):
Can not find the code by click the link provided.

Apologies! We fixed the corresponding access link (https://github.com/ddz-icb/temporal-dynamics-of-insulin-signaling).

Reviewer #2 (Remarks to the Author):

I thank the authors for the considerable time and effort dedicated to address my concerns and suggestions. I appreciate the thoroughness of the revisions and the additional data you have incorporated in response to my feedback.

In my opinion, the modifications you have made to the manuscript significantly strengthen the impact and relevance of the findings. I have no additional comments. Thank you again and congratulations for the nice work.

Thank you very much for the comment and suggestions!

Reviewer #4 (Remarks to the Author):

I think that this very interesting and groundbreaking manuscript has been thoroughly reviewed by all three reviewers and that the authors have gone to great length to address all the concerns addressed by the reviewers including reviewer 3.

Thank you very much for the comment and suggestions!