Global phosphoproteomic profiling of skeletal muscle in ovarian-hormone deficient mice

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Abstract

Background

Phosphorylation is important in skeletal muscle development, growth, regeneration, and contractile function. Alterations in the skeletal muscle phosphoproteome due to aging have been reported in males; however, studies in females are lacking. We have demonstrated that estrogen deficiency decreases muscle force which correlates with decreased myosin regulatory light chain phosphorylation. Thus, we questioned whether the decline of estrogen in females that occurs with aging might alter the skeletal muscle phosphoproteome.

Methods

C57BL/6J female mice randomly assigned to a sham-operated (Sham) or ovariectomy (Ovx) group to investigate the effects of estrogen deficiency on skeletal muscle protein phosphorylation in a resting, non-contracting condition. After 16 weeks of estrogen deficiency, the tibialis anterior muscle was dissected and prepped for label-free nano-liquid chromatography tandem mass spectrometry phosphoproteomic analysis.

Results

We identified 4,780 phosphopeptides in tibialis anterior muscles of ovariectomized (Ovx) and Sham-operated (Sham) control mice. Further analysis revealed 647 differentially regulated phosphopeptides (Benjamini – Hochberg adjusted p-value < 0.05 and 1.5-fold change ratio) that corresponded to 130 proteins with 22 proteins differentially phosphorylated (3 unique to Oxv, two unique to Sham, six upregulated, and 11 downregulated). Differentially phosphorylated proteins associated with the sarcomere, cytoplasm, and metabolic and calcium signaling pathways were identified.

Conclusion

Our work provides the first global phosphoproteomic analysis in females and how estrogen deficiency impacts the skeletal muscle phosphoproteome. Future work into specific phosphorylation events on sarcomeric proteins and phosphoproteins pertaining to calcium signaling may offer insight on the molecular mechanism(s) of skeletal muscle strength loss in females.

Introduction

Protein phosphorylation is one of the most well studied reversible, post-translational modifications due to its importance in signal transduction and disease (1–3). It is estimated that about 30% of the proteome in mammals is modified by phosphorylation, with more than 500,000 known phosphorylation sites identified to date (3, 4). The addition of one or more phosphate groups can have profound effects on the biological function of the modified protein. Phosphorylation can either activate or inactivate a protein as well as modulate molecular interactions and signaling (2). Thus, examining phosphorylation status of proteins in a given condition at a given time provides further understanding of their role in physiological or pathophysiological states.

Skeletal muscle mass and strength begin to decline with age, increasing the risk of poor balance, impaired mobility, falls, and overall mortality (5–7). Preclinical and clinical studies have shown that loss of muscle strength occurs earlier in females than males, and this decline in muscle strength in females is associated with the reduction of circulating

estrogen, specifically 17 β -estradiol (E2), as a result of menopause (8–10). To understand the impact of estrogen deficiency on molecular aspects of skeletal muscle function, rodent ovariectomy models were used to show that loss of estrogen significantly attenuates force generation by reducing the number of strong-binding myosin heads during contraction (10–11), which could be restored by supplementation of E2 (12). Similar effects of estrogen deficiency on myosin function have been indicated in muscles of postmenopausal women (13, 14). Protein phosphorylation, including myosin, has been shown to play a significant role in regulating signal transduction pathways that contribute to fiber-type differentiation, muscle hypertrophy, plasticity, regeneration, excitation-contraction coupling, and contractile function (15–25). Estrogen modulates myosin regulatory light chain (RLC) phosphorylation in striated muscle; however, in skeletal muscle, whether other proteins are sensitive to estrogen is yet to be determined (25–27). Broader phosphoproteomic analysis could aid in identifying candidate phosphoproteins to elucidate further how estrogen deficiency affects the molecular characteristics of muscle proteins and function in females.

Previous studies examining the skeletal muscle phosphoproteome have been predominantly performed with samples from males, with only limited global phosphoproteomic studies in females (28–31). Phosphoproteomic analysis of aged male compared to young male rat muscle via 2D gel electrophoresis and phosphostaining demonstrated altered phosphorylation in 22 muscle proteins (28); however, females were not included in that study, and 2D gel phosphoproteomics is limited in sensitivity, so the results may not fully represent the role of phosphorylation in muscle aging. Changes specifically in myosin phosphorylation and kinetics have been examined across young and aged humans (both male and female), and decreased myosin RLC phosphorylation and kinetics were only observed in aged females (31), suggesting potential estrogen-related differences; however, broader phosphoproteomics data on those differences have been lacking. We sought to investigate the functional landscape of the skeletal muscle phosphoproteome in estrogen-deficient females to identify whether broader scale changes beyond just those observed in RLC phosphorylation were occurring. We hypothesized that loss of estrogen in females would remodel the skeletal muscle phosphoproteome, affecting phosphoproteins of the sarcomere, the basic contractile unit of the muscle fiber critical for muscle function.

In this study, we used bilateral ovariectomy to model the loss of estrogen in menopause because it induces the deficiency of ovarian hormones, including the primary estrogen, E2. We performed label-free phosphoproteomic analysis on the tibialis anterior (TA) muscle of ovariectomized (Ovx) and control mice that had undergone a sham ovariectomy surgery (Sham) to determine how estrogen deficiency impacts the skeletal muscle phosphoproteome in females during a basal, non-contracting condition. Differentially phosphorylated proteins associated with the sarcomere, cytoplasm, and muscle-relevant canonical pathways, such as metabolic and calcium signaling pathways, were identified. These observations suggest that estrogen loss contributes to remodeling the skeletal muscle phosphoproteome, which could have downstream implications for the molecular function of proteins critical to muscle strength in aging females.

Materials And Methods

Animals

Female C57BL/6J (6 mo) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All mice were housed in groups of four to five with access to phytoestrogen-free food and water ad libitum. The room was maintained on a 14:10 h light/dark cycle. All experiments and procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Experimental Design

The study design to evaluate the skeletal muscle phosphoproteome in Ovx and Sham control mice is summarized in Fig. 1A. Female C57BL/6J mice were randomly assigned to a Sham or ovariectomy surgery. Vaginal cytology was performed 4 wk post-surgery, and uterine mass was measured at the time of sacrifice to confirm estrous cycling and successful ovariectomy. TA muscles from anesthetized mice (1.75% isoflurane and 200 ml O₂ per min) were dissected 16 wk post-surgery, immediately flash-frozen in liquid nitrogen, and stored at -80°C until sample preparation and phosphopeptide enrichment for nanoflow LC-MS/MS acquisition.

Sham and Ovariectomy surgeries

Anesthetized (1.75% isoflurane and 200 ml O₂ per min), mice received a subcutaneous injection of slow-release buprenorphine (1mg/kg) immediately prior to surgeries. Two dorsal lateral incisions were made to locate the ovaries, which were excised in mice assigned to the Ovx group, and located but not removed in mice assigned to the Sham group. The incisions were closed with 6-0 silk sutures, and 7 mm wound clips closed the skin incision.

Protein extraction, digestion, and phosphopeptide enrichment

Frozen TA muscles were pulverized into powder with a cryo-grinder (liquid nitrogen-cooled mortar and pestle), lysed (10 µl lysis buffer per mg of tissue) in protein lysis buffer (7M urea, 2M thiourea, 0.4M Tris pH7.5, 20% acetonitrile, four mM TCEP) with Complete Protease Inhibitor Cocktails (Roche; City, State), and sonicated for 5 s using a probe sonicator (Branson Digital Sonifier, manufacturer, City, State) set at 30% amplitude. After sonication, a 160 µl aliquot of each lysate was placed in the Barocycler® NEP2320 (Pressure Biosciences, South Easton, MA) at 37°C, with pressure cycles set at 35,000 psi for 20 s, then 0 psi for 10 s for 60 cycles for further protein homogenization. Once pressure cycling was complete, samples were transferred to a new 1.5 ml Eppendorf protein LoBind tube, and a 200 mM chloroacetamide stock solution was added for a final concentration of 8 mM to alkylate proteins (1:24 dilution) and incubated for 15 min. Samples were spun down at 15,000 x *g* for 10 min at 18°C. Two 1 µl aliquots of supernatant were used to determine protein concentration using the Bradford assay. For trypsin digestion, 500 µg total protein was digested with 20 µl Promega sequencing grade modified trypsin (0.625 µg/µl trypsin) and incubated at 37°C in a warm air incubator overnight (~ 16 h). Samples were acidified and extracted using Waters Oasis 1 CC HLB Solid Phase Extraction cartridge for clean-up, following the manufacturer's instructions. Peptides were eluted with 1.2 mL 80% acetonitrile and vacuum dried to remove acetonitrile. Lysates were stored at -80°C until phosphopeptide enrichment. Phosphopeptide enrichment was performed with a TiO₂ phosphopeptide enrichment kit (ThermoFisher Scientific). Eluted peptides were dehydrated using a speed-vac and desalted using homemade C18 Stage-tips (32).

Nanoflow LC-MS/MS

An Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) connected to an online Proxeon Easy nLC 1000 Nano-UPLC system (Thermo Scientific) was used for data-dependent acquisition tandem mass spectrometry (MS/MS) analysis, with a nano-liquid chromatography separation and electrospray ionization of eluting peptides. Peptides were separated at a flow rate of 300 nL/min over a 146 min gradient, consisting of 5–22% solvent B over 75 min, 22–35% solvent B over 45 min, 90% solvent B held for 20 min, and 5% solvent B held for 6 min. Solvent A was water with 0.1% formic acid, and solvent B was 80% acetonitrile with 0.1% formic acid. The column was packed using 3 µm C18 beads (ReproSil-Pur Basic) in a 100 µm x 50 cm PicoTip (Dr. Maisch GmbH). Precursor ions were detected by the orbitrap at a resolution of 120,000 at 200 *m/z* and a mass range of 380–1580 *m/z*. MS/MS spectra were acquired in the Orbitrap analyzer with an isolation window of 1.6 *m/z* after fragmentation at 30% higher energy collision dissociation energy at a resolution of 30,000.

Phosphoproteomic database search, phosphoprotein, and phosphopeptide quantification

The raw MS files were processed by Proteome Discoverer v2.4. MS/MS spectra were searched against the UniProtKB *Mus musculus* database (55,474 entries, UniProt UP000000589, downloaded November 2019) with the Sequest HT search engine embedded in Proteome Discoverer v2.4. Parameters were set as follows: MS1 tolerance of 15 ppm, MS/MS mass tolerance of 0.05 Da, trypsin (full) digestion with a maximum of two missed cleavages, minimum peptide length of 6, and maximum of 144 amino acids. Cysteine carbamidomethylation (57.02 Da) was set as a fixed modification, and methionine oxidation (15.99 Da), asparagine and glutamine deamidation (0.98 Da), acetylation of the N-terminus (42.01 Da), and phosphorylation of tyrosine, serine, and threonine (79.97 Da) were set as dynamic modifications. A false discovery rate (FDR) of 1% was set for peptide-to-spectrum matches using the Percolator algorithm (v3.02.1) and protein assignment. Phospho-localization scoring was performed with the IMP-ptmRS v2.0 node, and only phosphopeptides with a localization score > 0.8 were used for quantification. Precursor abundance quantification was based on area and normalized by total peptide amount. All peptides were used for normalization for protein quantification; however, only phosphorylated peptides were used for pairwise ratios and protein roll-up. Unique and razor peptides were used for protein quantification.

Label-free quantification (LFQ) of proteins and peptides was performed with normalized abundances using the Proteome Discoverer LFQ algorithms. The protein ratio was calculated as the geometric median of the peptide ratios, and the peptide ratios were calculated as the geometric median of all combinations of ratios from all the biological replicates in the study. Phosphopeptides and phosphoproteins with a 1% FDR confidence and normalized abundance (intensity) observed in at least three biological replicates in at least one of the groups were used for further analysis. Phosphoproteins, phosphopeptides, and phospho-sites were considered significant and differentially phosphorylated (proteins) or regulated (peptides and sites) if they had a Benjamini – Hochberg adjusted p-value < 0.05 and was defined as downregulated if they had a fold change ratio (Ovx/Sham) ≤ -1.5 or upregulated if they had a fold change ratio ≥ 1.5 .

Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome, and Gene Ontology (GO) enrichment analysis

Significant and differentially regulated phosphopeptides were mapped back to their precursor protein, and the list of phosphoproteins was used for overrepresentation analysis. Using the clusterprofiler and ReactomePA packages in R v4.1.1, KEGG and Reactome pathways and GO annotation terms for molecular functions, cellular components, and biological processes enriched in the dataset were identified (33–35). For GO molecular process enrichment analysis, hierarchical clustering was applied with average linkage to identify the top 5 clusters. Overrepresented pathways and GO annotation terms were considered significant if they had a Benjamini – Hochberg adjusted p-value < 0.05.

Ingenuity Pathway Analysis (IPA)

IPA (Qiagen, Redwood City CA, USA) was used to perform a core analysis on the phosphopeptides, analyzing associations of observed phosphoproteins with canonical pathways, molecular and cellular functions, physiological system development and functions, and upstream regulators using IPA's functional analysis algorithm and the curated Ingenuity Knowledge Base library. IPA's Downstream Effects analytics and prediction algorithm were used to compute an activation Z-score to predict the activation states of pathways, functions, and upstream regulators. Molecules from the dataset that met the cutoffs of a Benjamini-Hochberg adjusted p-value < 0.05 and 1.5-fold change ratio were considered for analysis.

Statistical analysis

Body and uterine masses were analyzed with Student's two-tailed t-tests (Sham vs. Ovx) using Graphpad Prism 9.1 (San Diego, CA, USA). All data are reported as the mean $\pm$ standard deviation (SD). Relative phosphoprotein and phosphopeptide quantification were analyzed in Proteome Discoverer v2.4 (ThermoFisher) using Student's t-tests.

Fisher's exact test was used in IPA to calculate p-values for the association or overlap between the identified molecules in the dataset and a given pathway/process/function. Benjamin-Hochberg's posthoc analysis was used to correct for multiple comparisons. The predicted activation states in IPA's pathways/functions/upstream regulators were measured as Z-scores. The Z-score measures how closely the observed expression pattern of the molecules in the dataset compared to the expected expression pattern based on the literature for a particular annotation. Significantly activated or inhibited state was accepted at a $-2 \geq Z\text{-score} \geq 2$. Significance was accepted at $\alpha < 0.05$ level.

Results

Comparative skeletal muscle phosphoproteomic analysis

To investigate the impact of estrogen deficiency on the skeletal muscle phosphoproteome in females, we employed an nLC-MS/MS label-free global phosphoproteomics profiling approach for relative quantification of phosphoproteins and phosphopeptides between Ovx and Sham female mice ($n = 4/\text{group}$; Fig. 1A). Successful removal of ovarian tissue was confirmed by examining body mass, uterine mass, and estrous cycling. 16 wk post-surgery, body mass and uterine mass between Sham and Ovx mice were significantly different ($p < 0.001$; Fig. 1B & C), with mean body masses of 26.2 ± 1.4 and 33.9 ± 1.0 g, respectively, and mean uterine masses of 200.1 ± 26.6 mg and 15.6 ± 6.2 mg, respectively. Vaginal cytology 4 wk post-surgeries further confirmed estrous cycling in Sham mice and persistent diestrus in Ovx mice.

After 16 weeks, tibialis anterior (TA) muscles were dissected, prepared via protein extraction, trypsin digestion, phosphopeptide enrichment, and analyzed by LC-MS/MS. Using label-free quantification as described in the Methods, phosphopeptides were identified and mapped to proteins, and abundance levels were compared between the Sham and Ovx groups. In total, 4,780 phosphopeptides comprising 5,493 phosphorylation sites were identified, 109 were unique to Ovx, 234 were unique to Sham, and 4437 were detected in both Ovx and Sham (Fig. 2A). Among these phosphopeptides, phosphorylation was most prevalent on serine (S) residues, representing 80% of the sites identified, followed by threonine (T, 17%) and tyrosine (Y, 4%), which is consistent with known distributions for S, T and Y phosphorylation (Fig. 2B)(36). Further analysis identified 647 significant and differentially regulated phosphopeptides, with 444 downregulated and 203 upregulated in Ovx compared to Sham (Fig. 2C). After filtering for robustness (abundance detected in at least three biological replicates in at least one of the Sham or Ovx groups), 189 phosphopeptides were identified that corresponded to 130 proteins (Fig. 2D) of which 22 were differentially phosphorylated: 3 unique to Ovx, two unique to Sham, and 11 downregulated and 6 upregulated in Ovx relative to Sham (Table 1). Four out of the 22 differentially phosphorylated proteins were sarcomeric proteins: tropomyosin alpha 3 (TPM3), obscurin (OBSCN), myosin heavy chain 2 (MYH2), and myozenin-2 (MYOZ2). Interestingly, TPM3 was unique only to Sham and absent in Ovx, and the other three were downregulated in Ovx compared to Sham mice. More, out of the 130 phospho-sites identified on the 22 differentially phosphorylated proteins, only 26 phospho-sites were identified as differentially regulated (Table 1). Taken as a whole, these data show that the skeletal muscle phosphoproteome is altered in Ovx female mice.

Table 1
Significantly differentially phosphorylated proteins and phospho-sites in Ovx relative to Sham mice.

Uniprot AcessionID	Gene Symbol	Description	Phosphorylation site (Score)	Log2 ratio	Differential regulation
Q99LI7	Cstf3	Cleavage stimulation factor subunit 3	S691(100)	6.64	Only Ovx
D3Z472	Phkg1	Phosphorylase b kinase gamma catalytic chain	S61(98.8)	6.64	Only Ovx
O54818	Tpd52l1	Tumor protein D53	S149(99.2)	6.64	Only Ovx
Q9WU42	Ncor2	Nuclear receptor corepressor 2	S149(100); S152(100)	4.79	Upregulated
A0A0R4J0Z3	Aqp4	Aquaporin 4, isoform CRA_a	S276(95.1); S285(99.9)	2.19	Upregulated
B1ARU1	Macf1	Microtubule-actin cross-linking factor 1	S1517(100); S1520(100)	2.17	Upregulated
G3X9Q6	Larp4	La-related protein 4	S642(97.7)	1.39	Upregulated
Q8BJS4	Sun2	SUN domain-containing protein 2	S12(100); S120(99.9); S122(97.8); S135(99.6); S281(100)	0.75	Upregulated
Q4FE56	Usp9x	Probable ubiquitin carboxyl-terminal hydrolase FAF-X	S1600(100); S2443(100)	0.59	Upregulated
A0A0R4J1P2	Tpm3	Tropomyosin alpha-3 chain OS = Mus musculus	Y60(100); T108(100); Y162(100); S206(100); Y221(100); T237(100); S245(100); T282(98.6)	-6.64	Only Sham
Q61193	Rgl2	Ral guanine nucleotide dissociation stimulator-like 2	S619(98.8)	-6.64	Only Sham
Q8C142	Ldlrap1	Low-density lipoprotein receptor adapter protein 1	S198(99)	-3.03	Downregulated
Q8BHL3	Tbc1d10b	TBC1 domain family member 10B	T138(99.1)	-2.21	Downregulated

Uniprot AcessionID	Gene Symbol	Description	Phosphorylation site (Score)	Log2 ratio	Differential regulation
A7E1Z5	Slc4a4	Anion exchange protein	S68(98.6)	-2.14	Downregulated
Q3U9G9	Lbr	Delta (14)-sterol reductase LBR	S101(100)	-2.07	Downregulated
Q8CAP3	Rad23a	Rad23a protein	S123(97.9); S216(95)	-1.93	Downregulated
P62754	Rps6	40S ribosomal protein S6	S235(100); S236(98.7); S240(100); S244(100)	-1.90	Downregulated
Q78JW9	Ubfd1	Ubiquitin domain-containing protein UBFD1	S131(99.9)	-1.50	Downregulated
Q9JJW5	Myoz2	Myozenin-2	T107(98.5); T111(100); S116(100); S158(100)	-1.35	Downregulated
G3UW82	Myh2	Myosin, heavy polypeptide 2, isoform CRA_d	T202(100); T218(100); T257(100); T381(100); Y413(100); T444(100); S650(100); T667(100); S745(100); T761(100); T918(100); T944(100); S955(100); T967(100); T986(100); T1000(100); T1026(100); S1044(100); S1095(100); S1135(100); S1147(100); S1165(99.2); T1195(100); S1206(100); S1240(100); S1246(100); S1268(100); T1289(100); S1306(100); S1309(100); T1316(100); S1360(100); S1373(100); Y1382(100); T1424(100); Y1467(98.8); S1483(100); Y1495(99.1); T1504(100); S1517(100); T1520(100); S1577(100); S1603(100); S1614(100); Y1650(100); S1717(100); T1728(100); S1729(97.4); T1733(100); T1739(100); S1742(100); T1767(100); T1782(99.4); S1835(100); T1861(100); S1901(100); S1922(100)	-1.06	Downregulated
E9QQ96	Obscn	Obscurin	S135(100); S236(100); S3254(100); S4708(100); S4893(100); T5568(100); S5632(100); T5636(100); S5639(100); S5665(97.8); T5670(99); S5683(97); S5686(100); S5687(100); S5711(100); S5916(98); T6134(100); S6236(100); S6445(100); T6451(100); S6455(100); S6551(100); T6680(100); S6837(100); S7083(100); S7134(100); S7147(100); S7149(100); S7167(98.6); S7223(100)	-1.03	Downregulated
Q920Q6	Msi2	RNA-binding protein Musashi homolog 2	S6(100); S12(97.7)	-1.00	Downregulated

The red residue site in the Phosphorylation site column denotes significantly regulated phospho-site in muscle of Ovx relative to Sham mice.

KEGG, Reactome, and GO overrepresentation analysis

To gain more insight into the differential impact of estrogen deficiency in the skeletal muscle phosphoproteome, differentially regulated phosphopeptides were mapped back to their precursor protein. The list of phosphoproteins was used in R for pathway and GO term overrepresentation analysis. The top 15 most overrepresented KEGG and Reactome pathways were related to calcium signaling, hypoxia-inducible factor (HIF) 1- α signaling, insulin signaling, muscle contraction, ion homeostasis, and mTOR signaling (Fig. 3A and B). GO molecular function (MF) was enriched for actin and actinin binding, mRNA binding, translation initiation factor binding, phosphatidylinositol phosphate binding, and structural constituent of muscle and post-synapse. These, in turn, resulted in strong enrichment in GO cellular components (CC) associated with the contractile apparatus and components of the muscle fiber, including the Z-disc, I-band, sarcoplasmic reticulum, T-tubules, sarcolemma, as well, as the cytoskeleton (Fig. 3C and D). The five main clusters from the GO biological process analysis emulated previous pathways and GO term enrichments. Clusters relating to calcium signaling and ion activities were highlighted in both KEGG (calcium signaling) and Reactome (muscle contraction) and are associated with calcineurin-NFAT signaling, which has been shown to be involved in gene regulation pertaining to skeletal muscle differentiation, fiber-type switching, and muscle hypertrophy. The metabolic signaling and activities cluster is associated with glucagon/insulin signaling in KEGG and pyruvate metabolism and TCA cycle in Reactome. The striated cell assembly development cluster is associated with GO MF and CC enrichment pertaining to maintenance of and structural components of the muscle cell, such as cytoskeleton – actin binding, sarcomere, structural constituent of muscle and post-synapse, and translation regulation – mRNA binding and translation initiation factor binding (Fig. 3E). Overall, all pathway and GO enrichment analyses show overrepresented proteins associated with calcium and metabolic signaling pathways and functions related to muscle cell maintenance and structural integrity.

Differentially overrepresented IPA canonical pathways

Next, to evaluate potential differential downstream effects from the changes in the phosphoproteome in Ovx mice, all 4,780 phosphopeptides were uploaded to IPA, 377 of which mapped to molecules characterized in the IPA database. The top 10 canonical pathways enriched in the dataset are shown in Fig. 4A. Consistent with the KEGG, Reactome, and GO analyses, the calcium signaling pathway was the most significantly enriched IPA annotated canonical pathway ($p < 0.05$, 12% overlapping molecules) with 50 observed molecules mapping to the pathway, 31 molecules downregulated, and 19 molecules upregulated in Ovx relative to Sham muscles out of IPA's 226 total annotated pathway molecules (Supplementary Fig. 1). IPA's activation Z-score algorithm was unable to predict the activation state of calcium signaling (i.e., pathway directionality – activation or inhibition). However, visualizing the mapping of observed phosphoproteins to the IPA “resting muscle cell” model indicated that phosphorylation of the SERCA, calsequestrin and ryanodine receptor proteins were altered in Ovx compared to Sham muscles (Fig. 4B). Two metabolic pathways also had significant predicted inhibition with IPA Z-scores of -2.12: glycolysis I ($p < 0.05$, 32% overlap, nine molecules downregulated and two molecules upregulated out of 25 annotated pathway molecules) and gluconeogenesis I ($p < 0.05$, 32% overlap, nine molecules downregulated and three molecules upregulated out of 25 annotated pathway molecules). These observations all indicate that essential metabolic and calcium signaling functions in skeletal muscle are affected in estrogen-deficient mice, which could, in turn, contribute to changes in muscle function overall.

IPA functional analysis

To further explore the biological significance of the molecular alterations induced by estrogen deficiency within the skeletal muscle phosphoproteome, we employed IPA functional analysis to predict the impact of phosphoproteome alterations on molecular and cellular functions and physiological system development and functions. The top 10 functional parent groups that mapped to molecules observed in the Ovx/Sham dataset are shown in Fig. 5A, with IPA's skeletal and muscular system development and function (SMSDF) group exhibiting the strongest association ($p < 0.05$) with 113 observed molecules mapping to this function. Deeper analysis into the physiological subfunctions assigned

by IPA to the SMSDF group predicted a significant inhibition of contractility of muscle in estrogen-deficient mice (Fig. 5B). Two other functional parent groups, cellular assembly and organization with 165 observed molecules mapping to this function ($p < 0.05$) and cellular function and maintenance with 179 observed molecules mapping to this function ($p < 0.05$), had three overlapping subfunctions with significant activation Z-scores. There was significant predicted inhibition in the formation of phagosomes (Fig. C) and vesicles (Fig. D) and significant predicted activation in disruption of cytoskeleton (Fig. 5E). In summary, estrogen deficiency perturbs skeletal muscle and cellular functions that may lead to impaired contractile function and compromised cellular and cytoskeleton integrity.

Upstream regulator analysis and mechanistic network of E2

Using IPA's activation Z-score prediction algorithm and Ingenuity Knowledge Base library, we examined kinase activity inferred by the changes in the phosphoproteome dataset; we identified the top four potentially inhibited or activated kinases, revealing potential inhibition of CDK6 (Table 2). We next sought to determine whether previously known E2-related alterations in protein phosphorylation could be observed through phosphoproteins directly detected in our dataset (Supplementary Fig. 2A). Overall, we identified nine proteins linked to E2 in IPA that were observed in our data—with increased phosphorylation on beta-catenin (CTNNB1), eukaryotic translation initiation factor 4E-binding protein 1 (EIF4EBP1), histone deacetylase 2 (HDAC2), and microtubule-associated protein tau (MAPT), and decreased phosphorylation on insulin receptor substrate 1 (IRS1), nitric oxide synthase 1 (NOS1), 40S ribosomal protein S6 (RPS6), SHC-transforming protein 1 (SHC1), and stromal interaction molecule 1 (STIM1). In addition, other functional effects of estrogen loss were represented indirectly in our phosphoprotein observations, including changes annotated in IPA as being directly (AMPK, IGF1, RPS6KB1, and D-glucose) or indirectly (TP53, PPARGC1a, and NR0B2) affected by estrogen deficiency (Supplementary Fig. 2B).

Table 2
Upstream kinases affecting the phosphoproteome in Ovx mice.

Kinase	Predicted Activation State	Activation z-score	Target Molecules in Dataset
CDK6	Inhibited	-2.0	ABI1, LPIN1, SRSF1, SYNPO2
PRKCE		-1.96	IRS1, RPS6, SHC1, VDAC1
PRKAA1		-1.347	EIF4EBP1, IRS1, PDHA1, RPS6, STIM1, YAP1
ROCK1		-1.091	INSR, IRS1, MYL2, MYLPF, RPS6
CAMK2A		1	ABI1, CAMK2A, CTNNB1, MAPT
ROCK2		1.029	MYL2, MYLPF, PPP1R12A, ROCK2
MAPK1		1.097	EIF4EBP1, IRS1, MAPKAPK2, MAPT, NCOR2, NEFH, NEFM, PKM, PLCG1, POLR2A, PXN, RPS6KA3, SEC16A, SHC1, YBX3
PTK2		1.262	ARHGEF11, GIT1, IRS1, MYL2, PLCG1, PXN, SHC1
Decreased or increased phosphorylation measurements of the target molecule are represented by green and red font color, respectively.			

To illustrate how the observations of differential phosphoproteins in TA muscle from Ovx/Sham muscle may connect to canonical effects of estrogen on cell signaling, we used IPA to build a mechanistic network (Fig. 6). This shows the direct relationships between E2 and the nine phosphoproteins observed, as well as two kinases, predicted to be altered

by estrogen deficiency (AMPK and RPS6KB1) but not directly observed in the dataset. Inhibition of E2 is associated with a predicted inhibition of AMPK that results in a predicted activation of Yes-associated protein1 (YAP1) and predicted inhibition of autophagy-related 9A (ATG9A). Both YAP1 and ATG9A had decreased phosphorylation observed in our dataset and were associated with maintaining muscle homeostasis. In addition, inhibition of E2 is directly related to the increase in phosphorylation that we observed of CTNNB1, EIF4EBP1, and HDAC2, which are all involved in transcription regulation. This upstream prediction analysis reveals how estrogen deficiency may modulate other regulators and co-activators of transcription factors important for maintaining muscle fiber integrity and function.

Discussion

Recent phosphoproteomic analyses of skeletal muscle have provided a greater understanding of the complex pathophysiology associated with skeletal muscle aging and strength loss. We identified in total 4,780 phosphopeptides comprising 5,493 distinct phosphorylation sites and corresponded to 2,932 phosphoproteins indicating that many skeletal muscle proteins had multiple phospho-sites. The majority of previous phosphoproteomic studies were conducted on muscle from males, and in this analysis of female skeletal muscle, we identified a number of proteins in agreement with those studies, showing that the majority of the phosphopeptides were from sarcomeric proteins and they were overrepresented amongst the peptides observed (29, 37, 38). For example, clinical phosphoproteomic analysis of the vastus lateralis muscle in a resting condition from three healthy males identified phosphoproteins related to the Z-disc, contractile apparatus, and calcium signaling proteins, among others—which is consistent with our results (29). Our three main findings from this study were alterations in phosphorylation of sarcomeric proteins, enrichment in phosphorylation of proteins associated with calcium signaling, and predicted inhibition of skeletal muscle function from the IPA downstream predictive modeling in estrogen deficient muscle.

We identified four significant and differentially phosphorylated sarcomeric proteins in our dataset, TPM3, OBSCN, MYH2, and MYOZ2 (Table 1), revealing decreased phosphorylation of all these proteins in muscle from OvX compared to Sham mice. In contrast, a sarcopenic male rat model showed increased phosphorylation of the sarcomeric proteins: myosin light chain 2 (MYL2), desmin (DES), and tropomyosin alpha in male muscles (28). The effect of phosphorylation on the four altered sarcomeric proteins identified in our dataset is poorly understood in skeletal muscle. However, studies examining the phosphorylation of TPM3 indicate that hyperphosphorylation contributes to the pathophysiology of myopathies and cardiomyopathies (39, 40). Conversely, decreased phosphorylation of these proteins may suggest loss of function leading to less active proteins and muscle dysfunction.

Relating observed phosphoproteomic alterations to functional outcomes is challenging, particularly given the known complexity of muscle structure, Z-disc, and contractile proteins. However, using enrichment analysis in conjunction with IPA's predictive modeling, we were able to determine pathways and GO terms overrepresented in our dataset and predicted activation states of canonical pathways, functions, and upstream regulators annotated by IPA's Knowledge Base. Enrichment of KEGG and Reactome pathways revealed overrepresentation in calcium and metabolic signaling consistent with IPA's enriched canonical pathways (Figs. 3 & 4). As most muscle phosphorylation literature stems from the cardiac muscle field, it is not surprising to see many KEGG pathways relating to cardiomyocytes/cardiomyopathy and Reactome pathways showing cardiac conduction enrichment. Cardiomyocytes and skeletal muscle fibers are both striated and their cellular structural components and contractile function are similar. Thus, it is reasonable that our data, comprised of analogous proteins, would be enriched in parallel pathways and functions related to cardiac muscles, and our intervention perturbing estrogen-mediated phosphorylation would suggest a compromise in muscle function as shown with the enrichment of cardiomyopathy pathway. Our enrichment and prediction analyses affirm that the phosphorylation alterations detected in OvX mice may be relevant to decrements in muscle function due to estrogen

loss. Not surprisingly, as calcium is critical for skeletal muscle function, our dataset showed significant enrichment in calcium signaling and calcium ion channel activity-related functions and pathways. Altered calcium handling has been shown in aging and diseased striated muscles (82–87). More specifically, in aging mouse hearts, estrogen deficiency has been shown to perturb intracellular Ca^{2+} homeostasis by increasing spontaneous Ca^{2+} release from the sarcoplasmic reticulum and reducing myofilament Ca^{2+} sensitivity (47). Therefore, it is likely that estrogen deficiency may alter calcium handling in skeletal as well as cardiac muscle, in line with our enrichment analyses.

IPA's functional analysis revealed predicted inhibition of skeletal muscle function and cellular and maintenance functions with estrogen deficiency (Fig. 5B). These predicted dysregulated functions suggest a potential impairment in contractile activity and mechanisms involved in skeletal muscle maintenance and repair. Muscle undergoes constant plasticity requiring gene regulation and cellular adaptations to stimuli, including contractile activity (e.g., in response to endurance exercise), loading and unloading conditions (e.g., resistance training and atrophy from disuse, respectively), changes in environmental factors such as nutrition or hypoxia, and injuries (e.g., exercise-induced or traumatic injuries). The predicted disruption in cellular maintenance and function were further related to vesicle and phagosome formation and cytoskeleton disruption, which may be attributed in part to our observation of decreased ATG9A phosphorylation, as ATG9A is critical for autophagosome formation and maintaining muscle homeostasis through autophagy-mediated proteolysis (48). The autophagy-mediated proteolysis in skeletal muscle is critical for maintaining muscle homeostasis under basal, non-contracting conditions as well as during physical activity. Appropriate autophagic flux is required to remove and degrade damaged proteins and cell organelles in muscle, and inhibition of autophagy induces atrophy and myopathies (49, 50). Estrogen deficiency, via ovariectomy, aging, or in a diseased state, decreases the ability of skeletal muscle to generate force and reduces recovery of muscle strength from injury (10, 51–56). Accordingly, the IPA predicted inhibition in contractility of muscle in estrogen-deficient mice supports what we and others have observed *in vitro* and *in vivo* in skeletal muscle and sheds light on underlying molecular mechanisms.

Conclusion

Our study is the first global phosphoproteomic profiling of the skeletal muscle phosphoproteome in female mice under two estrogenic conditions. The dataset combined with bioinformatic tools and computational predictive modeling demonstrates that estrogen deficiency is associated with distinct changes in the skeletal muscle phosphoproteome that may significantly affect muscle function and strength. Estrogen deficiency induced alterations in phosphoproteins of the Z-disc, contractile apparatus, and calcium and metabolic signaling pathways. The four differentially phosphorylated sarcomeric proteins (TPM3, OBSCN, MYH2, and MYOZ2) identified in this study showed overall decreased phosphorylation due to the loss of estrogen. Results support the concept that alterations in sarcomeric protein phosphorylation may play an important role in the phospho-signaling networks for force generation and may thereby contribute to muscle strength loss in estrogen-deficient females. In particular, altered phosphorylation of sarcomeric proteins sensitive to calcium suggests that disruption in calcium handling may be involved in the pathogenesis of skeletal muscle strength loss in females. Importantly, the results provide testable hypotheses for future studies to elucidate the molecular mechanisms underlying how estrogen deficiency affects skeletal muscle contractile function.

Declarations

Ethics approval and consent to participate

All experimental protocols and procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee. All methods were carried out in accordance with relevant University of Minnesota Institutional Animal Care and Use Committee guidelines and regulations.

Consent for publication

Not applicable

Availability of data and materials

The raw mass spectrometry data (Thermo RAW files) for proteins and phosphopeptides generated and analyzed during the current study have been deposited online and will be made available publicly through the Chorus project (<https://chorusproject.org/pages/index.html>).

Competing interests

The authors declare that they have no competing financial interests.

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Authors' contributions

MPP, TY, LH, TWM, LLP, and DAL contributed to experimental design and conceptual framework. MPP, TY, TWM, LH, and CV contributed to data collection and analysis. MPP, TY, and LH contributed to data curation and formal analysis. MPP prepared all figures and drafted the original manuscript. All authors contributed to the edits and revisions of the manuscript and have given approval to the final version of the manuscript.

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Figures

Figure 1

Experimental design and mouse characteristics.

Female mice were assigned to a surgical group and underwent their respective surgeries, Sham or ovariectomy, with body and uterine mass, measured 16 wk later at the time of sacrifice. (A) Schematic of experimental design – created with Biorender.com. Vaginal cytology was performed 4 wk post-surgeries. At 16 wk, tibialis anterior muscles were dissected and digested with trypsin for peptide extraction. The lysate underwent TiO_2 phosphopeptide enrichment for label-free phosphoproteomic analysis, and nLC-MS/MS was performed on the Orbitrap Fusion Tribrid mass spectrometer. (B) Body mass ($p < 0.001$) and (C) uterine mass ($p < 0.001$) of Sham and Ovx mice. Data were analyzed by a Student's t-test (Sham vs. Ovx); $n = 4/\text{group}$. Values represent means $\pm$ SD. *significantly different from Sham.

Figure 2

Characteristics of the phosphoproteome.

Proteome Discoverer (v2.4) was used for database search and identification of phosphopeptides and phosphoprotein analysis. (A) Venn diagram of all identified phosphopeptides identified in muscle from Sham and OvX mice. (B) Pie chart of modification sites there were phosphorylated on residues serine (S), threonine (T), and tyrosine (Y) in the dataset. (C) Volcano plot of differentially regulated phosphopeptides. Green and red dots represent downregulated and upregulated significantly regulated phosphopeptides (Benjamini – Hochberg adjusted p-value < 0.05 and 1.5-fold change ratio), respectively, whereas black dots represent non-significant phosphopeptide. (D) Significantly regulated phosphopeptides were mapped back to their precursor proteins. The list of phosphoproteins was submitted to Heatmapper. Heatmap of 130 phosphoproteins clustered according to average linkage using Pearson Correlation. Color scheme represents log₂ ratio of each phosphoprotein for each biological sample. Gray = missing data.

Figure 3

KEGG, Reactome, and GO term enrichment analyses.

Differentially regulated phosphopeptides that were quantified in at least three biological replicates or absent in all biological replicates (unique phosphopeptides) in at least one group were mapped back to their precursor protein, and the list of phosphoproteins was submitted for enrichment analysis using the R package clusterprofiler. The top 15 pathways overrepresented in the dataset are shown in (A) KEGG and (B) Reactome pathways. (C-D) Overrepresented GO terms in molecular function and cellular component in the dataset and (E) overrepresented GO terms in biological process with hierarchical clustering according to average linkage to identify the top 5 clusters are presented.

Figure 4

Changes in canonical metabolic pathways and calcium signaling pathways induced by ovariectomy

Phosphopeptides identified in the dataset were submitted to IPA's Downstream Effects analytics and prediction algorithm to analyze molecules associated with IPA's annotated canonical pathways. (A) The top 10 significantly associated canonical pathways from the dataset and predicted inhibition or activation state, with a $-2.0 \geq Z\text{-score} \geq 2.0$, respectively. (B) Calcium signaling pathway in a resting, i.e., non-contracting muscle cell and the phosphorylation state of proteins from the dataset is illustrated. Decreases or increases in protein phosphorylation measurements from Ovx muscle relative to Sham muscles are shown in green and red, respectively.

Figure 5

Changes in molecular, cellular, and physiological system development functional analyses in estrogen-deficient mice.

Phosphopeptides identified in the dataset were submitted to IPA's Downstream Effects analytics and activation Z-score prediction algorithm to analyze and predict molecular and cellular functions and physiological system development and functions. (A) The top 10 significantly enriched IPA functional parent groups from the dataset are summarized. Significant inhibition ($-2.0 \geq Z\text{-score}$) or significant activation ($Z\text{-score} \geq 2.0$) of subfunctions predicted from the dataset are shown in B) contractility of muscle, C) formation of phagosomes, D) formation of vesicles, and E) disruption in cytoskeleton. The number in the upper right box next to the molecule denotes the number of phosphopeptides observed in the dataset for the molecule. Green and red color of the molecule represents decreased or increased phosphorylation measurement of the protein in OvX relative to Sham mice, and the glow around the molecules indicates their activity status when opposite to the phosphoprotein measurement. The blue and orange color

of a subfunction or molecule represents the activation status – inhibition or activation, respectively. The color, orange, blue, yellow, or gray, of the edge (line) predicts the relationship between the two nodes (molecules), indicating activation, inhibition, inconsistent, or no prediction, respectively. The arrowhead or inhibition line at the end of the edge reflects the relationship proportionality (i.e., the inhibition line reflects the inverse proportionality, and the arrowhead reflects direct proportionality between two nodes).

Figure 6

Beta-estradiol (E2) mechanistic network.

Phosphopeptides identified in the dataset were submitted to IPA’s activation Z-score prediction algorithm to predict kinase activity inferred from the OvX/Sham dataset. A mechanistic network of E2 and its relationship to other regulators and the dataset was created. The number in the upper right box next to the molecule denotes the number of

phosphopeptides observed in the dataset for the molecule. E2 inhibition was predicted from the expression profile of nine molecules observed in our phosphoproteomics dataset (circled in black). See Figure 5 for explanation of color scheme, edges, and nodes.

Supplementary Files

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