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Beta$_2$-Adrenergic Stimulation Induces Resistance Training-Like Adaptations in Human Skeletal Muscle: Potential Role of KLHL41

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ABSTRACT

Skeletal muscle mass plays a pivotal role in metabolic function, but conditions such as bed rest or injury often render resistance training impractical. The beta$_2$-adrenergic receptor has been highlighted as a potential target to promote muscle hypertrophy and treat atrophic conditions. Here, we investigate the proteomic changes associated with beta$_2$-adrenergic-mediated muscle hypertrophy, using resistance training as a hypertrophic comparator. We utilize MS-based proteomics to map skeletal muscle proteome remodeling in response to beta$_2$-adrenergic stimulation or resistance training as well as cell model validation. We report that beta$_2$-adrenergic stimulation mimics multiple features of resistance training in proteome-wide remodeling, comprising systematic upregulation of ribosomal subunits and concomitant downregulation of mitochondrial proteins. Approximately 20% of proteins were regulated in both conditions, comprising proteins involved in steroid metabolism (AKR1C1, AKR1C2, AKRC1C3), protein-folding (SERPINB1), and extracellular matrix organization (COL1A1, COL1A2). Among overall most significantly upregulated proteins were kelch-like family members (KLHL) 40 and 41. In follow-up experiments, we identify KLHL41 as having novel implications for beta$_2$-adrenergic-mediated muscle hypertrophy. Treating C2C12 cells with beta$_2$-agonist for 96 h increased myotube diameter by 48% ($p < 0.001$). This anabolic effect was abolished by prior knockdown of KLHL41. Using siRNA, KLHL41 abundance was decreased by 60%, and the anabolic response to beta$_2$-agonist was diminished (+15%, i.e., greater in the presence of KLHL41, knock-down × treatment: $p = 0.004$). In conclusion, protein-wide remodeling induced by beta$_2$-adrenergic stimulation mimics multiple features of resistance training, and thus the beta$_2$-adrenergic receptor may be a target with therapeutic potential in the treatment of muscle wasting conditions without imposing mechanical load.

1 | Introduction

Muscle mass and function are important in a range of lifestyle and age-related conditions [1] and understanding mechanisms regulating muscle mass is vital for developing effective treatments that stimulate hypertrophy and counter muscle atrophic conditions. While resistance training is the classical approach to build muscle mass and enhance function, certain medical conditions, and movement-restricted situations (e.g., bed rest), render it unpractical. Furthermore, the long-term compliance to training regimens is poor [2]. With variable success, researchers have attempted to target the androgen receptor and growth differentiation factor pathways (e.g., myostatin inhibitors) but many developmental drugs fail due to unacceptable adverse effects—oncogenic in particular [3]. A re-emerging target is the beta$_2$-adrenergic receptor due to its involvement in regulating muscle mass and function. But while several studies have demonstrated the effectiveness of targeting the beta$_2$-adrenergic receptor to promote muscle hypertrophy, the molecular signature changes in response to prolonged selective beta$_2$-adrenergic agonist treatment remain elusive and is unknown in humans. However, one could speculate that the protein-wide remodeling induced by beta$_2$-adrenergic agonist shares multiple features with that induced by resistance training but without mechanical load.

There are several reasons why the beta$_2$-adrenergic receptor holds promise in muscle atrophic conditions. The beta$_2$-adrenergic receptor is widely expressed in skeletal muscle [4], and only a few weeks of treatment with selective beta$_2$-adrenergic agonist induces pronounced muscle mass accretion while also enhancing muscle function. For example, we recently showed that 4 weeks of daily treatment with a beta$_2$-adrenergic agonist increased muscle mass by 1.7 kg in young healthy males at high oral doses [5] and by 1 kg at inhaled doses [6]. And while not as efficient as resistance training, these and other [5, 7] data demonstrate the efficacy of targeting the beta$_2$-adrenergic receptor to induce skeletal muscle hypertrophy in the absence of exercise. Furthermore, beta$_2$-adrenergic agonists combined with resistance exercise induce an additive degree of muscle hypertrophy [6, 8]. This suggests the involvement of convergent pathways in mediating the hypertrophic response and coincides with experimental evidence from studies showing that prolonged beta$_2$-adrenergic stimulation also increases insulin-stimulated glucose disposal [9–11], just as would be expected from a period of resistance training [12]. Thus, there seems to be a degree of functional overlap in muscle remodeling between prolonged beta$_2$-adrenergic stimulation and resistance training. The identification of common targets mediating the hypertrophic response to resistance training and beta$_2$-adrenergic-induced hypertrophy may offer therapeutic strategies to combat muscle wasting and after periods of disuse such as in recovery from injury. Therefore, an unbiased exploration of skeletal muscle proteomics under these stimuli is warranted.

Here we utilized mass spectrometry (MS)-based proteomics to map skeletal muscle proteome remodeling in response to beta$_2$-adrenergic receptor agonist and resistance training. We show that beta$_2$-adrenergic stimulation mimics multiple features of resistance training in proteome-wide remodeling and we describe a member of the kelch-like family, KLHL41, as a potential regulator of beta$_2$-adrenergic receptor-mediated muscle hypertrophy.

2 | Methods

2.1 | Participants

For the current study, we analyzed stored human vastus lateralis muscle biopsies sampled from 13 and 12 active healthy men who had been treated with beta$_2$-adrenergic agonist [6] or performed a resistance training for a 4-week intervention period. Participants were young (mean ± SD age: 24 ± 3), non-obese (BMI: 22 ± 2 kg/m^2), lean (body fat: 16% ± 6%), and moderately trained (maximal oxygen uptake: 50 ± 16 mL O_2/min/kg) and gave their verbal and written informed consent to participate in the study. The study complies with the guidelines of the 2013 Declaration of Helsinki and was approved by the Regional Ethics Committee of Copenhagen (H-4-2014-002, H-1–2012-119).

2.2 | Beta$_2$-Agonist Treatment and Resistance Training

Participants treated with beta$_2$-adrenergic agonist were instructed to inhale a single daily dose of 4 mg terbutaline (8 × 0.5 mg, Bricanyl Turbuhaler, AstraZeneca, Cambridge, UK). The daily inhalations were supervised by video monitoring ensuring a compliance of 100% [6].

The participants subjected to supervised full-body resistance training performed as three weekly sessions consisting of 2–3 sets of leg press, lunges, leg extension, leg curls, bench press, incline bench press, row, lateral pulldowns, and military press. Load was continually adjusted between sets to reach volitional failure at ~10 repetitions. After each training session, participants received a protein-rich drink with carbohydrates (30 g of whey protein, Arla Foods, Viby J, Denmark; 35 g of carbohydrates, Maxim Sports Drink Orange, Orkla Care, Ishøj, Denmark) to stimulate postexercise protein synthesis. Mean compliance with the training protocol was > 90%.

2.3 | Muscle Biopsy Sampling

Muscle biopsies were collected under local anesthesia (20 mg/mL Xylocain without epinephrine; AstraZeneca, Cambridge, UK) through a small incision in the skin over the vastus lateralis. Biopsies were collected with a modified Bergström needle with suction. Upon collection, the muscle biopsy piece was rinsed in saline (9 mg/mL, Fresenius Kabi, Sweden) and quickly frozen in liquid nitrogen and stored at −80°C until analysis.

2.4 | Protein Extraction and Digestion

Protein was extracted from muscle biopsies using 250 μL of denaturation buffer comprising 7 M urea, 2 M thiourea in Tris-HCl, pH 8.0 with protease inhibitors (Complete mini-PI cocktail, Merck, Darmstadt, Germany) per mg tissue dry weight. After mixing end-over-end overnight at 4°C, insoluble material was removed by centrifugation (21 000 × g, 20 min) and protein concentrations estimated using the Pierce 660 nm protein assay. Sample volumes equivalent to 55 mg of protein were then sequentially treated with 10 mM DTT (overnight

at 4°C) and then 50 mM iodoacetamide (2 h at ambient temperature in the dark). Samples were cleaned up and then proteins digested with 2 mg of trypsin/LysC (Promega, Madison, Wisconsin) according to the SP3 method [13]. Following digestion, samples were acidified by the addition of 1% trifluoroacetic acid then desalted using C18 ZipTips (Merck) according to manufacturer's guidelines.

2.5 | High-pH Peptide Fractionation and Data-Dependent Mass Spectrometry

An experiment-specific peptide spectral library was generated using a Pierce High pH Reversed-Phase peptide fractionation kit (ThermoScientific, Waltham, USA). A pooled digest comprising 100 μg of protein was fractionated according to manufacturer's guidelines, then combined in 0.5 mL lo-bind Eppendorf tubes into four fractions by concatenation (i.e., 1 + 5; 2 + 6; 3 + 7 and 4 + 8). Following evaporation to dryness, peptides were reconstituted in 12 μL HPLC loading buffer and analyzed by DDA-MS using a Q-Exactive HF mass spectrometer fitted with a nanospray Flex ion source and coupled to an Ultimate 3000 RSLCnano (ThermoScientific). Briefly, peptide samples (~1 μg) were loaded onto a 20 mm × 75 μm PepMap 100 C18 trapping column, then separated over a segmented 90-min gradient at a flow rate of 300 nL/min, using a 250 mm × 75 μm PepMap 100 C18 analytical column held at 45°C. To compensate for the semi-stochastic nature of DDA-MS, each of the concatenated fractions was injected in triplicate: twice using a "fast" method with maximum IT of 28 ms and once using a "sensitive" method with maximum IT of 45 ms. MS1 spectra (370–1500 *m/z*) were acquired in profile mode at 60 K resolution followed by MS2 scans acquired in centroid mode at 15 K resolution using a Top10 DDA method, with 10-s dynamic exclusion. In addition to the fractionated samples, two further injections of pooled peptide sample were also analyzed using the "fast" DDA-MS method to detect any peptides that may have been lost during high pH fractionation.

2.6 | Mass Spectrometry—Data-Independent Acquisition

Individual peptide samples were analyzed by DIA-MS using the instrument configuration and HPLC gradient conditions described above. MS1 spectra (390–1240 *m/z*) were acquired at 120 K resolution, followed by MS2 scans across 25 DIA × 25 amu sequential windows over the range of 402.5–1027.5 *m/z*. MS2 spectra were acquired at a resolution of 30 K using an AGC target of 1e6, maximum IT of 55 ms.

2.7 | Raw Data Processing

Library files underwent processing using the MS-Fragger tool present within FragPipe v19.0 utilizing the SpecLib workflow with default configurations. These configurations encompassed a minimum requirement of seven amino acids for peptide length, allowing a maximum of two missed cleavages. Spectra within these files were subjected to exploration against a human FASTA file from UniProt (October 2021, 20420 entries). Consequently, the resulting library comprised a total of 1284 protein groups and 19695 precursor ions. MS data from individual samples was analyzed using DIA-NN version 1.8 in library-based search mode, utilizing the spectral library just described. Peptide quantification was conducted utilizing only proteotypic peptides, and the neural network was configured to a double-pass mode. The chosen quantification strategy was "Robust LC (high accuracy)" with default parameters, including the match between runs option enabled, a peptide length ranging from 7 to 30 amino acids, and a precursor false discovery rate of 1%. The protein groups file from the DIA-NN output was annotated and utilized for downstream bioinformatics analysis within the R environment.

2.8 | Bioinformatics

Following protein identification, 1207 protein groups (henceforth "proteins") were identified. Data were filtered for 70% valid values at both time points (pre/post) leaving 1132 proteins followed by median scaling. Missing values were imputed by drawing random numbers from a Gaussian distribution with a standard deviation of 30% in comparison with the standard deviation of measured protein abundances, and 1.8 standard deviation downshift from the mean. Proteins were annotated with gene ontology terms (GO:BP, GO:CC, and GO:MF) and keywords from the Uniprot database. Mitochondrial proteins were annotated with MitoCarta 3.0. Data were analyzed in R (v.4.3.2, Foundation for Statistical Computing, Vienna, Austria) by a series of two-tailed *t*-tests on $\log_2$ transformed values on each protein to estimate within-group changes. In addition, data were analyzed by a series of ANOVA's with time (pre/post) and treatment (beta_2-adrenergic agonist/resistance training) as fixed factors to estimate between-group changes. To control for multiple testing, the FDR method described by Storey and Tibshirani [14] was employed with the R package "qvalue" (v. 2.28.0). For gene set enrichment analysis, the R package clusterProfiler (v. 3.17) was used. Terms were simplified for more than 60% overlapping proteins, in which case the term with the lowest *p* value was used. Term *p* values were corrected with the Benjamin–Hochberg procedure.

2.9 | Cell Culture

C2C12 cells (CRL-1772, passage 5–8, ATCC) were cultured on ibiTreat 8-well chambered coverslips (Ibidi) with a density of 0.1×10^5 cells per well. Cells were proliferated in growth medium composed of Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, supplemented with 10% FBS and 1% Penicilin-streptomycin. When the cells reached 80% confluence, growth media were replaced by differentiation medium (DMEM 25 mM glucose supplemented with 2% horse serum and 1% penicilin–streptomycin). The medium was changed every other day, and the cells were incubated at 37°C in a humidified 5% CO_2 environment for 7 days. On the second day of differentiation, cells were transfected with either 25 nM KLHL41 siRNA or a scramble control siRNA using the TransIT-X2 Dynamic Delivery System (Mirus Bio). On Day 3 of differentiation, treatment with beta_2-adrenergic agonists began by exposing the cells to differentiation medium containing 1 μM terbutaline for a duration of 96 h.

2.10 | Immunofluorescence Analysis

On Day 7 of differentiation, C2C12 cells were fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100 in phosphate-buffered saline (PBS) and blocked with blocking solution (3%BSA/PBS). Cells were then stained; primary antibody anti-Myosin (M4276, Sigma-Aldrich) was diluted 1:400 with blocking solution and incubated overnight at 4°C. Subsequently, anti-mouse Alexa 568 conjugated secondary antibody was diluted 1:500 in blocking solution and incubated at room temperature for 1 h protected from light. Cell nuclei were stained with 4′,6-Diamidino-2-phenylindole (DAPI) at room temperature for 5 min. Fluorescence images were acquired using a laser scanning confocal microscope (LSM780, Zeiss, US) with a 20×/0.8 objective, and data was displayed and analyzed using ZEN 3.5 (blue edition) and ImageJ software. For the analysis, six fields were randomly selected per condition from three different wells. In each field, all myosin-positive multinucleated cells containing at least three nuclei were measured and the diameter per myotube was calculated as the mean of 10 measurements taken along the length of the myotubes using ImageJ software.

2.11 | Immunoblotting and SDS-PAGE

Cells were cultured in Matrigel-coated 12-well plates with a density of 0.5×10^5 cells per well, and the same procedure was performed as described for cell cultures above. Cells were harvested in 2% SDS on Day 7 of differentiation. Samples were immediately boiled at 95°C for 5 min and sonicated in Diagenode Bioruptor sonicator. After centrifugation at 16000 × *g* for 10 min, the pellet was discarded, and the supernatant was used for immunoblotting analysis. Total protein concentration in the samples was determined with a standard BSA kit (Millipore) and the protein concentration of each sample was adjusted to 1 μg/uL by the addition of 4×Laemmli buffer and 2% SDS. Equal amounts of protein were loaded in wells of pre-cast 4%–15% gels (Bio-Rad Laboratories, CA, US). Proteins were then separated according to their molecular weight by SDS-PAGE and semidry transferred to a PVDF membrane (Millipore A/S, Copenhagen, Denmark). The membrane was blocked for 15 min in 3% BSA in tris-buffered saline (TBS) containing 0.1% Tween20 (TBST) before an overnight incubation in primary antibody at 4°C and a subsequent incubation in horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Bands were visualized with ECL (Millipore) and recorded with a digital camera (ChemiDoc MP Imaging System, Bio-Rad Laboratories). Bands were quantified using Image Lab version 6.0 (Bio-Rad Laboratories) and determined as the total band intensity adjusted for background intensity. Primary antibody used was Anti-KBTBD10/KRP1 (ab252972, Abcam, Cambridge, MA, USA). Secondary antibody used was HRP-conjugated Goat Anti-Rabbit (1:5000; SouthernBiotech 4010-05).

2.12 | Statistics

Statistical analyses were performed in *R* with the rstatix package (v. 0.7.1). Changes in median intensities of cytosolic ribosomal proteins, initiation factors, and elongation factors (Figure 3c), and KLHL41 (Figure 4a) were analyzed by two-factor ANOVA with time (pre/post) and treatment (beta$_2$-adrenergic agonist/resistance training) as fixed factors. For cell culture experiments, two-factor ANOVA with treatment (beta$_2$-adrenergic agonist/control) and siRNA (control/siRNA^{control}/siRNA^{KLHL41}) as fixed factors was used. For univariate correlation analyses, the Pearson product–moment correlation coefficient was used (Figure 2i). Individual data are presented with *p* values to represent probability.

3 | Results

3.1 | Study Overview and Proteomic Workflow

We collected vastus lateralis muscle samples from 25 moderately active, young men before and after a 4-week period of either (1) daily treatment with inhaled beta$_2$-adrenergic agonist, terbutaline, or (2) resistance training three times weekly (Figure 1a).

Skeletal muscle biopsy samples were prepared for MS-based proteomics and measured by data independent acquisition method [15] (Figure 1b), which resulted in 1132 quantified proteins, spanning ~5 orders of magnitude (Figure 1c). On average, 13 ± 7 proteins were missing per sample, and these were imputed (Figure 1d). Myosin abundance distribution revealed relatively homogeneous composition of Type I (MYH7), Type IIa (MYH2) and Type IIx (MYH1) fiber composition (Figure 1e).

3.2 | Proteomic Signature of Prolonged Beta$_2$-Adrenergic Stimulation Mimics Multiple Features of Traditional Resistance Training

Using a false discovery rate (FDR) of 5%, the overall effect of the interventions (main effect of time) resulted in upregulation of 300 and downregulation of 67 proteins (Figure 2a). Specifically, a wide range of proteins of importance for cell structural integrity, assembly and myogenesis such as filamin c (FLNC), protein unc-45 homolog B (UNC45B), Ankyrin repeat domain-containing protein 2 (ANKRD2), and glutaminyl-tRNA synthase (QARS) were upregulated. Interestingly, QARS has been reported as a highly exercise responsive protein following high-intensity endurance exercise training [16]. One group of proteins upregulated with both interventions comprised three isoforms of the aldo-keto reductase family 1 (AKRC's), which play important roles in the interconversion of androgens and estrogens. Perhaps of particular interest is AKR1C3 which is involved in all major pathways leading to formation of testosterone and the potent androgen 5α-dihydrotestosterone [17], suggesting that androgenic metabolism may be involved in beta$_2$-adrenergic-induced hypertrophy.

Considering each intervention isolated suggested a larger proteomic remodeling with resistance training (144 upregulated and 61 downregulated proteins; Figure 2a) compared with beta$_2$-adrenergic stimulation (54 upregulated and 5 downregulated proteins; Figure 2a). Notably however, abundance changes for the 10 overall most up and downregulated proteins (Figure 2b) appeared to be largely equal in contribution from resistance training and beta$_2$-adrenergic stimulation. Specifically,

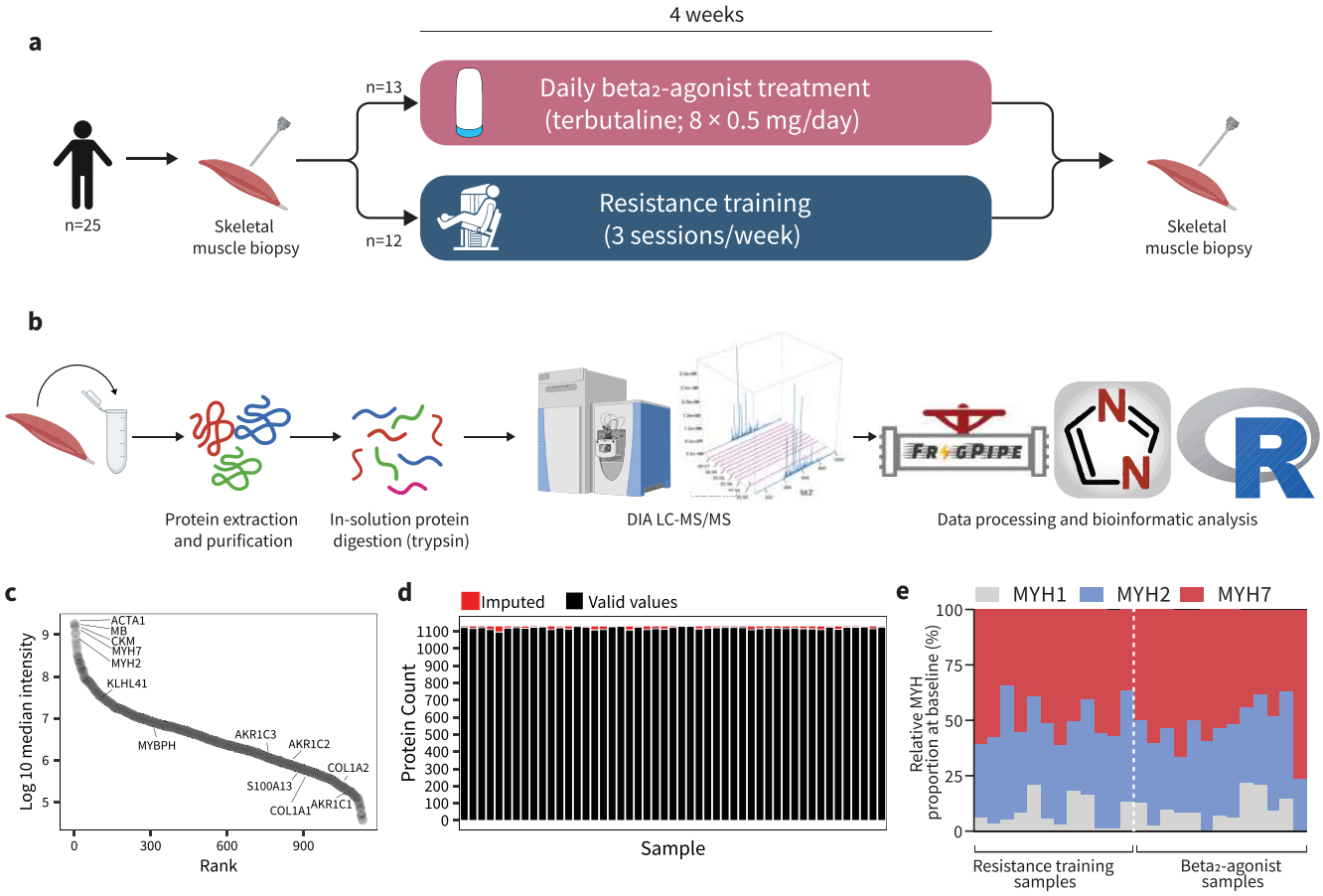

FIGURE 1 | Schematic overview of the study design and analysis workflow. (a) Twenty five healthy male participants completed either 4 weeks of resistance exercise training or prolonged beta$_2$-adrenergic stimulation. (b) Whole muscle tissue was lysed and trypsin digested before LC–MS/MS followed by computational analysis using Fragpipe and DIA-NN and bioinformatics using R. (c) Dynamic range of quantified proteins. (d) Valid values and imputed data. (e) Relative proportion at baseline of myosin isoforms of top 3 human skeletal muscle isoforms.

collagen component COL1A1 was markedly regulated by both interventions, whereas AKRC's featured prominently among the most regulated proteins for beta$_2$-adrenergic stimulation only (Figure 2b). Based on the number of significantly regulated proteins, the proteomic signatures with beta$_2$-adrenergic stimulation and resistance training comprised an overlap of ≈20% (Figure 2c), and abundance changes generally favored the same direction, even for proteins that were not significantly regulated (Figure 2d). In addition, full factorial analysis (intervention × time) revealed that only five proteins were differentially regulated between interventions (Figure 2a), indicating largely comparable proteomic remodeling with beta$_2$-adrenergic stimulation and resistance training.

Gene set enrichment analysis revealed overall enrichment of cellular compartments related to cytosolic ribosomes and extracellular matrix with a concomitant depletion of mitochondrial proteins, which was evident both as a main effect of time as well as for each intervention separately (Figure 2e). Concomitantly, biological processes related to translational capacity were increased while processes related to aerobic respiratory capacity were decreased in both interventions (Figure 2e).

Nonetheless, beta$_2$-adrenergic stimulation did not fully mimic the response to resistance training as several proteins showed distinct regulation. For example, MUSTN1, which has documented roles in cell differentiation and growth [18], and MYBPH, were both increased in response to resistance training but not to beta$_2$-adrenergic stimulation. In addition, the molecular chaperones SERPINH1 and CST3 responded more to resistance training. On the other hand, only beta$_2$-adrenergic stimulation increased abundance of RRAD, a $Ca_V1.2$ channel inhibitory protein which increases Ca^{2+} influx in response to PKA stimulation in cardiac muscle [19, 20]. Collectively, although the magnitude of the response was smaller, the above findings suggest that prolonged beta$_2$-adrenergic stimulation may induce partly overlapping adaptations to the proteomic landscape as resistance training.

3.3 | Prolonged Beta$_2$-Adrenergic Stimulation and Resistance Training Increase Translational Capacity but Impair Capacity for Aerobic Energy Production

We next conducted an in-depth investigation into remodeling of molecular machinery involved in protein translation. Proteins belonging to cytosolic ribosomal subunits revealed a modest but systematic increase with all identified cytosolic ribosomal proteins increasing with resistance training, and all but six proteins

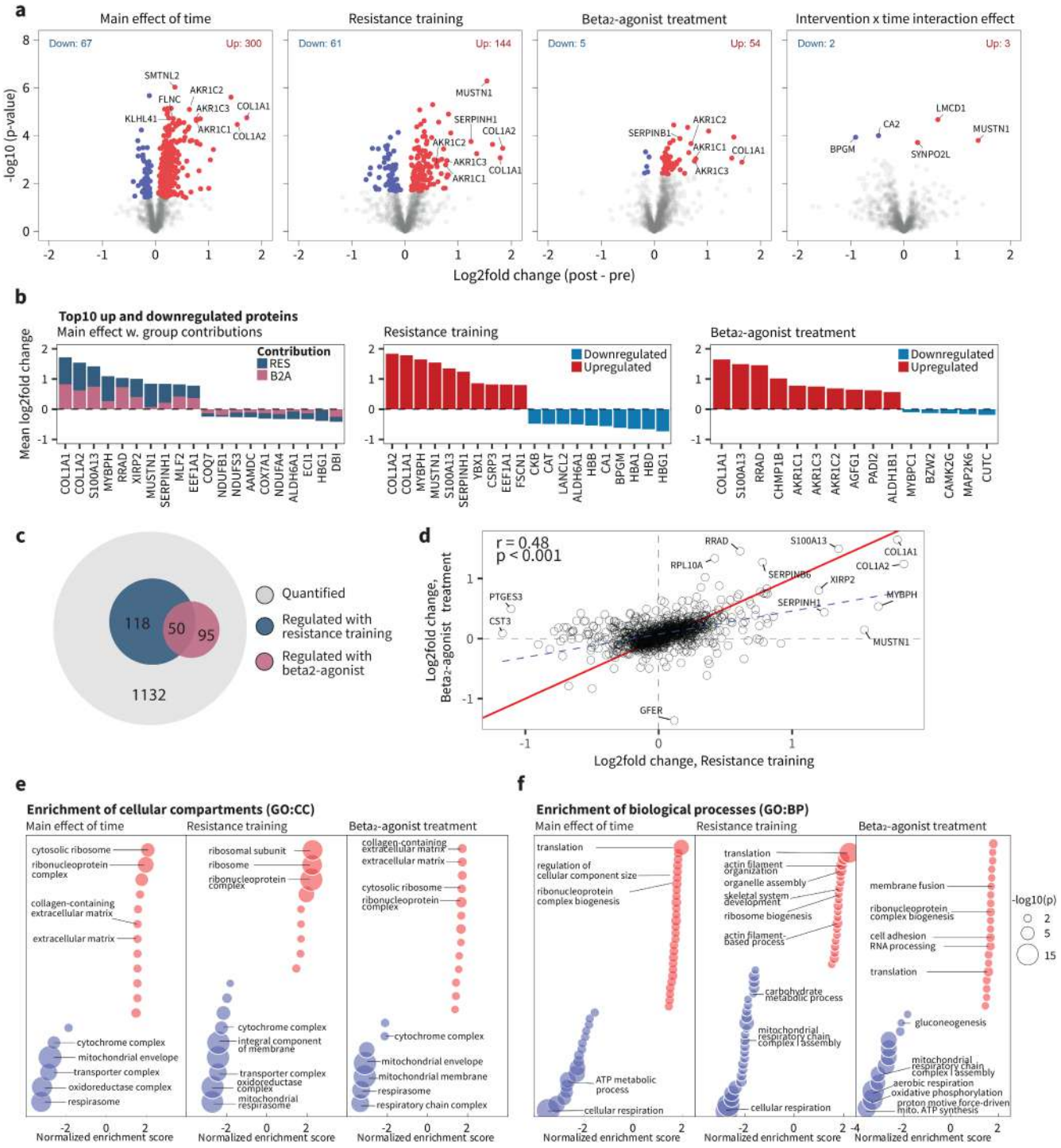

FIGURE 2 | Prolonged beta$_2$-adrenergic stimulation and resistance training produce convergent proteomic remodeling in skeletal muscle. (a) Volcano plots comparing protein abundance changes from pre to post as main effect of time, with resistance training, with beta$_2$-adrenergic agonist treatment, and interaction effect (intervention × time). Multiple comparison adjustment by permutation-based FDR with a 5% cutoff. (b) Top10 up and downregulated proteins (mean) for main effect and within each intervention. Contribution in main effect panel is area-proportionate fractional contribution to the main effect (i.e., within-group effect divided by two). (c) Venn diagram of total number of proteins quantified by LC/MS–MS as well as proteins regulated (up and down) in each intervention and overlap in regulation. (d) Pearson's correlation of protein abundance changes between interventions. Red line is identity line and dashed black line is regression line. (e) Bubble plots of significantly enriched (red) and depleted (blue) GO:CC terms with manual annotation of select terms. (f) Bubble plot of significantly enriched (red) or depleted (blue) GO:BP terms with manual annotation of select terms. For both panels (e) and (f), analysis by gene set enrichment analysis with clusterProfiler and filtering for GO-terms with semantic similarity > 60% in which case the most significant term was kept. B2A, beta$_2$-agonist treatment; RES, resistance training.

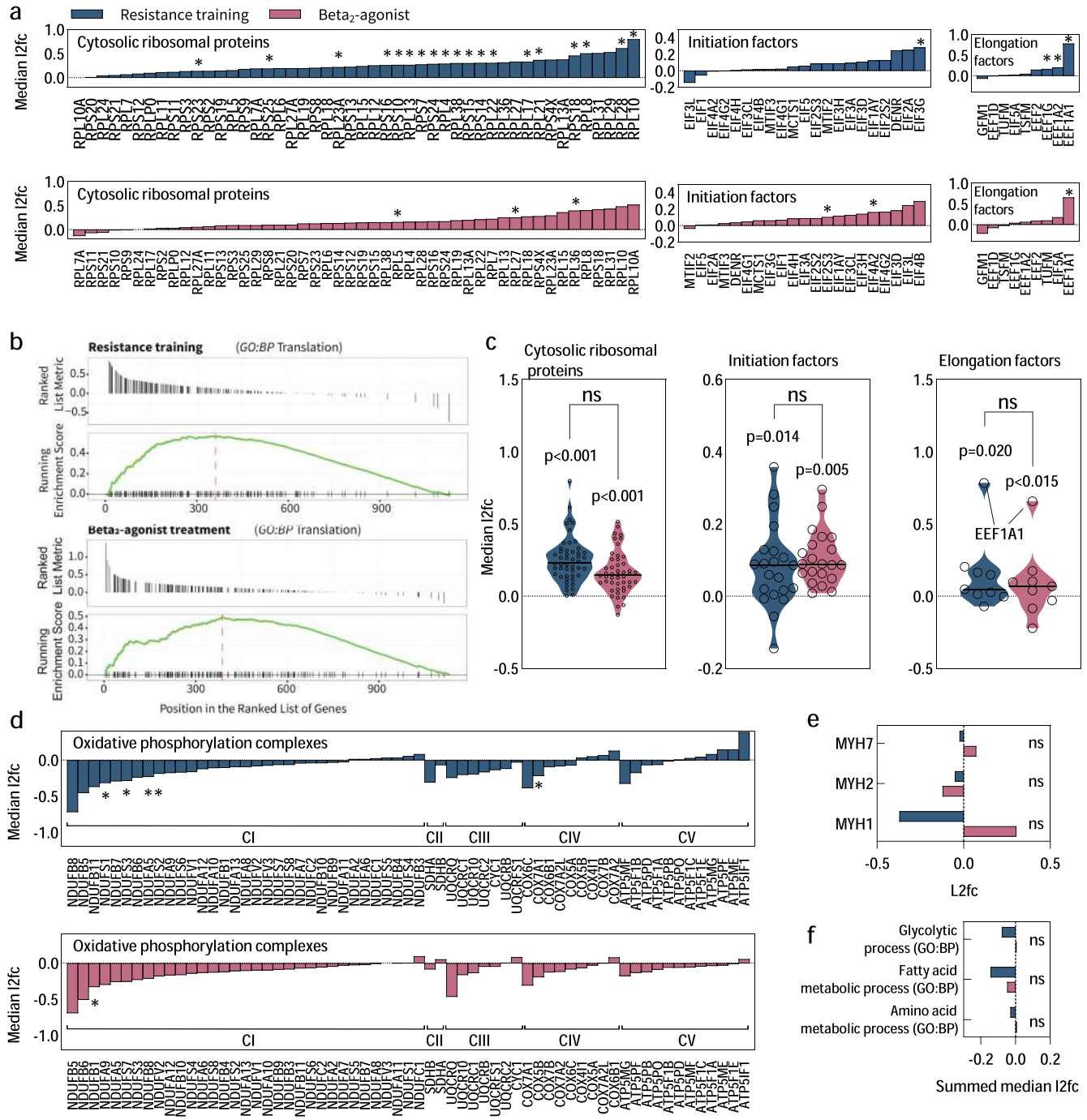

FIGURE 3 | Prolonged beta$_2$-adrenergic stimulation and resistance training increase translational capacity but impair mitochondrial capacity for aerobic energy production. (a) Median log2fold changes of proteins annotated as cytosolic ribosomal proteins, initiation factors, and elongation factors. (b) Running score plots of the GO:BP term "translation." (c) Individual values of proteins belonging to either cytosolic ribosomal proteins, initiation factors, or elongation factors. Horizontal solid line is mean. (d) Median log2fold changes in proteins annotated as belonging to mitochondrial oxidative phosphorylation complexes (CI–CV). (e) Median changes in abundance of myosin isoforms. (f) Median log2fold changes in abundance of proteins annotated to selected metabolic processes. *$q \leq 0.1$. L2fc, log2fold change.

increased with beta$_2$-adrenergic agonist treatment (Figure 3a). By extension, proteins annotated as involved in protein translation were generally located toward the tail-end (upregulated) of the ranked gene set (Figure 3b). The summed median log2-fold changes showed a consistent upregulation of proteins in the translational machinery and translation initiation and elongation factors that were not different between resistance training and beta$_2$-adrenergic stimulation (Figure 3c). Particularly eukaryotic elongation factor 1-alpha (eEF1A1) exhibited pronounced upregulation with both interventions. eEF1A1 chaperones aminoacylated tRNA's to the ribosomal A-site and is pivotal for the elongation of the nascent peptide chain during protein translation [21], which is in line with experimental evidence showing that eEF1A1 inhibitors decrease protein synthesis rates in mammalian cells [22] and thus corroborates the skeletal muscle hypertrophic effects of resistance training and beta$_2$-adrenergic stimulation.

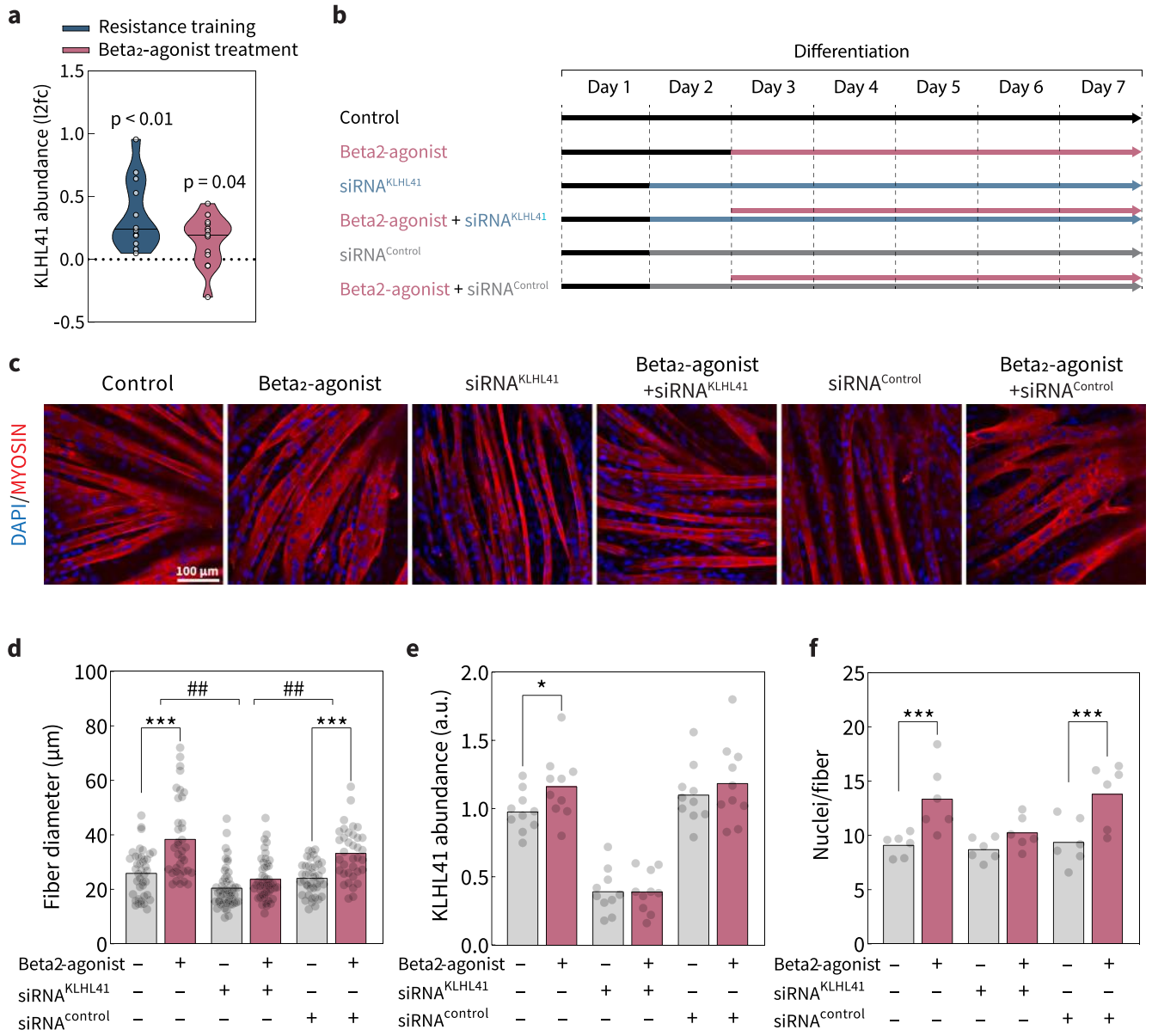

FIGURE 4 | KLHL41 may be important for beta$_2$-adrenergic agonist induced skeletal muscle growth. (a) Log2fold change in abundance of KLHL41 in human skeletal muscle samples. (b) Schematic presentation of the C2C12 workflow. Cells were proliferated until they reached 80% confluence and differentiated for 7 days. siRNA transfection was initiated on the second day of differentiation and beta$_2$-adrenergic agonist treatment (1 µM) was initiated on the third day of differentiation for 96 h. On the seventh day of differentiation, cells were fixed and stained for myosin (red) and DAPI (blue) and visualized. (c) Representative images. (d) Myotube diameter. (e) Abundance of KLHL41 assessed by western blotting. A.u., arbitrary units. (f) Number of nuclei per myofiber. *Significantly different ($p < 0.05$). *** Significantly different ($p < 0.001$). ##Interaction effect ($p < 0.01$).

In contrast to cytosolic ribosomal proteins, mitochondrial proteins of all oxidative phosphorylation complexes were systematically decreased with both resistance training and beta$_2$-adrenergic stimulation (Figure 3d).

Another often-observed effect of prolonged beta$_2$-adrenergic stimulation in rodents is a shift from a slow oxidative to fast glycolytic myofiber phenotype [23], but the present data do not indicate a shift in fiber type distribution based on myosin isoform abundances or alterations in proteins involved in glycolytic metabolic pathways (Figure 3e,f). This discrepancy may be related to the considerably larger doses of beta$_2$-adrenergic agonist administered in rodent studies. One study which administered a markedly higher dose of the beta$_2$-adrenergic agonist, salbutamol, during a resistance training intervention reported a shift toward a more glycolytic phenotype in humans [8], suggesting that the response may be dose-dependent.

3.4 | KLHL41 May Facilitate Beta$_2$-Adrenergic Induced Muscle Hypertrophy

We observed a pronounced overall increase (main effect $q < 0.001$) in content of two members of the kelch-like protein family KLHL40 and KLHL41 in response to the intervention (Figure 4a). A common feature between these two proteins is

that mutations in these genes are a hallmark characteristic of the skeletal muscle degenerative disorder *nemaline myopathy* [24, 25]—a congenital disorder causing severe muscular impairment characterized by the accumulation of nemaline bodies formed by misfolding and aggregation of the highly abundant skeletal muscle protein, nebuline [26]. KLHL41 and the closely related KLHL40, are known regulators of differentiation [27], and at least one report suggests that KLHL40 is an exercise responsive gene [28]. More importantly, a series of studies suggest a role of KLHL's in the development of structurally functional sarcomeres and aligned myofibrils [24, 29, 30] and knock-out of KLHL41 inhibits muscle growth and causes severe disarray in skeletal muscle sarcomeric structure in both mice [26] and zebrafish [25]. Based on these known roles of KLHL41 in muscle development combined with the consistent overall (main effect) regulation, we proceeded to investigate the role of KLHL41 in beta$_2$-adrenergic receptor induced muscle hypertrophy.

We treated C2C12 cells with beta$_2$-adrenergic agonist, terbutaline, beginning at the third day of differentiation and for 96 h (Figure 4b,c). Here, we observed that terbutaline not only induced a potent anabolic response (48% increase in myotube diameter, $p < 0.001$; Figure 4d) but also increased the abundance of KLHL41 by 19% ($p < 0.05$; Figure 4e). In addition, terbutaline increased myonuclear abundance (Figure 4f). This aligns with in vivo skeletal muscle hypertrophy incurred from resistance exercise training [31] and suggests a role of transcriptional as well as translational capacity in mediating beta$_2$-adrenergic induced skeletal muscle hypertrophy. Next, we treated C2C12 cells with siRNA for KLHL41, which decreased KLHL41 abundance by 60% ($p < 0.001$; Figure 4e). Notably, when we knocked down KLHL41 the increase in myotube diameter with beta$_2$-adrenergic agonist was diminished (+15% myotube diameter, $p = 0.088$; Figure 4d), and the increase was greater in the presence of KLHL41 (knock-down × treatment: $p = 0.004$) suggesting that KLHL41 is implicated in beta$_2$-adrenergic-induced hypertrophy.

The siRNA medium did not affect the experiments, as siRNA control did not inhibit beta$_2$-adrenergic induced growth or myonuclear addition (Figure 4d), although the beta$_2$-adrenergic induced increase in KLHL41 abundance with siRNA control was not significant (Figure 4e).

4 | Discussion

Here, we show that beta$_2$-adrenergic stimulation mimics multiple features of resistance training in muscle protein-wide regulation, particularly with respect to an increase in proteins involved in translational capacity and a concomitant decrease in mitochondrial protein abundance. In addition, we identify KLHL41 as a potential regulator of beta$_2$-adrenergic receptor induced muscle hypertrophy and myonuclear addition.

The beta$_2$-adrenergic receptor has long been recognized as a potential target to promote muscle growth and counter muscle atrophy [32–35]. We utilized MS-based proteomics for an in-depth characterization of beta$_2$-adrenergic induced proteomic remodeling. To strengthen our analysis, we included data from resistance training as a hypertrophic comparator. In doing so, we uncovered a ≈20% overlap in proteomic regulation, despite the lack of muscle mechanical stimulus in the beta$_2$-adrenergic stimulation group. For example, translational capacity, as measured by abundance of cytosolic ribosomal proteins, systematically increased with beta$_2$-adrenergic stimulation to a similar extent as with resistance training. The same was true for abundance of translational initiation and elongation factors, thus adding to the evidence suggesting increased translational capacity as a driver of skeletal muscle hypertrophy accrued from prolonged beta$_2$-adrenergic stimulation and that this mimics the response seen with resistance training.

We also found that proteins related to the extracellular matrix were among the most upregulated proteins with both beta$_2$-adrenergic stimulation and resistance training. These included components of Type 1 collagen, COL1A1 and COL1A2, and SERPINH1, a collagen-specific molecular chaperone required for correct folding of collagen [36]. The extracellular matrix plays a central role in maintenance of structural integrity to the sarcolemma and supports for lateral force transmission during contraction [37], and thus it is noteworthy that beta$_2$-adrenergic stimulation can elicit this adaptation in the absence of an intervention of mechanical muscle overload. Collectively, the coherence of protein regulation among beta$_2$-adrenergic stimulation and resistance training suggests overlap in molecular pathways and a possible contribution of the beta$_2$-adrenergic receptor in regulation of skeletal muscle mass. While the exact mechanisms cannot be inferred from our study, it is conceivable that exercise induced catecholamine release [38] contributes to the remodeling seen with resistance training, and that this may explain the partial overlap in regulation with prolonged beta$_2$-adrenergic stimulation without exercise.

A novel finding of the present study was the identification of KLHL41 as a protein with potential involvement in human skeletal muscle hypertrophy. KLHL41 was not among the highest observed log2fold changes. However, main effect analysis revealed it as one of the most significantly regulated proteins (eleventh most significant) and previous investigations provide strong biological rationale and coherence in findings of its importance in structural development of muscle fibers in animal and cell models. When we validated the MS-based data in a C2C12 model, we observed that partial knock-down of KLHL41 abolished the increase in myotube diameter seen with beta$_2$-agonist treatment, indicating that it may be involved in regulating beta$_2$-adrenergic receptor-mediated muscle hypertrophy. Beta$_2$-adrenergic receptor stimulation has shown therapeutic potential in a range of muscle atrophic conditions such as Duchenne's muscular dystrophy [39], spinal cord injury [40], and disuse mediated atrophy and strength loss [41] but with few identified mechanistic targets. Collectively, our results highlight KLHL41 as one potential target that warrants further investigation with respect to its involvement in beta$_2$-adrenergic induced muscle hypertrophy.

A downside of targeting the beta$_2$-adrenergic receptor is that of decreased mitochondrial protein content. Even though it has been reported that beta$_2$-adrenergic agonists increase fractional synthesis rate of mitochondrial proteins in rodents [42] and increases abundance of mitochondrial proteins in cell cultures [43], our data demonstrate a consistent decrease in proteins related to aerobic energy production. This was not only evident with gene set enrichment analysis, where mitochondrial terms were the

most depleted in both interventions, but also when examining individual proteins annotated as part of mitochondrial subunits. For example, with prolonged beta$_2$-adrenergic stimulation all but three proteins belonging to Complex I exhibited decreased abundance. These findings corroborate previous observations of selective beta$_2$-adrenergic agonists having detrimental effects on mitochondrial density and respiratory capacity [7]. However, this decrease is not necessarily indicative of impaired mitochondrial biogenesis, as both resistance training [44] and beta$_2$-adrenergic stimulation [42] are reported to increase markers of mitochondrial biogenesis. Rather, it may be the result of myofibrillar protein synthesis outpacing mitochondrial protein synthesis (as previously reviewed for resistance training [45]). Therefore, the decrease in mitochondrial content should not be perceived as a counter indication of beta$_2$-adrenergic stimulation, but rather a natural consequence of increased myofibrillar protein synthesis as seen with resistance training.

4.1 | Perspective

The findings from this study provide novel insights into the role of beta$_2$-adrenergic receptors in promoting muscle hypertrophy, paralleling the effects typically achieved through resistance training. This discovery is particularly significant in the field of medicine, as it highlights a potential therapeutic pathway for individuals unable to engage in traditional resistance exercise due to injury or other constraints. Further research should seek to address the concomitant strength outcomes with beta$_2$-adrenoceptor mediated hypertrophy. For example, one study showed that beta$_2$-adrenergic stimulation during a resistance training period induced greater muscle hypertrophy compared to placebo, while strength gains were comparable between groups [8]. In addition, further research into optimizing beta$_2$-adrenergic receptor targeting strategies may elaborate on the functional role of KLHL41 in vivo to address its potential role in interventions to combat muscle wasting conditions.

In summary, we have provided the first in-depth insights into the molecular underpinnings of skeletal muscle adaptation to prolonged beta$_2$-adrenergic receptor stimulation in humans and shown that the proteome-wide remodeling mimics multiple features of traditional resistance training. Specifically, we demonstrate systematic increases in translational capacity as an important driver of beta$_2$-adrenergic receptor mediated skeletal muscle hypertrophy at the expense of mitochondrial protein content. We further identify KLHL41 as a protein involved in beta$_2$-adrenergic receptor-mediated muscle hypertrophy. In conclusion, these data support the therapeutic potential of beta$_2$-adrenergic stimulation to combat muscle wasting conditions and suggest KLHL41 as a potential regulator of beta$_2$-adrenergic receptor-mediated muscle hypertrophy.

Author Contributions

Søren Jessen: conceptualization, data curation and analysis, investigation, project management and writing. **Júlia Prats Quesada**, **Andrea Di Credico**, **Roger Moreno-Justicia**, **Richard Wilson**, **and Glenn Jacobson:** data curation, investigation, and editing. **Jens Bangsbo:** project supervision, funding acquisition, and editing. **Atul S. Deshmukh:** conceptualization, project management and supervision, funding acquisition, and editing. **Morten Hostrup:** conceptualization, project management and supervision, funding acquisition, and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD045147. All other data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.