Striated muscle-specific serine/threonine-protein kinase beta (SPEGβ) segregates with high- versus low-responsiveness to endurance exercise training

Running Head: SPEGβ segregates with responsiveness to endurance training

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Keywords:

Artificial selection model; Co-immunopreciptiation; Endurance training; Exercise capacity; Label-free quantitation; Liquid chromatography mass spectrometry; Responsiveness to exercise; Skeletal muscle.

35 Supplementary data.

36 https://doi.org/10.6084/m9.figshare.9995087 (https://figshare.com/s/1684cf1d68caa238fbdb)

Abstract

Bi-directional selection for either high- or low-responsiveness to endurance running has created divergent rat phenotypes of high-response trainers (HRT) and low-response trainers (LRT). We conducted proteome profiling of HRT and LRT gastrocnemius of 10 female rats (body weight 279 ± 35 g; n=5 LRT and n=5 HRT) from generation 8 of selection. Differential analysis of soluble proteins from gastrocnemius was conducted using label-free quantitation. Genetic association studies were conducted in 384 Russian international-level athletes (age 23.8 ± 3.4 y; 202 males and 182 females) stratified to endurance or power disciplines. Proteomic analysis encompassed 1,024 proteins, 76 of which exhibited statistically significant ($P<0.05$, FDR <1 %) differences between HRT and LRT muscle. There was significant enrichment of enzymes involved in glycolysis/ gluconeogenesis in LRT muscle but no enrichment of gene ontology phrases in HRT muscle. Striated muscle-specific serine/threonine-protein kinase beta (SPEGβ) exhibited the greatest difference in abundance and was 2.64-fold greater ($P=0.0014$) in HRT muscle. Co-immunoprecipitation identified 24 potential binding partners of SPEGβ in HRT muscle. The frequency of the G variant of the rs7564856 polymorphism that increases *SPEG* gene expression, was significantly greater (32.9 vs 23.8%; OR = 1.6, P = 0.009) in international-level endurance athletes (n=258) compared to power athletes (n=126) and was significantly associated (β = 8.345, P = 0.0048) with a greater proportion of slow-twitch fibres in vastus lateralis of female endurance athletes. Co-immunoprecipitation of SPEGβ in HRT muscle discovered putative interacting proteins that link with previously reported differences in transforming growth factor-β signalling in exercised muscle.

Introduction

Exercise training has a positive impact on health and is broadly considered to be effective in the prevention of chronic diseases, including type 2 diabetes mellitus and cardiovascular disease (56). In humans, maximum aerobic capacity (VO_2max) is a strong and independent indicator of mortality (40), and also modifies risk associated with factors collectively termed the metabolic syndrome, including insulin resistance, abdominal obesity, atherogenic dyslipidaemia, hypertension, and a proinflammatory and prothrombotic state (16). An individual's VO_2max is a product of their genetic heritage as well as their recent history of habitual activity or exercise training. However, there is broad inter-individual variation in the response of humans to exercise training (5, 22). The Health, Risk Factors, Exercise Training and Genetics (HERITAGE) family study reported 47 % of the variance in responsiveness (improvement in VO_2max) to training may be attributable to genetic and other familial factors (4). In Caucasian men and women, the average increase in VO_2max after 20-weeks supervised training, was 400 ml/min, but the magnitude of improvement in VO_2max was broadly distributed, for example the VO_2max of some individuals increased by more than 1,000 ml/min (relative increase of approximately 50 %). In contrast, other individuals failed to show a measurable change in VO_2max or responded negatively and exhibited a decrease in VO_2max in response to the standardised exercise stimulus. Moreover, changes in insulin sensitivity varied widely and 42 % of the 596 HERITAGE participants that underwent an intravenous glucose tolerance test exhibited no change or a decrease in insulin sensitivity after exercise training (7). Such an inability to respond to aerobic exercise may have serious health consequences and investigation of the mechanisms underpinning this phenomenon is needed to identify targets and candidate biomarkers for more personalised therapies.

In humans a mixture of genetic and environmental factors contribute to the broad range of responsiveness to exercise training and this presents challenges to identifying the underlying mechanistic links between exercise and improvements in health outcomes. The genetic factors that contribute to VO_2max (and therefore disease risk) interact with environment factors and can be divided into intrinsic and acquired components. The intrinsic component governs an individual's baseline aerobic capacity and disease risk-profile in the non-trained sedentary state, whereas the acquired component governs the individual's responsiveness to an environment of high physical-activity such as regular endurance training. The intrinsic and acquired components of exercise capacity are each selectable traits and we have used bi-directional artificial selection in rats to develop models of either high- versus low-intrinsic running capacity or high- versus low-responsiveness to endurance training. Selection on intrinsic running capacity has generated high-capacity runners (HCR) that resemble endurance-trained individuals and low-capacity runners (LCR) that have a significantly heightened disease risk profile, including significantly poorer cardiovascular function (e.g. (57)), peripheral insulin sensitivity (e.g. (47)) and life expectancy (e.g. (30)). Selection on the acquired component of exercise capacity has generated high-response to training (HRT) and low-response to training (LRT) rats (31, 48) that do not differ in their intrinsic running capacity but have significantly different

responses to a standardised regimen of endurance training. After 8 weeks of moderate endurance exercise, the maximal running capacity of HRT rats increases by on average 50 %, whereas LRT rats either fail to respond positively or decrease in maximal running capacity on average by 50 %. Lessard et al (33) reports LRT exhibit primary metabolic defects including poor glucose tolerance and elevated plasma triacylglyceride levels in the untrained sedentary state, and when exposed to endurance training LRT fail to promote skeletal muscle angiogenesis and exhibit an altered inflammatory response to acute exercise. It is not yet clear whether common molecular targets from hypothesis-led literature on muscle responses to exercise training are differentially regulated in HRT/LRT muscle. For example, Lessard et al (33) reports no difference in the response of signalling proteins (e.g. AMPK and Akt) or mitochondrial capacity between HRT/LRT rats exposed to exercise training. In contrast, Marton et al (37) conclude there are significant differences in markers of mitochondrial biogenesis in the muscles HRT and LRT rats exposed to controlled exercise training.

Wider analysis of molecular differences that regress with low- versus high-responsiveness to endurance training may provide further mechanistic insight to the regulators of the training response and the elevated disease risk that is associated with a lack of adaptation to endurance training. Nevertheless, finding the common denominators that underpin differences in the change in exercise capacity in response to endurance training is challenging because the genetic underpinning of training responsiveness is highly convoluted. Previous attempts to find predictors of exercise responsiveness have been performed at the transcript level, which offers powerful bioinformatic analysis through reverse engineering of nucleotide data and has highlighted complex relationships such as the repression of negative regulators (e.g. miRNA) that target selective transcription factors (28). Proteomic analysis offers an alternative and pragmatic approach to discovering new information that may be complementary to other omic approaches (59), and we (9, 10) have previously used proteomics to highlight differences in HCR/LCR muscle that segregate with intrinsic running capacity. The proteome/ protein complement of a tissue is what defines that tissue and is the net result of complex upstream events involving genetic and environmental interactions. The proteome is often regarded as a product downstream of transcriptional processing, translational regulation and protein degradative processes. However, proteins that already exist within the cell are what 'sense' and transduce environmental stimuli, and so also reside upstream of gene transcription and other regulatory processes. To gain new insight to muscle proteome differences associated with the responsiveness to endurance training, we conducted high-definition mass spectrometry ($HDMS^E$; (8)) profiling of proteins in HRT and LRT gastrocnemius. We report comprehensive differences between HRT and LRT muscle, including a greater abundance of straited muscle-specific serine/threonine protein kinase beta (SPEGβ) in HRT gastrocnemius. We identify putative binding partners of SPEGβ in rat muscle that link with previously reported differences in transforming growth factor-β (TGF-β) signalling. Moreover, genetic analysis in international-level Russian athletes found the G variant of the rs7564856 polymorphism that increases *SPEG* gene expression, was significantly greater in endurance than power athletes, which offers some external validation of the potential role of SPEGβ in modulating muscle responsiveness to endurance training.

Methods

Rat model of artificial selection on responsiveness to endurance exercise training.

High-response trainer (HRT) and low-response trainer (LRT) rats were generated from a large-scale bi-directional selection programme on the response to endurance exercise described in Koch et al (31). Briefly, genetically heterogeneous rats (n= 152) from the N:NIH outcrossed stock were used to develop the HRT and LRT strains by selective mating of males and females that exhibited either the greatest (HRT) or least (LRT) response (improvement in exercise capacity) to a standardised and progressive regimen of endurance training. The maximal running capacity (maximum distance run; DIST) of each animal was measured using a speed-ramped treadmill test to exhaustion that was performed prior to (DIST1) and after (DIST2) an 8-week programme of 24 exercise sessions beginning when the animals were approximately 11-13 weeks of age. The volume of endurance training progressed each session by increments of 1 m/min running speed and 0.5 min duration, beginning at 10 m/min for 20 min in the 1st week and finishing at 21 m/min for 31.5 min in the 8th week. All exercise tests and training sessions were performed on a motorised treadmill at a 15 ° incline and the response to training was calculated as the change in maximal running distance (ΔDIST = DIST2 - DIST1). At each generation, 10 male and 10 female rats that represented the extremes of training response were bred to develop HRT and LRT strains. Rats used in the current work were females from generation 8 of selection and were shipped from the U.S. to the University of Jyväskylä, Finland, at 10 months of age. The animals were housed in standard conditions (temperature 22 °C, humidity 50 ± 10 %, light from 8.00 a.m. to 8.00 p.m.) and had free access to tap water and food pellets (R36; Labfor, Stockholm, Sweden). The study was approved by the National Animal Experiment Board, Finland (Permit number ESLH-2007-06894/Ym-23) and at the age of 17 months the maximal running capacity of the rats was tested according to the speed-ramped protocol used previously at the University of Michigan. Two weeks after the maximal running test 5 LRT and 5 HRT rats (body mass 290 ± 30 g and 268 ± 40 g, respectively) were euthanized and gastrocnemius muscles were excised, snap-frozen in liquid nitrogen and stored at -80 °C before being shipped to Liverpool John Moores University, UK, for proteomic analysis.

Processing of rat muscle samples

Muscles were pulverised in liquid nitrogen then homogenised on ice in 8 volumes of 1 % NP-40, 50 mM Tris pH 7.4 containing Complete™ protease inhibitor (Roche Diagnostics, Lewes, UK). Samples were incubated on ice for 15 min then centrifuged at 1,000 rpm, 4 °C for 5 min. Supernatants were cleared by centrifugation (12,000 g, 4 °C for 45 min) and protein concentrations were measured using the Bradford assay (Sigma, Poole, Dorset, UK). Each sample was adjusted to 5 µg/µl and in-solution tryptic digestion was performed in preparation for label-free quantitation (LFQ). Aliquots containing 100 µg protein were precipitated in 5 volumes of acetone for 1 h at -20 °C. Pellets were resuspended in 0.1 % (w/v) Rapigest SF (Waters; Milford, MA) in 50 mM ammonium bicarbonate and incubated at 80 °C for 15 min. DTT was added (final

concentration 1 mM) and incubated at 60 °C for 15 min followed by incubation whilst protected from light in the presence of 5 mM iodoacetamide at 4 °C. Sequencing grade trypsin (Promega; Madison, WI) was added at an enzyme to protein ratio of 1:50 and digestion allowed to proceed at 37 ˚C overnight. Digestion was terminated by the addition of 2 µl concentrated TFA and peptide solutions were cleared by centrifugation at 13 000 g for 15 min. Samples were diluted 1:1 with a tryptic digest of yeast alcohol dehydrogenase 1 (100 fmol/ µl) to enable the amount of each identified protein to be quantified, as described previously (51).

Label-free quantitation (LFQ) by high-definition – mass spectrometry ($HDMS^E$)

Peptide mixtures were analysed by nanoscale reverse-phase ultra-performance liquid chromatography (UPLC; nanoACQUITY, Waters, Milford, MA) and online ion-mobility mass spectrometry (IMS; SYNAPT G2-S, Waters, Manchester, UK). Samples (200 ng tryptic peptides) were loaded in aqueous 0.1 % (v/v) formic acid via a Symmetry C_{18} 5 µm, 2 cm x 180 µm trap column (Waters, Milford, MA). Separation was conducted at 35 °C through an HSS T3 C_{18} 1.8 µm, 25 cm x 75 µm analytical column (Waters, Milford, MA). Peptides were eluted using a gradient rising to 40 % acetonitrile 0.1 % (v/v) formic acid over 90 min at a flow rate of 300 nl/min. Additionally, a Lockmass reference (100 fmol/ µl Glu-1-fibrinopeptide B) was delivered to the NanoLockSpray source of the mass spectrometer at a flow rate of 500 nl/ min, and was sampled at 60 s intervals. For all measurements, the mass spectrometer was operated in a positive electrospray ionisation mode at a resolution of >25,000 FWHM. Prior to analysis, the time of flight analyser was calibrated with a NaCsI mixture from m/z 50 to 1990. $HDMS^E$ analyses were conducted within the Tri-wave ion guide. Accumulated ions were separated according to their drift time characteristics in the N_2 gas-filled mobility cell prior to collision induced dissociation (CID) alternating between low (4 eV) and elevated (14-40 eV) collision energies at a scan speed of 0.9 s per function over 50-2000 m/z. Analytical data were LockMass corrected post-acquisition using the doubly charged monoisotopic ion of the Glu-1-fibrinopeptide B. Charge reduction and deconvolution of potential parent-fragment correlation was achieved in the first instance by means of retention and drift time alignment, as described previously (35).

$HDMS^E$ spectra were aligned using Progenesis QI for Proteomics (QI-P; Nonlinear Dynamics, Newcastle, UK). Prominent ion features (approximately 1200 per chromatogram) were used as vectors to match each dataset to a common reference chromatogram. An analysis window of 10 min - 100 min and 50 m/z - 1650 m/z was selected, which encompassed a total of 47,109 features (charge states of +2, +3 or +4) and 3,924 of these features were separated by IMS. Protein identifications and quantitative information were extracted using the dedicated algorithms in ProteinLynx GlobalSERVER (PLGS) v3.0 (Waters, Milford, MA). Peak lists were searched against the UniProt database restricted to 'Rattus' (8,071 entries). The initial ion-matching requirements were ≧1 fragment per peptide, ≧3 fragments per protein and ≧1 peptide per protein. The enzyme specificity was trypsin allowing 1 missed cleavage, carbamidomethyl of cysteine (fixed) and oxidation of methionine (variable). Parent- and fragment-ion ppm errors were calculated empirically and decoy databases were used to calculate the identification error rate. Scoring of the database searches was refined

198 by correlation of physicochemical properties of fragmented peptides from theoretical and experimental data.
199 Peptide identifications were imported to QI-P and filtered to exclude peptides with scores less than 5.5 (34).
200 In total, 16, 749 peptides were identified and 1,018 had been resolved by IMS.

201 ## Co-IP and GeLC-MS/MS

202 Co-immunoprecipitation (Co-IP) experiments were performed using a rabbit anti-SPEG polyclonal Ab
203 (HPA018904; Sigma-Aldrich, Poole, Dorset, UK). Negative control Co-IP experiments were conducted using
204 rabbit anti-NDRG2 monoclonal Ab (Ab174850; Abcam plc) or by incubating samples with Protein-A
205 Dynabeads (Thermoscientific, Runcorn, UK) only. Protein A dynabeads were suspended in
206 phosphate-buffered saline with 0.05 % Tween-20 (PBS-T) and rotated for 30 min at room temperature with 1
207 µg of polyclonal antibody in 50 µl of PBS-T. The bead-antibody complex was washed five times in 50 µl of
208 PBS-T and incubated with 500 µg of muscle protein for 3 h at 4 °C on sample mixer. The bead-Ab-sample
209 complexes were washed 3 times in PBS-T, and proteins were extracted from the beads by two sequential
210 incubations in 5 µl of LDS sample buffer (NuPAGE; Thermo Scientific, Runcorn, UK) for 4 min each at 95 °C.
211 Samples were electrophoresed through 7 % Tris-Acetate pre-cast gels (NuPAGE; Thermo Scientific, Runcorn,
212 UK) and stained for 1 h with Colloidal Coomassie blue (BioRad, Deeside, UK). Each gel lane was cut into 7 x 5
213 mm segments and each segment was diced in to 1 mm^3 pieces and tryptic in-gel digestion was performed as
214 described previously (20). Each segment was processed separately in preparation for nanoscale
215 reverse-phase UPLC (NanoAcquity; Waters, Milford, MA) and online ESI QTOF MS/MS (Q-TOF Premier;
216 Waters, Manchester, UK). Peptides were desalted using C_{18} ZipTips (Millipore, Billercia, MA, USA) and loaded
217 by partial-loop injection on to a 180 µm ID x 2 cm long 100 Å, 5 µm BEH C_{18} Symmetry trap column (Waters,
218 Milford, MA) at flow rate of 5 µl/min for 3 min in 2.5 % (v/v) ACN, 0.1% (v/v) FA. Separation was conducted
219 at 35 °C via a 75 µm ID x 25 cm long 130 Å, 1.7 µm BEH C_{18} analytical reverse-phase column (Waters, Milford,
220 MA). Peptides were eluted using a linear gradient that rose to 37.5 % ACN 0.1% (v/v) FA over 90 min at a
221 flow rate of 300 nl/min. Eluted peptides were sprayed directly in to the MS via a NanoLock Spray source and
222 Picotip emitter (New Objective, Woburn, MA). Additionally, a LockMass reference (100 fmol/µl
223 Glu-1-fibrinopeptide B) was delivered to the NanoLock Spray source of the MS and was sampled at 240 s
224 intervals. For all measurements, the MS was operated in positive ESI mode at a resolution of 10,000 FWHM.
225 Before analysis, the TOF analyser was calibrated using fragment ions of [Glu-1]-fibrinopeptide B from m/z 50
226 to 1990. Peptide MS were recorded between 350 and 1600 m/z. Data-dependent MS/MS spectra were
227 collected over the range 50–2000 m/z. The 5 most abundant precursor ions of charge 2+ 3+ or 4+ were
228 selected for fragmentation using an elevated (20–40 eV) collision energy. A 30-s dynamic exclusion window
229 was used to avoid repeated selection of peptides for MS/MS.

230 MS/MS spectra were searched against the UniProt database restricted to Rattus (8,071 sequences) using
231 Mascot Distiller (www.matrixscience.com) and a locally implemented Mascot server (v.2.2.03;
232 www.matrixscience.com). Enzyme specificity was trypsin (allowing 1 missed cleavage), carbamidomethyl

modification of cysteine (fixed), deamidation of asparagine and glutamine (variable), oxidation of methionine (variable) and m/z errors of 0.3 Da.

Genetic association studies in human athletes

Genetic association studies were conducted in 384 Russian international-level athletes that have been reported in previous studies (e.g. (2, 42)). Participants (age 23.8 ± 3.4 y; 202 males and 182 females) included in the current cohort were of Caucasian Eastern European descent and were stratified into 2 groups. Group 1 (n = 258) included endurance athletes (3-10 km runners, biathletes, 5-10 km skaters, cross-country skiers, marathon runners, 0.8-25 km swimmers, rowers/kayakers, race walkers, 1.5-10 km speed skaters and triathletes). Of those, 35 female endurance athletes were also involved in the muscle biopsy study. Group 2 (n = 126) comprised power athletes (50-100 m swimmers, sprint cyclers, 100-400 m runners, 500-1000 m speed skaters and short-trackers, track and field jumpers, heptathletes / decathletes, and throwers). The study was approved by the Ethics Committee of the Physiological Section of the Russian National Committee for Biological Ethics and Ethics Committee of the Federal Research and Clinical Center of Physical-chemical Medicine of the Federal Medical and Biological Agency of Russia. Written informed consent was obtained from each participant. The study complied with the guidelines set out in the Declaration of Helsinki and ethical standards in sport and exercise science research.

Venous blood samples (4 ml) were collected in EDTA-coated tubes (Vacuette EDTA, Greiner Bio-One, Austria) and were transported to the laboratory at 4 °C. DNA was extracted from leukocytes on the same day using a commercial kit according to the manufacturer's instructions (Technoclon, Russia). DNA quality was assessed by agarose gel electrophoresis and HumanOmni1-Quad BeadChips (Illumina Inc, USA) were used for genotyping of 1,140,419 single nucleotide polymorphisms (SNPs). In addition, Human OmniExpress BeadChips (Illumina Inc, USA) were used for genotyping of > 700,000 SNPs in the 35 female athletes that also gave muscle samples for fiber-type analysis. The assay required 200 ng of DNA sample as input with a concentration of at least 50 ng/µl. Exact concentrations of DNA in each sample were measured using a Qubit Fluorometer (Invitrogen, USA). All further procedures were performed according to the instructions of Infinium HD Assay.

Evaluation of muscle fiber composition in human athletes

Samples of the vastus lateralis muscle of 35 female athletes were obtained with the Bergström needle biopsy procedure under local anaesthesia with 1 % lidocaine solution. Serial cross-sections (7 µm thick) were cut from frozen muscle samples using a microtome (Leica Microsystems, Wetzlar, Germany) and were thaw-mounted on Polysine glass slides. Sections were air-dried for 15 min at RT and then washed in 3 x 5 min incubations in PBS before being incubated (RT for 1 h) in PBS containing primary Ab against slow or fast isoforms of myosin heavy chain (M8421, 1:5000; M4276; 1:600, respectively; Sigma-Aldrich, USA). Muscle sections were washed for 3 x 5 min in PBS and then incubated (RT for 1 h) in PBS containing secondary Ab

conjugated with FITC (F0257; 1:100; Sigma-Aldrich). Sections were washed in PBS (3 × 5 min), placed in mounting media and covered with a cover slip prior to imaging using a fluorescent microscope (Eclipse Ti-U; Nikon, Japan). All analysed images contained >100 fibers and the ratio of the number of stained fibers to the total fiber number was calculated. Fibers stained in serial sections with antibodies against slow and fast isoforms were considered as hybrid fibers.

Statistical analysis

Data are presented as mean and standard deviation unless otherwise stated. Differences in protein abundance measured by LFQ of LRT and HRT samples (n = 5 in each group) were investigated by one-way analysis of variance, corrected using q-values (53) to a false discovery rate of 1 %. Functional enrichment testing was performed using the Database for Annotation, Visualisation and Integrated Discovery (DAVID; https://david.ncifcrf.gov).

Genetic variations in or near the human *SPEG* gene were investigated using the Genotype Tissue Expression (GTEx) database (17). Statistical analysis of genotype data was conducted using PLINK v1.90, R (version 3.4.3), and GraphPad InStat (GraphPad Software, Inc., USA) software. Genotype distribution and allele frequencies between athletes in Group 1 or Group 2 were compared using χ^2 tests. Quantitative trait (proportion of slow-twitch muscle fibers) SNP association was tested in a linear additive model. P values < 0.05 were considered statistically significant.

Results

LRT and HRT rats had a maximum running capacity (DIST1) of 852 ± 176 m and 642 ± 98 m, respectively, at ~11 weeks of age. After 8 weeks endurance training, the maximum running capacity (DIST2) of LRT (539 ± 107 m) had decreased by 49 % (ΔDIST -313 ± 144 m), whereas the maximum running capacity of HRT rats had increased by 44 % (ΔDIST +376 ± 111 m). The running capacity of the animals was retested at 17 months of age and there was no difference between LRT (114 ± 122 m) and HRT (173 ± 102 m) groups.

LFQ encompassed 1,024 proteins that were confidently (FDR <1 %) identified in each of the HRT and LRT samples (n = 5, per group). Protein identifications and normalised abundance data are available at https://doi.org/10.6084/m9.figshare.9995087. Differential analysis of proteins quantified using three or more peptides revealed the relative abundance of 76 proteins differed significantly ($P<0.05$, $q<0.01$) between HRT and LRT groups (Figure 1). Thirteen proteins were more abundant in HRT muscle (Table 1), whereas 63 proteins were more abundant in LRT (Table 2 and Figure 2a). There was significant enrichment of proteins associated with the KEGG metabolic pathways glycolysis and gluconeogenesis in LRT muscle and 12 enzymes involved in muscle glycogen/glucose metabolism (Figure 2b) were more abundant compared to HRT muscle. In contrast, there was no significant enrichment of gene ontology phrases or KEGG metabolic pathways amongst the 13 proteins that were more abundant in HRT muscle. The protein most enriched in gastrocnemius of HRT rats was striated muscle-specific serine/threonine-protein kinase beta (SPEGβ; also known as striated muscle preferentially expressed gene). Co-IP GeLC-MS/MS identified 24 potential binding partners of SPEGβ in HRT muscle (Table 3). There was no significant enrichment of gene ontology amongst the potential SPEGβ binding partners and we were unable to identify binding partners that were specific to either HRT or LRT.

Six SNPs (rs13386459, rs907683, rs72965313, rs4674396, rs745027, rs7564856) located in close proximity (i.e. in high linkage disequilibrium) to the *SPEG* gene were significantly ($P<5*10^{-8}$-10^{-13}) associated with *SPEG* gene expression in human skeletal muscle. Of those, the rs7564856 was available for genotyping in the athletic cohorts using micro-array analysis. According to GTEx data, the G allele of the rs7564856 SNP was reported to be associated ($P = 2.7*10^{-8}$) with increased expression of the *SPEG* gene and may be favourable for endurance sports. The G allele of rs7564856 was significantly greater (32.9 vs 23.8%; OR = 1.6, $P = 0.009$) in endurance athletes compared to power athletes (Figure 3). We also found the G allele was significantly ($\beta = 8.345$, $P = 0.0048$) associated with increased proportion of slow-twitch muscle fibers in female endurance athletes.

Discussion

We have used robust HDMS^E profiling to compare the abundance of more than 1,000 proteins in gastrocnemius of rats artificially selected (31) as high-responders (HRT) or low-responders (LRT) to endurance training. Stringent differential analysis (P values filtered to 1 % FDR) identified widespread differences that co-segregate with exercise responsiveness and highlighted potential targets for future mechanistic research. SPEGβ exhibited the greatest difference in abundance and was 2.64-fold greater in HRT muscle. SPEGβ has been highlighted in at least two (18, 43) earlier non-targeted proteomic analyses of acute muscle responses to exercise. In the current work, proteins that co-immunoprecipitated with SPEGβ in HRT muscle include novel targets involved in c-Jun N-terminal kinase (JNK) signalling, which has recently emerged as a regulator of the adaptive response of muscle to exercise training (32). In humans, the G allele of SNP rs7564856 near the SPEGβ locus was associated with differences in endurance performance and muscle fiber type. These findings provide new insight to the role of SPEGβ in muscle and heighten interest in SPEGβ as a target for mechanistic research in molecular exercise physiology.

Lessard et al (33) reports hyperactivation of JNK in sedentary LRT muscle and alterations to normal TGF-β signalling in response to endurance training. Follow-up analyses (32) found JNK phosphorylates the linker region of similar to mothers against decapentaplegic homolog 2 (SMAD2) and, thereby, inhibits canonical myostatin-TGFβ signalling. In human muscle, JNK phosphorylation of the SMAD2 linker region is particularly robust after resistance rather than endurance exercise (32). Phosphorylation of the SMAD2 linker region potentiates muscle adaptation to resistance exercise but activation of JNK in response to endurance exercise (i.e. as in LRT muscle) appears to be an inappropriate response and is associated with blunted adaptation to endurance training (33). The events upstream of exercise-induced phosphorylation of SMAD2 by JNK have not yet been elucidated. Interestingly, several of the proteins that co-immunoprecipitated with SPEGβ in HRT muscle (Table 3) are implicated in JNK and TGFβ signalling and could represent viable candidates to investigate differences in TGFβ-mediated responses to endurance exercise. Caveolin-1 (CAV1) co-immunoprecipitated with SPEGβ and a greater expression of CAV1 mRNA was previously highlighted by transcriptome profiling of HRT/LRT muscle (28). CAV1 inhibits TGFβ-mediated activation of SMAD2 by promoting degradation of TGF-β receptors (32). Similarly, ventricular zone-expressed PH domain-containing protein homolog 1 (VEPH1) may also interfere with TGFβ-targeted signalling by impeding nuclear accumulation of SMAD2 (49). Serine/threonine-protein kinase (TAOK3), which was initially named JNK inhibitory kinase (55), downregulates the activity of JNK (26) and, therefore, may be the most likely of the putative SPEGβ interacting partners to play a mechanistic role in modulating muscle adaption to endurance training.

Targeted investigations of SPEGβ binding partners in skeletal muscle report SPEGβ interacts with myotubularin and co-localises with other proteins of the junctional sarcoplasmic reticulum (SR), including the dihydropyridine receptor, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), and the Z-band protein,

desmin (1). Accordingly, skeletal muscles of SPEG knockout mice exhibit centronuclear myopathy (1) and deficits in force that are associated with impaired RyR1-meditated Ca^{2+} release from the SR (23). Similar protein-protein interactions also occur in cardiac muscle (46), where SPEGβ phosphorylates SERCA2a and promotes SERCA2a oligomerisation, which is associated with enhanced Ca^{2+} transport activity (45). In the current analysis, SERCA2 co-immunoprecipitated in control experiments as well as with SPEGβ, and so was removed from the list (Table 3) of putative SPEGβ interacting partners. However, dynamin 2 (DNM2) was amongst the putative interaction partners of SPEGβ. Overexpression of DNM2 increases resting $[Ca^{+2}]_i$ and is associated with impaired contractile properties and centronuclear myopathy (14). Valosin-containing protein (VSP, also known as transitional endoplasmic ATPase) belongs to the AAA+ ATPase family and immunoprecipitated with SPEGβ. VSP is required for lysosomal network dynamics, and upon its inhibition lysosomal network and autophagy are impaired (25).

Pathway analysis of proteins enriched in HRT muscle did not find significant enrichment of functional groups, but manual interrogation of Table 1 reveals the protein list is punctuated by features that are common to exercise-trained muscle. For example, HRT muscle has a greater abundance of the fatty acid translocase (CD36), mitochondrial superoxide dismutase (Mn SOD) and 10 kDa heat shock protein (CH10) that are also more abundant in diaphragm of exercise-trained rats (52). Consistent with these findings, gene expression of CD36 is also greater in HRT muscle (28). However, SPEG was not among the genes annotated on the array used in the previous (28) transcriptomic analysis. Notably, there was no overlap between proteins enriched in HRT muscle and those previously reported (9) to be enriched in the soleus proteome of HCR rats selected for high intrinsic running capacity. This is consistent with the difference in genetic heritability of training responsiveness vs intrinsic capacity in rats (31) and humans (6).

HRT muscle had greater abundance of proteins associated with store-operated Ca^{2+} entry (SOCE; (44)), including cGMP-gated cation channel alpha-1 (CNGA1), inositol monophosphatase (IMP), stromal interaction molecule 1 (STIM1) and 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma-1 (PLCG1). SOCE may assist in maintaining the myoplasmic pool of Ca^{2+} during repeated contractions and in doing so may modulate fatigue (41). STIM1 localises to the longitudinal SR and enables rapid activation (13) of SOCE. Muscles of STIM1-haploinsufficent animals have impaired refilling of internal Ca^{2+} stores when subjected to repeated stimulation, fatigue sooner and achieve smaller tetanic forces. In hepatocytes, PLCG1 can activate SOCE independently of STIM1 (36) but the role of PLCG1 in skeletal muscle has not yet been reported. CNGA1 is activated by SOCE (58) at physiological extracellular concentrations, which enables propagation of Ca^{2+} currents through CNGA1 (15). Maintenance of phosphatidylinositol 4,5-bisphosphate (PIP2) levels by IMP supports both SOCE and Ca^{2+} oscillating signals (3). Ca^{2+} signalling is indeed diversely involved in exercise-related adaptations. Oscillations in, and moderate rises to, $[Ca^{2+}]_i$ upregulate mitochondrial biogenesis (24) and ATP production (19), whereas a prolonged elevation in $[Ca^{2+}]_i$ is associated with muscle weakness and atrophy (38). Although speculative, exercise-related perturbations in $[Ca^{2+}]_i$ in LCR muscle may

result in prolonged increase in $[Ca^{2+}]_i$ culminating in negative effects that eventually lead to a low-responsiveness phenotype. Increased $[Ca^{2+}]_i$ results in oligomerisation and overexpression of VDAC1, which leads to the formation of apoptosomes and cell death (27). VDAC1 is 1.27-fold more abundant in LRT and the S100a1 Ca^{2+}-binding protein, which is enriched in slow twitch fibers (12), and was 1.4-fold more abundant in LRT. Accounting for a lesser percentage of slow-twitch fibers in LRT gastrocnemius makes the relative difference per fiber more pronounced.

LRT gastrocnemius exhibited pronounced enrichment of enzymes involved in glucose metabolic processes (Figure 2b), which may link with the previously (33) reported difference in myofiber profile between HRT and LRT. In the plantaris of LRT rats, the proportion of type I slow-twitch fibers (7 %) is similar to sedentary Wistar rats (50) but significantly less than in HRT muscle (20 %), whereas no difference in myosin heavy chain (MyHC) type IIa myofiber abundance is observed (33). The current proteomic analysis focused on soluble muscle proteins and it is not possible to report the myofiber profile of HRT/LRT gastrocnemius muscle using the current data. The 1.4-fold greater abundance of sarcoplasmic/endoplasmic reticulum ATPase-1 and parvalbumin in LRT muscle alongside the conspicuous enrichment of glycolytic enzymes may suggest a faster twitch myofiber profile in LRT compared to HRT gastrocnemius. However, some mitochondrial proteins were also more abundant in LRT compared to HRT muscle (Table 2).

A potential difference in myofiber profile between HRT/LRT gastrocnemius raises the question of whether differences in SPEGβ protein abundance may also be a consequence of a difference in MyHC profile. Drexler et al (12) reports comparative proteome analysis of mouse fast-twitch extensor digitorum longus (EDL) and slow-twitch soleus and reports SPEGβ amongst those proteins enriched in EDL. Murgia et al (39) reports deep proteome analysis of individual myofibers extracted from vastus lateralis of younger and older human adults. We extracted SPEGβ abundance data from Murgia et al (39) and investigated differences due to either MyHC fiber type or age using 2-way analysis of variance. The abundance of SPEGβ was not different ($P=0.8144$) between muscles of younger and older adults, but SPEGβ abundance was significantly ($P=0.0288$) different across different myofiber types. SPEGβ abundance was 65 % greater ($P=0.0129$) in MyHC IIx than type I fibers while fibers containing MyHC IIa were intermediate and SPEGβ abundance was 21 % greater ($P=0.7463$) in MyHc IIa containing fibers compared to MyHC type I (Tukey HSD post-hoc analysis). Therefore, potential differences in myofiber profile between HRT and LRT gastrocnemius seem to be an unlikely explanation for the greater abundance of SPEGβ in HRT muscle.

SPEGβ is serine/threonine protein kinase homologous to proteins of the myosin light chain kinase family (54). In C2C12 myoblasts, expression of the alpha isoform of SPEG co-occurs with myoblast differentiation and the emergence of myosin heavy chain expression (21). Whereas, SPEGβ is solely detected in adult muscle *in vivo* (21), suggesting expression of the beta isoform may be instigated during postnatal maturation in response to contractile activity. SPEGβ is a phosphoprotein and we (18) reported greater phosphorylation of SPEGβ in rat heart 0-3 h after an incremental exercise test to VO_2max. Similarly, Potts et al (43) reports phosphorylation

of SPEGβ occurs in mouse skeletal muscle 1 h after a protocol of maximal-intensity contractions. In the current work, the abundance of SPEGβ is greater (2.64-fold, P=0.0014) in gastrocnemius of rats that exhibit high-responsiveness to endurance training. Although we did not investigate the phosphorylation status of SPEGβ in HRT/LRT gastrocnemius, the evidence to date suggests SPEGβ functions as a mechanosensitive kinase in striated muscle. In the future, it would be interesting to investigate whether either SPEGβ abundance or phosphorylation status changes in response to long-term endurance training. We were unable to find data reporting SPEGβ abundance in trained versus untrained muscle, but we did identify that the frequency of the G variant of SNP rs7564856 (that increases the expression of the SPEG gene) is greater in endurance compared to power athletes. The G allele of SNP rs7564856 was also significantly associated with increased proportion of slow-twitch muscle fibers in female endurance athletes. In addition, in 130,000 UK Biobank participants, rs7564856 is associated ($P=1.63*10^{-11}$) with the pulmonary ratio of forced expiratory volume in the first one second (FEV1)/ forced vital capacity (FVC) (29), which is a surrogate indicator of VO_2max (11).

In conclusion, artificial selection for high responsiveness to endurance training is associated with a greater abundance of SPEGβ in rat gastrocnemius. Co-immunoprecipitation of SPEGβ in HRT muscle discovered putative interacting proteins that may link with previously reported differences in TGFβ signalling in exercised muscle. In humans, genetic polymorphisms near the *SPEG* gene locus are associated with higher expression of SPEGβ, which may favour endurance phenotypes. These findings alongside recent reports of acute phosphorylation of SPEGβ in cardiac (18) and skeletal muscle (43) in response to acute exercise support the hypothesis that SPEGβ is an important component in the adaptational response of muscle to exercise and warrants further mechanistic study.

Acknowledgements

The exercise rat models are funded by the Office of Research Infrastructure Programs grant P40OD021331 from the National Institutes of Health. The rat models for low and high exercise response to training are maintained as an international resource with support from the Department of Physiology & Pharmacology, University of Toledo College of Medicine, Toledo, OH. Contact LGK Lauren.Koch2@UToledo.Edu or SLB brittons@umich.edu for information on the exercise rat models. The Russian study was supported in part by grant from the Russian Science Foundation (Grant No. 17-15-01436: "Comprehensive analysis of the contribution of genetic, epigenetic and environmental factors in the individual variability of the composition of human muscle fibers"; DNA sample collection, genotyping and determination of muscle fiber composition of Russian subjects).

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Figure 1 – Differential analysis of $HDMS^E$ data

Volcano plot presenting the $\log_2$ fold-difference in abundance between HRT versus LRT gastrocnemius and the statistical significance determined by one-way ANOVA (n=5, per group). Proteins that were statistically different (P<0.05) and had a false discovery rate <1 % are highlighted in red. Proteins that exhibit a >1-fold difference in abundance are annotated by UniProt protein identifiers.

Figure 2 – Enzymes of the glycolytic pathway are more abundant in LRT gastrocnemius

(A) Heat map of 76 proteins that differed significantly (P<0.05, q<0.01) in abundance between HRT and LRT gastrocnemius (n = 5, per group). LRT muscle exhibited significant enrichment of proteins associated with the KEGG metabolic pathways glycolysis and gluconeogenesis. (B) Twelve enzymes involved in glycogen/glucose metabolism were more abundant in LRT muscle.

Figure 3 – SPEG rs7564856 G allele frequency is greater in international-level endurance athletes

Genetic association studies in Russian international-level athletes of Caucasian Eastern European descent, stratified into endurance athletes (n = 258) and power athletes (n=126). The frequency of the G allele of rs7564856 reported to be associated with increased expression of the *SPEG* gene was significantly (P = 0.009) greater in endurance compared to power athletes.

Table 1 – Proteins more abundant in HRT gastrocnemius

Description	Accession	Score	Peptides	Delta	P value
Striated muscle-specific serine/threonine-protein kinase	Q63638	109	18 (4)	2.64	1.44E-03
Nuclear autoantigenic sperm protein	Q66HD3	44	7 (3)	2.35	2.1E-04
1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma-1	P10686	57	9 (4)	1.72	1.3E-04
ATP synthase protein 8	P11608	24	4 (3)	1.67	4.85E-09
cGMP-gated cation channel alpha-1	Q62927	83	11 (3)	1.53	1.81E-03
Stromal interaction molecule 1	P84903	38	6 (4)	1.42	7.78E-06
Ras-related protein Rab-35	Q5U316	106	14 (4)	1.4	5.3E-04
Alcohol dehydrogenase [NADP+]	P51635	80	13 (6)	1.28	1.1E-04
10 kDa heat shock protein, mitochondrial	P26772	65	10 (7)	1.27	9.1E-04
Inositol monophosphatase	P97697	123	18 (10)	1.26	1.9E-04
Platelet glycoprotein 4	Q07969	28	5 (4)	1.25	7.7E-04
Superoxide dismutase [Mn], mitochondrial	P07895	51	10 (6)	1.21	4.8E-04
Cytochrome c oxidase subunit 6C-2	P11951	69	11 (5)	1.15	9.4E-04

Protein description and Accession relate to the Swiss-Prot database entry identified from MSe searches performed in TransOmics via GLPS. Delta is the fold difference relative to LRT. Values are reported for proteins quantified using 3 or more peptides and exhibiting significant ($P<0.05$) differences in abundance at a false discovery rate of < 1%.

Table 2 – Proteins more abundant in LRT gastrocnemius

Description	Accession	Score	Peptides	Delta	P value
Membrane/ cytoskeletal/ vesicle/ microtubule					
Ras-related protein Rab-27B	Q99P74	38	6 (3)	1.96	1.25E-04
Ras-related protein Rab-9A	Q99P75	43	6 (6)	1.72	2.08E-04
Tripartite motif-containing protein 72	A0JPQ4	261	28 (22)	1.56	3.37E-05
Signal-induced proliferation-associated 1-like protein 1	O35412	327	52 (21)	1.41	1.24E-04
Growth arrest-specific protein 8	Q499U4	51	8 (6)	1.38	9.09E-09
Myosin-Ie	Q63356	96	16 (6)	1.37	8.65E-07
Potassium voltage-gated channel subfamily A member 5	P19024	65	12 (7)	1.25	1.03E-05
Protocadherin Fat 3	Q8R508	279	52 (17)	1.23	1.84E-04
Annexin A4	P55260	169	25 (14)	1.16	5.92E-05
Cofilin-1	P45592	104	15 (8)	1.15	6.96E-04
Annexin A6	P48037	523	67 (44)	1.12	2.36E-04
Dynein heavy chain 12, axonemal	Q923J6	797	132 (57)	1.11	6.2E-05

Microtubule-associated protein 1B	P15205	188	30 (15)	1.11	6.94E-04
Glucose metabolic processes					
Glyceraldehyde-3-phosphate dehydrogenase	P04797	473	62 (34)	1.77	1.58E-03
L-lactate dehydrogenase A chain	P04642	450	78 (40)	1.47	1.56E-06
Fatty acid-binding protein, epidermal	P55053	34	4 (3)	1.38	1.09E-03
Glucose-6-phosphate isomerase	Q6P6V0	485	65 (46)	1.37	5.45E-08
Glycogen phosphorylase, brain form (Fragment)	P53534	631	83 (23)	1.34	7.83E-05
6-phosphofructokinase, muscle type	P47858	477	63 (35)	1.31	1.14E-03
Glycogen phosphorylase, muscle form	P09812	1275	188 (104)	1.28	3.39E-06
Fructose-bisphosphate aldolase A	P05065	554	76 (43)	1.28	1.02E-05
Beta-enolase	P15429	687	101 (26)	1.27	7.6E-04
Glycogen [starch] synthase, muscle	A2RRU1	284	41 (28)	1.26	2.7E-05
Triosephosphate isomerase	P48500	368	58 (43)	1.25	1.13E-05
Phosphoglycerate kinase 1	P16617	512	79 (54)	1.25	4.55E-05
Aldose reductase	P07943	209	30 (22)	1.24	5.43E-05
Phosphoglycerate mutase 2	P16290	290	40 (15)	1.24	8.11E-05

Mitochondrion					
Acyl-CoA synthetase family member 2, mitochondrial	Q499N5	36	7 (5)	1.41	1.69E-04
Trans-2,3-enoyl-CoA reductase	Q64232	41	5 (3)	1.37	1.35E-03
NADP-dependent malic enzyme	P13697	70	11 (7)	1.3	1.59E-03
Citrate synthase, mitochondrial	Q8VHF5	213	25 (19)	1.3	1.49E-03
Voltage-dependent anion-selective channel protein 1	Q9Z2L0	293	35 (28)	1.27	6.57E-05
ADP/ATP translocase 1	Q05962	275	35 (8)	1.21	7.6E-05
Phosphatidylethanolamine-binding protein 1	P31044	106	11 (9)	1.2	1.23E-03
NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	Q66HF1	378	47 (28)	1.15	1.52E-03
Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	P08503	153	23 (14)	1.1	6.77E-04
Calcium handling					
Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	Q64578	1060	176 (64)	1.41	1.03E-07
Protein S100-A1	P35467	37	4 (3)	1.4	2.53E-05
Parvalbumin alpha	P02625	272	45 (31)	1.39	5.71E-06
Chaperones/ protein folding					
UDP-glucose:glycoprotein glucosyltransferase 1	Q9JLA3	138	24 (7)	1.7	1.6E-06

Alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase A	Q08834	52	9 (3)	1.35	4.46E-04
Heat shock protein HSP 90-alpha	P82995	296	45 (16	1.31	1.45E-05
Heat shock cognate 71 kDa protein	P63018	486	52 (17)	1.28	1.56E-04
T-complex protein 1 subunit beta	Q5XIM9	64	9 (7)	1.26	1.13E-03
Peptidyl-prolyl cis-trans isomerase A	P10111	123	13 (10)	1.12	2.12E-04
Signal transduction					
RAC-beta serine/threonine-protein kinase	P47197	110	20 (9)	1.39	4.53E-04
Calcium/calmodulin-dependent protein kinase type II beta chain	P08413	63	10 (4)	1.29	1.08E-04
14-3-3 protein epsilon	P62260	202	25 (15)	1.14	2.42E-04
Cell Stress					
Glutathione S-transferase Mu 2	P08010	337	37 (12)	1.5	3.08E-06
Macrophage migration inhibitory factor	P30904	36	7 (7)	1.17	2.16E-04
Dual oxidase 1	Q8CIY2	72	14 (4)	1.15	6.68E-05
Skeletal muscle-specific					
Carbonic anhydrase 3	P14141	249	43 (31)	1.26	1.43E-03
Creatine kinase M-type	P00564	549	94 (58)	1.21	5.66E-04

Four and a half LIM domains protein 1	Q9WUH4	229	30 (23)	1.19	1.68E-03
Post-transcriptional processing					
Protein mago nashi homolog	Q27W02	67	9 (3)	1.42	1.73E-03
Serine/threonine-protein kinase PRP4 homolog	Q5RKH1	64	12 (6)	1.29	1.87E-03
Protein turnover (ribosome/ proteasome)					
26S protease regulatory subunit 4	P62193	98	15 (6)	1.22	6.32E-05
NSFL1 cofactor p47	O35987	126	19 (12)	1.2	1.72E-03
Elongation factor 2	P05197	376	52 (34)	1.13	3.82E-04
Lipoprotein					
Apolipoprotein A-I	P04639	82	12 (7)	1.22	5.22E-07
Hemopexin	P20059	262	35 (27)	1.14	6.22E-04
Uncharacterised					
Coiled-coil domain-containing protein 146	Q66H60	110	19 (7)	1.39	5.73E-05
Coiled-coil domain-containing protein 67	Q5U3Z6	132	26 (10)	1.35	1.03E-03

Protein description and Accession relate to the Swiss-Prot database entry identified from MSe searches performed in Progenesis via GLPS. Fold difference relative to HRT. Values are reported for protein quantified using 3 or more peptides and exhibiting significant differences in abundance at a false discovery rate of < 1%.

Table 3 – Putative protein-interaction partners of SPEGβ in skeletal muscle

Accession	Description; protein identifier	MOWSE score	Sequence coverage (%)
C0HL12	Adhesion G protein-coupled receptor B1; AGRB1	29	3.1
Q5U2S6	Ankyrin repeat and SOCS box protein 2; ASB2	35	3.8
P70673	ATP-sensitive inward rectifier potassium channel 11; KCJ11	68	5.9
Q3KR97	Brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 1; BI2L1	31	4.5
P41350	Caveolin-1; CAV1	64	32.6
P01026	Complement C3; CO3	35	4.4
Q01205	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial; ODO2	51	7.3
P39052	Dynamin-2; DYN2	36	4.5
M0R8U1	Dynein heavy chain 5, axonemal; DYH5	30	2.5
Q5U4E6	Golgin subfamily A member 4; GOGA4	40	4.6
P97636	Interleukin-18; IL18	29	13.4
O35790	N-acetylglucosaminyl-phosphatidylinositol de-N-acetylase; PIGL	34	5.2

P51839	Olfactory guanylyl cyclase GC-D; GUC2D	31	0.8
Q8K4M9	Oxysterol-binding protein-related protein 1; OSBL1	30	3.5
Q505J8	Phenylalanine--tRNA ligase alpha subunit; SYFA	29	2.2
P33568	Retinoblastoma-associated protein; RB	34	1.4
Q53UA7	Serine/threonine-protein kinase TAO3; TAOK3	137	8.1
P02770	Serum albumin; ALBU	46	5.6
A4ZYQ5	Solute carrier family 2, facilitated glucose transporter member 7; GTR7	28	2.1
Q63638*	Striated muscle-specific serine/threonine-protein kinase; SPEG	68	1.1
P46462	Transitional endoplasmic reticulum ATPase; TERA	116	18.7
Q6AY56	Tubulin alpha-8 chain; TBA8	31	11.1
Q5PQS3	Ventricular zone-expressed PH domain-containing protein homolog 1; MELT	32	3.1
Q8K3Y6	Zinc finger CCCH-type antiviral protein 1; ZCCHV	30	4