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1 Running Head: Human Muscle Proteome Responses to a High Fat Diet and Exercise

2

3 **Dynamic Proteome Profiling of Individual Proteins in Human Skeletal Muscle**
4 **Following A High Fat Diet & Resistance Exercise**

5

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8

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14

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17

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35 **Abbreviations:**

36 4E-BP1, Eukaryotic translation initiation factor 4E-binding protein 1; ACTS, Actin Alpha 1;
37 ALBU, Albumin; AMPK, 5' AMP-activated protein kinase; ANKR2, ankyrin repeat domain
38 protein; CAH3, carbonic anhydrase 3; CRYAB, alpha B-crystallin; D₂O, Deuterium oxide;
39 DPP, Dynamic Proteome Profiling; FAT/CD36, fatty acid translocase; FFA, Free fatty acids;
40 FFM, Fat free mass; FLNC, Filamin-C; GPR56, G protein-coupled receptor 56; HFLC, High
41 Fat Low Carbohydrate; HSPB1, heat shock protein; IL-6, Interleukin-6; IQR, inter-quartile
42 range; MAFbx, Muscle Atrophy F-box; MyHC, myosin heavy chain; MPE, molar per-cent
43 enrichment; MuRF1, Muscle-specific RING Finger protein-1; mTOR, mechanistic target of
44 rapamycin; OGTT, Oral Glucose Tolerance Test; ODPB, Pyruvate dehydrogenase E1
45 component subunit beta; PAI-1, Plasminogen activator inhibitor type 1; PDK4, Pyruvate
46 dehydrogenase lipoamide kinase isozyme 4; PEBP1, Phosphatidylethanolamine-binding
47 protein 1; PGC-1 α , Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
48 PRDX6, Peroxiredoxin-6; PROF1, Profilin-1; REX, Resistance Exercise; RKIP, RAF kinase
49 inhibitor protein; TNNC2, Troponin C; TNF- α , Tissue Necrosis Factor- α ; TRFE, Transferrin

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69 **Abstract**

70 It is generally accepted that muscle adaptation to resistance exercise training is underpinned
71 by contraction-induced increased rates of protein synthesis and dietary protein availability.
72 Utilising Dynamic Proteome Profiling (DPP), we investigated the contributions of both
73 synthesis and breakdown to changes in abundance on a protein-by-protein basis in human
74 skeletal muscle. Age-matched, overweight males consumed nine days of a high-fat, low-
75 carbohydrate (HFLC) diet during which time they either undertook three sessions of
76 resistance exercise (REX) or performed no exercise. Precursor enrichment and the rate of
77 incorporation of deuterium oxide (D₂O) into newly synthesised muscle proteins were
78 determined by mass spectrometry. Ninety proteins were included in the DPP with 28 proteins
79 exhibiting significant responses to REX. The most common pattern of response was an
80 increase in turnover, followed by an increase in abundance with no detectable increase in
81 protein synthesis. We provide novel evidence demonstrating that the contribution of synthesis
82 and breakdown to changes in protein abundance induced by REX differ on a protein-by-
83 protein basis. We also highlight the importance of the degradation of individual muscle
84 proteins following exercise in human skeletal muscle.

85

86 Word Count: 181

87

88 Key words: Muscle protein synthesis, protein degradation, proteome,

89

90 Skeletal muscle displays remarkable plasticity with the capacity to alter its phenotype in
91 response to contractile activity and nutrient availability (1, 2). Resistance exercise (REX)
92 increases muscle size and contractile strength and is an important intervention to prevent the
93 muscle loss associated with disuse, ageing or disease-related cachexia (2). Exercise-induced
94 gains in muscle mass are commonly attributed to a greater protein synthetic response. Indeed,
95 a single bout of REX increases muscle protein fractional synthetic rate ~20% above basal
96 levels (3-5), a response further augmented with amino acid ingestion (6-8).

97 In humans the synthesis and degradation of muscle proteins *in vivo* has been extensively
98 investigated via metabolic labelling experiments using sterile intra-venous infusions of stable
99 isotopes such as [²H₅]-phenylalanine (5, 9). However, the acute elevations in muscle protein
100 synthesis estimated by these techniques do not always correlate with muscle hypertrophy
101 responses induced by chronic REX (10, 11). Additionally, the acute nature of amino acid
102 tracer methods (i.e. time course of < 12 hours) means changes in protein turnover during
103 fasted (sleeping) periods and over several days are not captured. Metabolic labelling with
104 deuterium oxide (D₂O) offers an elegant solution to investigate the chronic effects of
105 exercise-nutrient interactions on rates of muscle protein synthesis and degradation because it
106 can be administered via drinking water to free-living humans over prolonged periods (weeks
107 to months) allowing measurement under ‘real world’ conditions of habitual diet and activity
108 patterns (12). Using these techniques, Brook and colleagues recently reported an 18%
109 increase in the synthesis rate of mixed myofibrillar proteins during the first 3 wk of a REX
110 intervention that accounted for most of the observed increase in muscle mass (13). However,
111 that study did not measure individual protein responses to exercise, so the relationship
112 between an average increase in synthesis across mixed myofibrillar proteins and selective
113 changes in myofibrillar protein abundance was not clear. A greater understanding of protein
114 specific responses, including rates of synthesis, degradation and abundance, is important
115 given that suppression of the rate of renewal of some proteins can result in aggregation, gain-
116 of-function toxicity and a loss of proteome quality (proteostasis), a hallmark of ageing (14).

117 In a comprehensive study of muscle protein responses to exercise, we combine state-of-the-
118 art measurements of protein synthesis rates with well-established label-free profiling methods
119 (15-17) that are considered the “gold-standard” for measuring relative changes in protein
120 abundance in humans. For the first time, we report the anabolic effects of REX on the
121 synthesis, breakdown and abundance of individual muscle proteins in human skeletal muscle.

122 Specifically, we investigated whether a short-term high-fat, low carbohydrate (HFLC) diet
123 impairs rates of muscle protein synthesis, degradation and abundance, as such diets are often
124 promoted for rapid weight loss. However, a common shortcoming of HFLC diets is that they
125 fail to confer protection against the debilitating loss of skeletal muscle mass (18) and
126 therefore provide a suitable model to measure synthesis and degradation rates of individual
127 muscle proteins.

128 **Materials and Methods**

129 *Subjects* Sixteen sedentary, untrained, otherwise healthy male subjects were recruited for this
130 study (Table 1). Participants were provided with oral and written information about the
131 purpose, nature and potential risks involved with the study, and written informed consent was
132 obtained prior to participation. The study was approved by the Australian Catholic University
133 Human Research Ethics Committee and conformed with the policy statement regarding the
134 use of human subjects in the latest revision of the Declaration of Helsinki. The trial was
135 registered with the Australian New Zealand Clinical Trials Registry (ACTRN 369316). The
136 study employed a between-subjects design where subjects were pair-matched for fat free
137 mass and strength and allocated to either an exercise ($n = 8$, REX) or non-exercise ($n = 8$,
138 HFLC) group for the experimental trial. Previous studies measuring individual muscle
139 proteome responses in humans have been performed in only 3-6 participants (19, 20).
140 Moreover, we did not allow any missing values in our proteomic analyses (proteins that could
141 not be measured in all 16 participants at all time points were excluded).

142
143 *Preliminary Testing and Control Diet:* Preliminary Testing VO_{2peak} and Maximum Strength
144 were determined two-weeks prior to the commencement of experimental trials (described
145 subsequently). VO_{2peak} was determined during an incremental test to volitional fatigue on a
146 Lode cycle ergometer (Groningen, The Netherlands) (21). Quadriceps strength was
147 determined during a series of single repetitions on a pin-loaded leg extension (Cybex,
148 Massachusetts, USA) and leg press (Synergy Leg Press, Queensland, Australia) machine until
149 the maximum load lifted was established (1 RM) (6). Body composition (fat mass and fat free
150 mass (FFM)) was measured using whole-body dual-energy x-ray absorptiometry (DXA)
151 scans (GE Lunar iDXA Pro, enCORE software Version 16) under standardized conditions.
152 VO_{2peak} , strength and body composition were determined two weeks prior to experimental
153 trials during which time subjects maintained their habitual diet and physical activity patterns.
154 Following preliminary testing, participants were instructed to refrain from exercise training,

155 vigorous physical activity, alcohol and caffeine consumption for the 72 h before commencing
156 the 9-day high fat diet and exercise experimental period (described subsequently). During this
157 time, participants were also provided with standardized prepacked meals for breakfast, lunch,
158 dinner and snacks that provided an energy availability of 45 kcal·kg fat-free mass (FFM)
159 $^{-1}\cdot\text{day}^{-1}$ consisting of 6.1 g carbohydrate/kg FFM (55% caloric intake), 1.7 g protein/ kg FFM
160 (15%) and 1.5 g fat/ kg FFM (30%) (Figure 1).

161

162 *High Fat Low Carbohydrate (HFLC) Diet.* On the next morning following the diet and
163 exercise control period, participants reported to the laboratory after a ~10-h overnight fast.
164 After resting in the supine position for ~15 min, a catheter was inserted into the antecubital
165 vein of one arm and a baseline blood sample (~5 mL) was taken. Under local anaesthesia (2–
166 3 mL of 1% Xylocaine) a resting biopsy (Day 1) was obtained from the *vastus lateralis* using
167 a 5-mm Bergstrom needle modified with suction. An oral glucose tolerance test (OGTT) was
168 then conducted using a 75 g 300 mL glucose solution with blood samples obtained every 30
169 min up to 2 h. Participants were then provided their HFLC diet to be consumed as their only
170 caloric intake for the next 9 days consisting of 0.8g carbohydrate kg/ FFM (8% total caloric
171 intake), 1.7 g protein/ kg FFM (15%) and 3.9 g fat/ kg FFM (77%) (Figure 1). This diet
172 followed guidelines previously reported (22). Total energy intake was based on an energy
173 availability of 45 kcal·kg FFM $^{-1}\cdot\text{day}^{-1}$ to ensure participants were in energy balance, and
174 meal plans were created using Foodworks 7.0 ® Xyris Software (Melbourne, Australia).
175 Menu construction and the preparation of meals and snacks were undertaken by food service
176 dietitians and sports dietitians (Dineamic, Camberwell, Victoria). Meal plans were
177 individually developed for each participant to integrate individual food preferences and BM.
178 All meals and snacks were supplied to subjects. Participants were required to keep a food
179 checklist to note their compliance to the dietary instructions and their intake of any additional
180 food or drinks. Every 2 days, participants met with a dietician to receive new food parcels
181 and check their adherence to the previous days' diet. Additional muscle biopsies were
182 obtained on Day 3 and 6 of the HFLC diet. Each muscle biopsy was taken under fasted
183 conditions from a separate site 2-3 cm distal from the same leg and moving in a proximal
184 direction with successive biopsies with all samples stored at -80°C until subsequent analysis.
185 Blood samples were obtained every morning in EDTA tubes via venepuncture from the
186 antecubital vein on alternate arms. All blood samples were immediately centrifuged at 1,000
187 g at 4°C for 15 min, with aliquots of plasma frozen in liquid N₂ and stored at -80°C. The

188 morning following the HFLC diet, an additional muscle biopsy was obtained and participants
189 then underwent another OGTT as previously described.

190

191 *Deuterium Labelling Protocol* Deuterium labelling of newly synthesized proteins was
192 achieved by oral consumption of D₂O (Sigma Aldrich, Castle Hill, Australia) based on
193 previous work (19). To achieve an appropriate target enrichment of 1–2%, participant's
194 consumed 50 ml of 99.8 atom % D₂O four times a day for a total of 200 mL per day for all
195 days of the HFLC intervention commencing after the first muscle biopsy on Day 1. All 50
196 mL doses were provided in individually sealed bottles purchased from Sigma Aldrich and
197 participants were instructed to consume each dose at least 3-4 h apart.

198

199 *Resistance Exercise* Participants in the REX group performed a resistance exercise session on
200 Day 1, 4 and 7 of the HFLC diet. The resistance exercise session consisted of 4 X 8-10
201 repetitions of leg press at 80% 1-RM, 4 X 8-10 repetitions of leg extension at 80% 1-RM, and
202 4 sets of dumbbell squats. Each set was separated by a 3-min recovery period during which
203 time participants rested.

204

205 *Gas chromatography-mass spectrometry* Body water enrichment of D₂O was measured in
206 plasma samples against external standards constructed by adding D₂O to phosphate buffered
207 saline over the range from 0.0 to 3.0 % in 0.5 % increments. The D₂O enrichment of aqueous
208 solutions was determined after exchange with acetone (23). Samples were centrifuged at
209 12,000 g, 4 °C for 10 min and 20 µL of plasma supernatant or standard was reacted overnight
210 at room temperature with 2 µL of 10 N NaOH and 4 µL of 5 % (v/v) acetone in acetonitrile.
211 Acetone was then extracted in to 500 µL chloroform and water was captured in 0.5 g Na₂SO₄
212 prior to transferring a 200 µL aliquot of chloroform to an auto-sampler vial. Samples and
213 standards were analysed in triplicate using an Agilent 5973N mass selective detector coupled
214 to an Agilent 6890 gas chromatography system. A CD624-GC column (30 m x 0.25 mm x
215 1.40 µ m) was used in all analyses. Samples (1 µL) were injected using an Agilent 7683 auto
216 sampler. The temperature program began at 50 °C and increased by 30 °C/min to 150 °C, and
217 was held for 1 min. The split ratio was 50:1 with a helium flow of 1.5 mL/min. Acetone
218 eluted at approximately 3.0 min. The mass spectrometer was operated in the electron impact
219 mode (70 eV) and selective ion monitoring of m/z 58 and 59 was performed using a dwell
220 time of 10 ms/ion.

221

222 *Muscle processing* Muscle samples (~60 mg) were pulverised in liquid nitrogen then
223 homogenised on ice in 10 volumes of 1% Triton X-100, 50 mM Tris pH 7.4 containing
224 Complete™ protease inhibitor (Roche Diagnostics, Lewes, UK) using a PolyTron
225 homogeniser. Samples were incubated on ice for 15 min then centrifuged at 1,000 g, 4°C for
226 5 min. Supernatants containing soluble/ sarcoplasmic proteins were decanted and stored on
227 ice while the myofibrillar pellet was resuspended in 0.5 ml of homogenisation buffer and then
228 centrifuged at 1,000 g, 4°C for 5 min. The washed myofibrillar pellet was then solubilised in
229 0.5 ml of 7 M urea, 2 M thiourea, 4 % CHAPS, 30 mM Tris pH 8.5 and cleared by
230 centrifugation at 12,000 g, 4°C for 45 min. The protein concentrations of both the
231 myofibrillar fraction and sarcoplasmic fraction were measured using the Bradford assay
232 (Sigma, Poole, Dorset, UK). Aliquots containing 100 µg protein were precipitated in 5
233 volumes of acetone for 1 hour at -20°C. Pellets were resuspended in 0.1% (w/v) Rapigest SF
234 (Waters; Milford, MA, USA) in 50 mM ammonium bicarbonate and incubated at 80°C for 15
235 min. Samples were washed with 0.1% (w/v) Rapigest SF (Waters; Milford, MA, USA) in 50
236 mM ammonium bicarbonate using spin columns with 5 kDa molecular weight filters and
237 adjusted to a final volume of 100 µL. DTT was added (final concentration 1 mM) and
238 incubated at 60°C for 30 min followed by incubation (30 min) while being protected from
239 light in the presence of 5 mM iodoacetamide at 4°C. Sequencing grade trypsin (Promega;
240 Madison, WI, USA) was added at a protein ratio of 1:50 and digestion allowed to proceed at
241 37°C overnight. Digestion was terminated by the addition of 2 µL concentrated TFA and
242 peptide solutions were cleared by centrifugation at 15,000 g for 15 min.

243

244 *Liquid chromatography-mass spectrometry* Label-free liquid chromatography-mass
245 spectrometry (LC-MS) analysis was performed using nanoscale reversed-phase ultra-
246 performance liquid chromatography (nanoACQUITY, Waters, Milford, MA) and online
247 electrospray ionisation (ESI) quadrupole - time of flight mass spectrometry (Q-TOF Premier,
248 Waters, Manchester, UK). Samples (400 ng tryptic peptides) were loaded in aqueous 0.1%
249 (v/v) formic acid via a Symmetry C18 5 µm, 2 cm x 180 µm trap column (Waters, Milford,
250 MA). Separation was conducted at 35 °C through a BEH C18 1.7 µm, 25 cm x 75 µm
251 analytical reverse phase column (Waters, Milford, MA). Peptides were eluted using a
252 gradient rising to 37 % acetonitrile 0.1% (v/v) formic acid over 90 min at a flow rate of 300
253 nL/min. For all measurements, the mass spectrometer was operated in a data-dependent
254 positive ESI mode at a resolution of >10,000 FWHM. Prior to analysis, the time of flight
255 analyser was calibrated using fragment ions of [Glu-1]-fibrinopeptide B from m/z 50 to 1990.

256 Mass spectra for LC-MS profiling were recorded between 350 m/z and 1600 m/z using MS
257 survey scans of 0.45 s duration with an inter-scan delay of 0.05 s. In addition, equivalent
258 data-dependent tandem mass spectrometry (MS/MS) spectra were collected from each D0
259 (control) sample. MS/MS spectra of collision-induced dissociation fragment ions were
260 recorded for the 5 most abundant precursor's ions of charge 2+ or 3+ detected in the survey
261 scan. Precursor fragmentation was achieved by collision induced dissociation (CID) at an
262 elevated (20-40 eV) collision energy over a duration of 0.15 s per parent ion with an inter-
263 scan delay of 0.05 s over 50-2000 m/z. Acquisition was switched from MS to MS/MS mode
264 when the base peak intensity (BPI) exceeded a threshold of 750 counts per second, and
265 returned to the MS mode when the TIC in the MS/MS channel exceeded 50000 counts/s or
266 when 1.0 s (5 scans) were acquired. To avoid repeated selection of peptides for MS/MS the
267 program used a 30 s dynamic exclusion window.

268

269 *Progenesis protein profiling* Progenesis QI for proteomics (QI-P; Nonlinear Dynamics,
270 Newcastle, UK) was used to perform label-free quantitation consistent with our previous
271 work (15-17). Prominent ion features (>600 per chromatogram) were used as vectors to warp
272 each dataset to a common reference chromatogram. An analysis window of 15 min - 105 min
273 and 350 m/z - 1500 m/z was selected, which encompassed a total of 7,018 features with
274 charge states of +2 or +3. Log transformed MS data were normalised by inter-sample
275 abundance ratio and differences in relative protein abundance were investigated using non-
276 conflicting peptides only. MS/MS spectra (48, 601 queries) were exported in Mascot generic
277 format and searched against the Swiss-Prot database (2016.7) restricted to 'Homo Sapiens'
278 (20,272 sequences) using a locally implemented Mascot (www.matrixscience.com) server
279 (version 2.2.03). The enzyme specificity was trypsin allowing 1 missed cleavage,
280 carbamidomethyl modification of cysteine (fixed), deamination of asparagine and glutamine
281 (variable), oxidation of methionine (variable) and an m/z error of ± 0.3 Da. The Mascot
282 output (xml format), restricted to non-homologous protein identifications was recombined
283 with MS profile data and peptides modified by deamination or oxidation were removed prior
284 to quantitative analysis.

285

286 *Measurement of protein synthesis rates* Protein synthesis rates were calculated using mass
287 isotopomer distribution analysis consistent with previous work (19). Mass isotopomer
288 abundance data were extracted from MS only spectra using Progenesis Quantitative
289 Informatics (QI; Nonlinear Dynamics, Newcastle, UK). Peak picking was performed on ion

290 features with +1, +2 or +3 charge states within an analysis window of 15 min - 105 min and
291 350 m/z - 1500 m/z. The abundance of M0-M3 mass isotopomers were collected over the
292 entire chromatographic peak for each non-conflicting peptide that was used for label-free
293 quantitation in the aforementioned Progenesis QI-P analysis. Mass isotopomer information
294 was exported from Progenesis QI and processed in R version 3.3.1 (24) according to
295 published methods (19). Briefly, the incorporation of deuterium in to newly synthesised
296 protein causes a decrease in the abundance (Figure 1) of the monoisotopic (M0) peak relative
297 to the abundances of the M1, M2 and M3 isotopomers that contain 1, 2 or 3 'heavy' isotopes
298 (e.g. ^{13}C , D_2O , ^{15}N , etc.). Over the duration of the experiment, changes in mass isotopomer
299 distribution follow a non-linear bi-exponential pattern due to the rise-to-plateau kinetics in
300 precursor enrichment (body water D_2O enrichment measured in plasma samples by GC-MS)
301 and the rise-to-plateau kinetics of D_2O -labelled amino acids in to newly synthesised protein.
302 Therefore, a machine learning approach was taken employing the Nelder-Mead method to
303 optimise for the rate of change in the relative abundance of the monoisotopic (M0) peak. The
304 rate of change in mass isotopomer distribution is also a function of the number (n) of
305 exchangeable H sites and this was accounted for by referencing each peptide sequence
306 against standard tables reporting the relative enrichment of amino acids by tritium in mice
307 (25) or deuterium in humans (26) to give the synthesis rate (k) for each peptide.
308 Supplementary tables S1 and S2 report the raw mass spectrometry data for each peptide
309 analysed in the myofibrillar and sarcoplasmic fractions, respectively. Peptide synthesis rates
310 across each protein were averaged to give a synthesis rate for that protein in each individual
311 participant.

312

313 *Estimation of protein breakdown rates.* The rate of change in the abundance of a protein is
314 dependent on the difference between its rate of synthesis k_s and rate of breakdown k_d . We
315 assumed each of these was constant and used first-order kinetics following the standard
316 formula:

$$A = A_0 e^{(k_s - k_d)(t - t_0)}$$

317 where A is the abundance at time t and A_0 is the abundance at time t_0 . Using protein
318 abundance data at times t and t_0 the net rate of change in abundance can be calculated by
319 rearranging the above to give:

$$k_s - k_d = \frac{1}{t - t_0} \ln \frac{A}{A_0}$$

320 Converting differences in abundance between D0 and D9 to rates of change in abundance
321 enables the rate of breakdown of each protein to be calculated as the difference between its
322 rate of synthesis and its rate of change in abundance. Supplementary table S3 summarises the
323 mean (n=8 in each group) abundance, synthesis and degradation data for each protein
324 investigated in the sarcoplasmic and myofibrillar fractions.

325

326 *Plasma Analysis* Plasma glucose concentration was measured via an enzymatic reference
327 method with hexokinase (Melbourne Pathology, Melbourne, Australia). Plasma insulin,
328 leptin, adiponectin, plasminogen activator inhibitor type 1 (PAI-1), tumour necrosis factor α
329 (TNF- α) and interleukin-6 (IL-6) were measured on 96-well plates utilizing commercially
330 available and customised Milliplex Human magnetic bead panels (Millipore, Massachusetts,
331 USA) following the kit-specific protocols provided by Millipore. Analytes were quantified in
332 duplicate using the Magpix system utilising xPONENT 4.2 software. Concentrations of all
333 analytes were determined on the basis of the fit of a standard curve for mean fluorescence
334 intensity versus pg/mL. Two quality controls with designated ranges were run with each
335 assay to ensure validity of data generated. For the quantification of active ghrelin, whole
336 blood samples (1 ml) were transferred to eppendorf tubes containing 1 mg 4-(2-aminoethyl)-
337 benzene sulfonyl fluoride (AEBSF; Sigma Aldrich, Castle Hill, Australia). Samples were then
338 incubated at room temperature for 30 mins to clot and then centrifuged at 3,000g for 15 min
339 at 4°C. Plasma samples were then acidified with 4 μ L 1N Hydrochloric acid and then stored
340 at -80°C. A sandwich ELISA (Millipore, Massachusetts, USA) incorporating
341 spectrophotometric absorbance was used to measure active ghrelin levels. C-reactive protein
342 levels were quantified via enzyme immunoassay (IBL-International, Männedorf,
343 Switzerland). Plasma FFA concentrations were determined by an enzymatic colorimetric
344 method (Wako Diagnostics, Tokyo, Japan).

345

346 *Immunoblotting* Approximately 30 mg of muscle was homogenized in ice-cold buffer as
347 previously described (27). Lysates were centrifuged at 12,000 g for 30 min at 4 °C and the
348 supernatant was transferred to a sterile microcentrifuge tube and aliquoted to measure protein
349 concentration using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Lysate
350 was then re-suspended in 4X Laemmli sample buffer with 40 μ g of protein loaded onto 4–

351 20% Mini-PROTEAN TGX Stain-Free™ Gels (BioRad Laboratories, Gladesville, Australia).
352 Post electrophoresis, gels were activated on a Chemidoc according to the manufacturer's
353 instructions (BioRad Laboratories, Gladesville, Australia) and then transferred to
354 polyvinylidene fluoride (PVDF) membranes. After transfer, a stain-free image of the PVDF
355 membranes for total protein normalization was obtained before membranes were rinsed
356 briefly in distilled water and blocked with 5% non-fat milk, washed with 10 mM of Tris-
357 HCl, 100 mM of NaCl, and 0.02% Tween 20, and incubated with primary antibody (1:1000)
358 overnight at 4 °C. Membranes were then incubated with secondary antibody (1:2000), and
359 proteins were detected via enhanced chemiluminescence (Thermo Fisher, Scoresby,
360 Australia) and quantified by densitometry (ChemiDoc™ XRS+ System; BioRad
361 Laboratories, California, USA). Exercise and Non-Exercise pre- and post-samples were run
362 on the same gel. Primary antibodies used were phospho- 4E-BP1Thr37/46 (#2855),
363 mTORSer2448 (#2971), p70S6KThr389 (#9205), AMPK α Thr172 (#2531) and total 4E-BP1
364 (#9644), mTOR (#2972), p70S6K (#9202) and AMPK α (#2532). All antibodies were
365 purchased from Cell Signaling Technology (Danvers, USA). Volume density of each target
366 phospho protein band was normalized to its respective total protein content, while the total
367 protein band was normalised to the total protein loaded into each lane using stain-free
368 technology (28), with data expressed in arbitrary units.

369

370 *RNA Extraction, Quantification, Reverse Transcription and Real-Time PCR* Skeletal muscle
371 tissue RNA extraction was performed using a TRIzol-based kit according to the
372 manufacturer's protocol (Invitrogen, Melbourne, Australia). In brief, approximately 20 mg of
373 frozen skeletal muscle was homogenized in TRIzol with chloroform added to form an
374 aqueous RNA phase. This RNA phase was then precipitated by mixing with isopropanol
375 alcohol and the resulting pellet was washed and re-suspended in 50 μ L of RNase-free water.
376 Extracted RNA was quantified using a NanoDrop 2000 Spectrophotometer (Thermo
377 Scientific, Scoresby, VIC, Australia). Reverse transcription and real-time Polymerase Chain
378 Reaction (RT-PCR) was performed as previously described (29). In brief, first-strand
379 complementary DNA (cDNA) synthesis was performed using commercially available
380 TaqMan Reverse Transcription Reagents (Invitrogen, Melbourne, Australia). Quantification
381 of mRNA in duplicate was performed using a CFX96 Touch™ Real-Time PCR Detection
382 System (Bio Rad, California, USA). TaqMan-FAM-labelled primer/probes for Atrogin-1
383 (Hs01041408_m1), Myostatin (Hs00976237_m1), Muscle Ring Finger-1 (MuRF-1;
384 Hs00822397_m1), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

385 (PGC-1 α ; Hs01016719_m1), Pyruvate dehydrogenase lipoamide kinase isozyme 4 (PDK4;
386 Hs01037712_m1), IL-6 (Hs00174131_m1), G protein-coupled receptor 56 (GPR56;
387 Hs00938474_m1), and Fatty Acid Translocase Cluster of Differentiation 36 (FAT/CD36;
388 Hs00354519_m1) were used in a final reaction volume of 20 μ l. 18S ribosomal RNA (Cat.
389 No. 4333760T) was used to normalize threshold cycle (CT) values and was stably expressed
390 between post-exercise and between the different exercise conditions (data not shown). The
391 relative amounts of mRNAs were calculated using the relative quantification ($\Delta\Delta$ CT) method
392 (30).

393

394 *Statistical analysis*

395 For all DPP analyses, baseline differences between the independent groups were investigated
396 by one-way ANOVA of Pre samples taken prior to deuterium oxide administration using *R*
397 software for statistical computing. Within subject differences that occurred among Pre, Day
398 3, Day 6 and Day 9 samples were investigated using repeated measures ANOVA and
399 separate tests were performed to investigate differences in either REX or HFLC groups. To
400 control the false discovery rate (FDR), P-value distributions were used to calculate q-values
401 and a criterion FDR of <1% was set. This statistical approach considers the biological
402 variation across each protein and is, therefore, more sophisticated than arbitrarily
403 implementing a threshold based on fold-change. Statistics for remaining analyses were
404 performed using SigmaPlot (Version 12, Systat Software). Normal distribution and equal
405 variance of the data were tested using Shapiro-Wilk and F tests, respectively. Two-way
406 ANOVA followed by Student Newman Kuel's post-hoc tests were performed to determine
407 differences between REX and HFLC groups, and time (Pre, D3, D6, and Post). All data in
408 text and figures are presented as mean \pm SEM with P values <0.05 indicating statistical
409 significance.

410

411 **Results**

412 *Effects of High Fat Low Carbohydrate Diet and Resistance Exercise on Glucose Metabolism,* 413 *Free Fatty Acids and Plasma Markers of Inflammation and Appetite Control*

414 Consumption of HFLC diet did not result in differences in plasma insulin or glucose in
415 response to an OGTT in either group (Figure 2A, B) although REX tended to lower glucose
416 area under the curve between D0 and D9 (Figure 2C, P = 0.150). The HFLC diet increased
417 fasting plasma non-esterified free fatty acids (FFA) in both groups, measured on the four
418 mornings muscle biopsies were taken (P<0.05, Figure 3A).

419 There were main effects for time for IL-6 and TNF- α ($P < 0.05$; Figure 3). IL-6 increased
420 $\sim 390\%$ at Day 6 compared to Post in participants that performed REX ($P < 0.05$). Similarly,
421 TNF- α was highest at Day 6 in both groups and was $\sim 205\%$ and $\sim 140\%$ above Pre in REX
422 and HFLC groups, respectively ($P < 0.05$, Figure 3). There were non-significant increases in
423 C-reactive protein, Pre-to-Post intervention (Figure 3). We also investigated the effects of our
424 HFLC diet on the appetite and energy balance regulating hormones ghrelin and leptin. We
425 observed non-significant decreases in these markers following our dietary intervention. There
426 were also no changes in the concentrations of adiponectin or plasminogen activator inhibitor
427 type 1 (PAI-1), circulating factors previously associated with insulin resistance (31, 32),
428 between the REX and HFLC groups or throughout the dietary intervention (Figure 3).

429 *Dynamic proteome profiling of human muscle proteins*

430 On average, the molar per-cent enrichment (MPE) of D₂O in body water increased at a rate of
431 0.135 ± 0.005 % per day and reached a peak of 2.14 ± 0.08 % on day 9. Label-free proteome
432 profiling was conducted on proteins that had one or more unique peptides that were measured
433 in all 16 participants at each of the four experiment time points. In the myofibrillar fraction,
434 38 proteins were analysed and included each of the major components of the muscle thick
435 and thin myo-filaments. Analysis of the sarcoplasmic fraction encompassed 88 proteins with
436 the majority of these having biological functions associated with the generation of precursor
437 metabolites and belong to KEGG pathways including glycolysis/glycogenolysis, the
438 tricarboxylic acid cycle and pyruvate metabolism.

439

440 The rate constant (k) of synthesis of each protein was calculated by fitting mass isotopomer
441 data to a non-linear model (Figure 4) described previously in (19). After filtering to remove
442 peptides with relatively poor fitting data ($R^2 < 0.99$), 31 of the 38 myofibrillar proteins and 60
443 of the 88 sarcoplasmic proteins were retained (Supplementary tables report raw (S1 and S2)
444 and summary (S3) mass spectrometry data). Figure 5 displays the rank order of synthesis
445 rates for the 91 proteins investigated in the muscle of both groups of participants. Pyruvate
446 dehydrogenase E1 component subunit beta (ODPB) had the lowest synthesis rate (0.08 ± 0.08
447 % per day), whereas carbonic anhydrase 3 (CAH3) exhibited the greatest synthesis rate (23.8
448 ± 5.8 % per day). The median and inter-quartile range (IQR) synthesis rate was 2.29 % per
449 day (IQR $1.4 - 3.2$ %/d) in the myofibrillar fraction and 3.1 % per day (IQR $0.9 - 3.9$ %/d) in
450 sarcoplasmic fraction. There was no difference ($P = 0.317$) in the rate of synthesis of proteins
451 analysed in the myofibrillar versus sarcoplasmic fractions. There was no association ($\rho - 0.08$,

452 P=0.3925) between the rate of synthesis of a protein and its relative abundance in either the
453 myofibrillar or sarcoplasmic fractions.

454

455 *The effect of High Fat Low Carbohydrate Diet and Resistance Exercise on muscle protein*
456 *abundances*

457 Prior to the diet and exercise intervention there were no differences in muscle protein
458 abundance between groups. The relative proportions of myosin heavy chain (MyHC)
459 isoforms, which are commonly used to phenotype skeletal muscle, were 60 % MyHC 1, 31 %
460 MyHC 2a and 9 % MyHC 2x in REX participants and were similar to participants in the
461 HFLC group (63 % MyHC 1, 28 % MyHC 2a and 9 % MyHC 2x). Within-subject analysis of
462 changes in protein abundance over the duration of the study period did not reveal any
463 significant changes in protein abundance in the muscle of HFLC participants. In contrast, 17
464 proteins exhibited significant differences in abundance between baseline (D0) and D9 in the
465 muscle of the REX group. With the exception of troponin C, the changes in protein
466 abundance were reported from analysis of the sarcoplasmic fraction. Figure 6 presents a
467 volcano plot of protein abundance data from REX participants and displays the fold change in
468 protein abundance between D0 and D9 of the intervention. In total, 11 proteins increased and
469 6 decreased in abundance in response to the resistance exercise training and diet intervention.

470

471 *The effect of High Fat Low Carbohydrate Diet and Resistance Exercise on the synthesis of*
472 *muscle proteins*

473 The average rate of synthesis of myofibrillar proteins was 1.58 ± 0.08 % per day in
474 participants that performed REX. This synthesis response was significantly greater than the
475 1.09 ± 0.06 % per day in the muscle from HFLC only participants ($P < 0.001$, Figure 7). This
476 effect of REX was not detected in the sarcoplasmic fraction where the average synthesis rate
477 was similar between groups (REX = 2.15 ± 0.15 % /d, HFLC = 2.05 ± 0.17 % /d). When
478 considered on a protein-by-proteins basis, 13 proteins (7 myofibrillar and 7 sarcoplasmic) had
479 significantly greater rates of synthesis in the REX group ($P < 0.05$, Figure 8).

480

481 *Protein responses to High Fat Low Carbohydrate Diet and Resistance Exercise*

482 Figure 9 summarises data from the REX group comparing within subject changes in protein
483 abundance with between group analysis of differences in protein synthesis rate against the
484 HFLC group and illustrates the variety of different responses of muscle proteins to resistance
485 exercise. Proteins positioned in the upper left quadrant were less abundant after REX but

486 exhibited a greater rate of synthesis in exercised versus non-exercised muscle. Conversely,
487 the abundance of proteins in the lower right quadrant increased in response to resistance
488 exercise but their rate of synthesis was less than that measured in non-exercised muscle that
489 did not exhibit any change in protein abundance.

490

491 Table 2 categorises exercise responsive proteins (categories i-vi) according to the pattern of
492 the response observed in the REX group. The most common response to exercise was an
493 increase in turnover rate without any significant change in abundance, and proteins that
494 exhibited this response were mostly of myofibrillar origin (10 proteins). The second and third
495 most numerous groups of proteins exhibited significant changes in abundance that were
496 primarily attributable to differences in breakdown rate (i.e. protein abundance changed but
497 there was no significant difference in synthesis rate). Proteins that increased in abundance
498 (Table 2 category ii) included cytoskeletal binding proteins (ACTS, FLNC and PROF1) and
499 cytoplasmic or membrane-bound vesicle proteins (ALBU, PRDX6, TRFE) that have anti-
500 oxidant (PRDX6, ALBU) functions, whereas glycolytic enzymes were common amongst
501 proteins that decreased in abundance (Table 2 category iii). Two other glycolytic enzymes
502 exhibited unique responses including beta enolase, which was significantly less abundant
503 after exercise training despite its rate of synthesis being significantly greater in exercised
504 versus non-exercise trained muscle. Conversely, aldolase A did not change in abundance in
505 REX muscle but its rate of synthesis was significantly less than reported in the HFLC group.

506

507 *Modulation in gene and protein markers of translation initiation mitochondrial biogenesis,*
508 *and muscle proteolysis following HFLC diet*

509 The expression of the ubiquitin E3 ligases Muscle-specific RING Finger protein1 (MuRF1)
510 and Muscle Atrophy F-box (MAFbx/atrogen-1) decreased ~160% post-intervention in the
511 REX group only, resulting in a ~115-230% differential expression between groups post-
512 intervention (P<0.05; Figure 9). Myostatin decreased by 140% post-intervention in the REX
513 cohort and compared to the HFLC (~200%, P<0.05; Figure 10). REX also attenuated PPAR δ
514 mRNA expression compared to the non-exercise group by ~40% post-intervention (Figure 9),
515 while the transcriptional co-activator PGC-1 α decreased by ~130% in both groups post-
516 intervention (Figure 9). There were no changes in FAT/CD36, GPR56, PDK4 or IL-6 mRNA
517 expression (Figure 9). Western blot analyses on key mTOR related signalling substrates that
518 regulate translation initiation processes revealed a ~50% increase in total mTOR abundance
519 in REX compared to HFLC post-intervention (P<0.05; Figure 11). The phosphorylation state

520 or total amount of p70S6K, 4E-BP1 and AMPK were unaltered between groups or post-
521 intervention (Figure 10).

522

523 **Discussion**

524 In an effort to find therapeutic strategies to combat conditions such as sarcopenia, the
525 anabolic response of muscle to resistance exercise and nutrition has been a longstanding
526 focus of many clinical investigations. Previous application of peptide mass spectrometry and
527 deuterium oxide has enabled measurement of synthesis rates for numerous proteins in human
528 muscle (20). For the first time we report fully integrated proteomic profiling in association
529 with deuterium labelling to simultaneously measure the rate of synthesis, net abundance and
530 rate of breakdown of human muscle proteins in response to a diet-exercise intervention.
531 Muscle anabolism induced by resistance exercise is typically regarded as being driven by
532 synthetic processes, but using dynamic proteome profiling (DPP), we provide novel data to
533 show that exercise-induced changes in muscle protein occur through several different patterns
534 of response involving modulation of both synthetic and degradative processes. Such
535 multiplicity in the adaptive response of the proteome (Figure 8) has not previously been
536 captured and is a unique aspect of the current investigation.

537

538 New techniques have recently been developed incorporating D₂O labelling with proteomic
539 analyses including peptide mass spectrometry, which measure the rate constant (k) for
540 synthesis on a protein-by-protein basis in the muscle of animals (33-36) and humans (20).
541 Collectively, these works and our current data (Figure 5) demonstrate that there is a broad
542 range of different synthesis rates of muscle proteins. These results highlight the advantages of
543 interrogation of protein-specific synthetic responses (as opposed to the average gross
544 synthesis of mixed proteins from whole muscle or muscle fractions) to discover novel
545 therapeutic targets and provide mechanistic insight into protein metabolism. Moreover,
546 results from these studies that show that protein synthesis rates differ, providing evidence for
547 the selective breakdown of proteins at different rates in order to maintain the same relative
548 abundance of proteins in tissue (i.e. maintenance of muscle mass). Chronic resistance training
549 (i.e., 16 weeks) is associated with selective changes in protein abundance, such as a shift
550 toward a greater proportion of the type IIa myosin heavy chain isoform (37). Resistance
551 exercise increases the synthesis of mixed myosin heavy chains (38) but our data (Figures 4
552 and 8) is the first to shown this effect is confined to the fast-twitch IIa (MYH2). This ability

553 to measure the synthesis of key proteins such as MYH2 will be important to the future study
554 of the effects of resistance training in older adults with muscle wasting conditions.

555 In the current study, we reasoned that, when investigated at a protein-specific level, the
556 average rate of breakdown can be estimated from the difference between its measured change
557 in relative abundance and its rate of synthesis over that time period. That is, because the
558 synthesis, abundance and degradation of the protein are intimately connected then any change
559 in abundance that is not explained by the measured rate of synthesis during that period may
560 be a function of the rate of breakdown of that protein. Eleven proteins (Table 2) exhibited
561 negative values for the calculated rate of breakdown (i.e. the measured increase in protein
562 abundance was greater than the accumulative increase predicted from the measured synthesis
563 rate). While this may be attributable to technical errors associated with the measurement of
564 protein abundance and/or synthesis rate, it may also reflect secretion and delivery to muscle
565 of pre-existing protein, which would have the effect of increasing protein abundance to a
566 greater extent than predicted by synthesis alone. ALBU and TRFE each exhibited ‘negative’
567 breakdown rates and, while abundant in muscle (15), these proteins are predominantly
568 expressed in the liver, secreted into the plasma and become resident in the muscle interstitial
569 space, which would have the effect of concentrating their abundance in a process distinct
570 from the synthesis process capture by deuterium oxide labelling. Similarly, the increases in
571 ACTS and TNNC2 could be explained by the translocation of soluble, pre-myofibril
572 complexes from the sarcoplasmic fraction and incorporation into mature myofibrils through
573 the process of myofibril assembly (39).

574 The potential involvement of myofibril assembly brings context to other findings from our
575 DPP data. Pre-myofibrils contain thin filament proteins (e.g. actin, troponin and
576 tropomyosin), costameric proteins (e.g. desmin and filamin) and z-band/z-body proteins (e.g.
577 alpha-actinin) that were responsive to resistance exercise (Table 2). Desmin has largely been
578 associated with lateral force transmission from contracting sarcomeres to the muscle exterior
579 (40) and is already known to increase in human muscle after resistance training (41). Our
580 findings raise the question whether part of this response is associated with myofibril
581 assembly, often an understudied area in the context of exercise adaptation. We also report
582 increases in the abundance of ankyrin repeat domain protein 2 (ANKR2) and 27 kDa heat
583 shock protein (HSPB1) in the sarcoplasmic fraction, and a greater turnover of alpha B-
584 crystallin (CRYAB) specifically in the myofibrillar fraction of exercised muscle. These

585 findings are consistent with earlier work (42, 43) and the responses observed for these
586 proteins may be necessary to stabilise cytoskeleton and myofibril structures or prevent
587 protein denaturation (44). Similarly Alpha B-crystallin translocates to and stabilises
588 myofibrils in response to contractile stress (45) and this event is evidenced in our DPP data
589 by the calculated 'negative' breakdown rate for CRYAB.

590

591 The greater (1.58 %/d) synthesis of myofibrillar proteins in exercised muscle (Figure 6)
592 matches the 1.6 %/d rate reported by Brook et al (13) during the first three weeks of a similar
593 resistance training stimulus. A potential limitation of our experimental design is that we did
594 not include a healthy 'control' diet so we cannot compare the effect of a HFLC diet on
595 muscle protein synthesis to other dietary interventions. Nevertheless, the average synthesis
596 rate of myofibrillar proteins (1.09 %/d) in HFLC participants is less than reported in
597 untrained muscle (1.35 %/d) by Brook et al (13). Of note was that the HFLC diet alone did
598 not alter the abundance of muscle proteins suggesting there was little effect on muscle protein
599 synthesis response. When combined with resistance exercise there was a decrease in the
600 abundance of four glycolytic enzymes (Table 2, categories iii and v) and a lower synthesis
601 rate for aldolase A. This contrasts with the increase in glycolytic metabolism that has
602 previously been associated with resistance training (46, 47) although we also report two
603 glycolytic enzymes (Table 2, category i) exhibited greater turnover in exercised muscle. This
604 response may be associated with maintaining enzyme pool efficiency (19, 48) and a similar
605 response has been reported previously in mouse heart during isoprenaline-induced cardio-
606 toxicity (37).

607

608 MEK-ERK signalling has previously been associated with anabolic responses to resistance
609 exercise (49, 50) and may have contributed to the differences between HFLC and REX
610 groups. Degradation of phosphatidylethanolamine-binding protein 1 (PEBP1; also known as
611 RAF kinase inhibitor protein RKIP) was greater in exercised muscle and resulted in
612 significantly less RKIP abundance, which may enhance signalling through the MEK-ERK
613 pathway (51). RKIP is also a component in the diacylglycerol (DAG)-sensitive PKC pathway
614 that increases IL-6 expression in response to saturated fatty acids (52) and we observed
615 greater IL-6 mRNA expression and plasma concentration of IL-6 in resistance-exercised
616 individuals. New data from the model organism *C. Elegans* also implicates RKIP in protein
617 breakdown (53) and RKIP/PEBP1 was reported to be an inhibitor of starvation-induced
618 autophagy in mammalian cells (54). Here, mRNA expression of ubiquitin E3 ligases

619 (Atrogin-1 and MuRF-1) was lower in exercised muscle, which may indicate the ubiquitin-
620 proteasomal system is not the primary mechanism for the observed greater breakdown of
621 selected proteins (Table 2, categories i, ii and iii). Other processes that could be responsible
622 for the breakdown of these proteins include chaperone-assisted selective autophagy (CASA).
623 Filamin-C (FLNC), which is an actin-binding protein that may be involved in mechano-
624 transduction and intracellular signalling (55) and Ulbricht and colleagues recently
625 demonstrated increased FLNC mRNA and protein levels after maximal eccentric exercise in
626 human skeletal muscle (56). As filamin-C is thought to be damaged during eccentric muscle
627 contraction it may require removal by autophagy in exercised muscle (57). CASA is
628 implicated in these responses and inhibition of CASA may also be responsible, in part, for the
629 increased abundance (Table 2 category ii) detected with DPP and RKIP.

630

631 In conclusion, the response of human muscle protein turnover to resistance exercise training
632 differs on a protein-by-protein basis (Table 2). Of the proteins that were altered by the
633 exercise stimulus, the most common response was an increase in turnover (i.e. the increase in
634 synthesis rate was matched by an increase in the rate of breakdown resulting in no
635 measurable change in protein abundance). We report that changes in protein abundance in
636 response to resistance exercise are underpinned by regulation of either synthetic or
637 degradative processes, or a combination of both. While such concepts are not new, it has been
638 challenging to collect empirical data to support this paradigm in human tissue. We show DPP
639 can provide data on the synthesis, abundance and breakdown of individual proteins. As such,
640 use of DPP in future studies has the potential to significantly advance knowledge of exercise-
641 induced adaptations as well as patho-biochemical processes associated with chronic human
642 muscle-wasting diseases and muscle loss with ageing. For example, the application of DPP in
643 chronic interventions could uncover how the response of enhanced turnover reported here is
644 converted into protein accretion and myofibre hypertrophy, and whether increases in
645 synthesis continue are sustained (and for how long) or if the rate of breakdown subsides with
646 longer period of resistance exercise training.

647

648

649 **Conflict of Interest Statement**

650 The authors declare that the research was conducted in the absence of any commercial or
651 financial relationships that could be construed as a potential conflict of interest.

652 **Author contributions**

653 DMC, JGB and JAH designed the study, DMC and WJS conducted the experiment, DMC,
654 JGB and MAP performed the biochemical assays and data analyses, DMC, JGB and JAH
655 wrote the manuscript and all authors approved the manuscript prior to submission.

656

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684 **Figure legends**

685 **Table 1.**

686 Baseline Characteristics of the participants in the REX and HFLC groups.

687

688 **Table 2.**

689 Pattern of muscle proteome responses of individual proteins observed in the REX group
690 between and D0 and D9. For the purpose of comparison/ calculation, changes in abundance
691 have been converted to rate of change in abundance.

692

693 **Figure 1.** D₂O-labeling protocol and peptide enrichment. (A) After 3 days of dietary control
694 participants consumed a high-fat low-carbohydrate (HFLC) diet (8% Carbohydrate, 77% Fat,
695 15% Protein) and undertook three sessions of resistance exercise (REX), or consumed the
696 HFLC with no exercise (HFLC). Participants ingested deuterium oxide (200 ml/ d) during a
697 9-day intervention period, venous blood samples were collected daily and samples of *vastus*
698 *lateralis* were collected prior to (i.e. morning of day 0) and during (days 3, 6 and 9) the
699 intervention. Blood and muscle samples were analysed by mass spectrometry to determine
700 precursor enrichment and the rate of incorporation of deuterium in to newly synthesised
701 protein, respectively.

702

703 **Figure 2.** The effects of high-fat low-carbohydrate diet and resistance exercise on blood
704 glucose homeostasis. Differences in plasma insulin (A), glucose (B) and glucose area under
705 the curve (C) in participants who performed resistance exercise (REX) or consumed a high
706 fat low-carbohydrate (HFLC) diet only. Data are presented as mean ± SEM; n = 8 in REX/ n
707 = 7 in HFLC, a = P < 0.05 to 'Rest' within group, d = P < 0.05 to '90 min' within group, e =
708 P < 0.05 to '120 min' within group; 2-way ANOVA.

709

710 **Figure 3.** Metabolite and hormone responses to high-fat low-carbohydrate diet and resistance
711 exercise. Differences in plasma concentrations of Free fatty acids (A), Adiponectin (B), PAI-
712 1 (C), IL-6 (D), TNF- α , (E), Leptin (F) and C-reactive protein (G) and (H) Ghrelin. Data
713 represent resistance trained (REX) participants who performed exercise and consumed a high
714 fat low carbohydrate diet and control (HFLC) participants who consumed the high fat low
715 carbohydrate diet only. Data are presented as mean ± SEM; n = 8 in REX/ n = 7 in HFLC, *
716 = P < 0.05 between REX and HFLC, # = P < 0.05 interaction for time; 2-way ANOVA.

717

718 **Figure 4.** (A) Example mass spectra of peptide LAQESIMDIENEK ($[M+2H]^{2+} = 760.3837$
719 m/z) from the type IIa isoform (MYH2) of myosin heavy chain. The relative abundances of
720 m_0 (monoisotopic), m_1 , m_2 , m_3 and m_4 mass isotopomers at D0, D3, D6 and D9 is
721 presented. The incorporation of deuterium in to newly synthesised protein is evident in the
722 proportional increase in the ‘heavy’ isotopomers (m_1 , m_2 , m_3 and m_4) and relative decrease
723 in the fractional abundance of the monoisotopic peak (i.e. $m_0/\sum m_i$). The mean \pm SD and
724 coefficient of variation (%CV) is reported for exercise trained participants ($n = 8$). (B) The
725 rate constant of synthesis was calculated by fitting mass isotopomer data ($m_0/\sum m_i$) to a non-
726 linear model of the rise in precursor enrichment and incorporation of deuterium in to the
727 product peptide, taking in to account the amino acid composition of the peptide. (C) The
728 fractional synthesis rate (k) of MYH2 in exercised muscle (0.0187 ± 0.0032) was
729 significantly ($P=0.016$) greater than control (0.0096 ± 0.0017) based on mass isotopomer
730 distribution analysis of 5 peptides investigated in $n=8$ participants per group.

731

732

733 **Figure 5.** Rank order of protein synthesis rates in human skeletal muscle. (A) The mean rate
734 constant (k) for the synthesis of each protein ranked from least to greatest from D0 to D9.
735 Data represent proteins analysed in the myofibrillar pellet (blue) and sarcoplasmic/ soluble
736 muscle fraction (orange) of both diet control (HFLC) and resistance exercised (REX)
737 participants. Labels indicate the UniProt knowledgebase identifiers for a sub-selection of
738 proteins. The size (radius) of each data point represents the mean normalised abundance of
739 each protein in baseline (day 0) samples. (B) Boxplots presenting the average (median and
740 interquartile range) rate constant of synthesis of proteins analysed in the myofibrillar pellet
741 and soluble muscle fraction from D0 to D9. Independent two-tailed t-test found no significant
742 ($P=0.317$) difference in the average rate of synthesis of proteins in myofibrillar ($n=31$) vs
743 sarcoplasmic ($n=60$) proteins.

744

745 **Figure 6.** The effects of resistance exercise on muscle protein abundance. Volcano plot
746 presenting pre- (day 0) versus post-intervention (day 9) changes (Fold change (Log2)) in
747 resistance trained (REX) participants. There were no significant differences in protein
748 abundance in the muscle of control participants who consumed the high-fat low-carbohydrate
749 (HFLC) diet only. P values were calculated from log-transformed data using within-subject
750 (repeated measures) one-way analysis of variance. Proteins that exhibited a statistically
751 significant ($p<0.05$) change in abundances are coloured red if the calculated false discovery

752 rate (FDR) is $< 5\%$ or blue if FDR is $> 5\%$ and less than 10% . Labels represent the Uniport
753 Knowledgebase identifier of each of the statistically significant proteins.

754

755 **Figure 7.** The effect of three bouts of resistance exercise on the average synthesis rate of
756 human muscle proteins. Data are presented using violin plots overlaid with box plots that
757 represent the average (median and interquartile range) rate of synthesis of mixed myofibrillar
758 or soluble muscle proteins. Exercise participants performed resistance training (REX) and
759 consumed a high-fat low-carbohydrate diet, whereas control (HFLC) participants consumed
760 the high-fat low-carbohydrate diet only ($n = 8/\text{group}$). Two-tailed independent t-tests found
761 the synthesis rate of mixed myofibrillar proteins was significantly ($P < 0.001$) greater in REX
762 compared to HFLC. There was no significant ($P = 0.531$) difference in the rate of synthesis of
763 sarcoplasmic proteins between groups.

764

765 **Figure 8.** The effects of resistance exercise on the synthesis rate of muscle proteins in REX
766 participants only. Volcano plot presenting differences (Fold-difference (Log2)) in the rate of
767 synthesis of proteins between participants who performed resistance exercise (REX) and or
768 those who consumed a high-fat low-carbohydrate (HFLC) diet only. P values were calculated
769 from log-transformed data ($n = 8/\text{group}$) using between-subject one-way analysis of variance.
770 Proteins that exhibited a statistically significant ($p < 0.05$) change in abundances are coloured
771 red if the calculated false discovery rate (FDR) is $< 5\%$ or blue if FDR is $> 5\%$ and less than
772 10% . Labels represent the Uniport Knowledgebase identifier of each of the statistically
773 significant proteins.

774

775 **Figure 9.** Dynamic proteome responses to resistance exercise in the myofibrillar (A) and
776 sarcoplasmic (B) fractions. Summary data presented are from resistance exercised (REX)
777 participants only. There were no changes in protein abundance in HFLC control participants
778 who consumed a high-fat low-carbohydrate diet only. Upper left quadrant = decrease in
779 abundance over the duration of the experiment but greater rate of synthesis compared to
780 HFLC participants. Upper right quadrant = proteins that increased in abundance in the muscle
781 of REX participants and protein synthesis rate was also greater in REX compared to HFLC.
782 Lower left quadrant = Decrease in abundance after REX while synthesis rate is also less than
783 HFLC participants. Lower right quadrant = increase in abundance after REX despite the
784 synthesis rate being less than HFLC participants.

785

786 **Figure 10.** Differences in mRNA expression of (A) Atrogin-1, (B) MuRF-1, (C) Myostatin,
787 (D) FAT/CD36, (E) GPR56, (F) PDK4, (G) PGC1- α , (H) PPAR- δ and (I) IL-6. Data
788 represent REX participants who performed resistance exercise and consumed a high fat low
789 carbohydrate diet and HFLC participants who consumed the high fat low carbohydrate diet
790 only (mean \pm SEM; n = 6 in REX/ n = 5 in HFLC, * = P < 0.05 between REX and HFLC, a =
791 P < 0.05 to 'Rest' within group; 2-way ANOVA).

792

793 **Figure 11.** Differences in phosphorylation status of (A) 4E-BP1Thr37/46, (C) AMP
794 KinaseThr172, (E) mTORSer2448, (G) p70S6KThr389, and total abundance of (B) 4E-BP1,
795 (D) AMPK α , (F) mTOR and (H) p70S6K. Data represent REX participants who performed
796 resistance exercise and consumed a high fat low carbohydrate diet and HFLC participants
797 who consumed the high fat low carbohydrate diet only (mean \pm SEM; n = 5 in REX/ n = 4 in
798 HFLC, * = P < 0.05 between REX and HFLC; 2-way ANOVA).

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Table 1.

Baseline Characteristics of the participants

	REX (n = 8)	HFLC (n = 8)
Age (y)	36.9 ± 1.9	36.9 ± 2.1
Body Mass (kg)	88.8 ± 4.2	85.5 ± 3.4
BMI (kg m ⁻²)	26.9 ± 1.0	26.4 ± 1.1
Fat Free Mass (kg)	60.4 ± 2.0	57.8 ± 2.6
Fat Mass (kg)	24.9 ± 2.9	24.5 ± 2.1
VO _{2peak} (ml/kg/min)	39.7 ± 1.9	36.5 ± 2.2
VO _{2peak} (L/min)	3.5 ± 0.1	3.1 ± 0.2
Leg Extension 1-RM (kg)	79.8 ± 5.0	76.5 ± 6.6
Leg Press 1-RM (kg)	221.0 ± 6.0	217.6 ± 16.1

819

820 Values are given as mean ± SEM.

821 **Table 2**

Protein identifier (UniProt ID)	Protein name	Abundance change (%/d)	Fractional synthesis rate (%/d)	Fractional breakdown rate (%/d)
(i) Increased turnover, no change in abundance				
ACON	Aconitase	-3.34	3.46	6.8
ACTN2	Alpha-actinin 2	+1.83	4.54	2.71
CRYAB	Alpha B-crystallin	+3.89	4.38	0.49
DESM	Desmin	-1.55	4.25	5.8
LDHA	Lactate dehydrogenase	-3.34	9.63	12.97
MLRS	Myosin regulatory light chain 2	+2.82	1.25	-1.57
MYH2	Myosin heavy chain 2	-0.11	1.88	1.99
MYL1	Myosin light chain 1/3	+1.55	2.53	0.98
PGM1	Phosphoglucomutase 1	-1.45	1.09	2.54
TPM2	Tropomyosin beta	2.65	0.93	-1.72
(ii) Significant increase in abundance with no difference in synthesis rate				
ACTS	Actin, alpha skeletal muscle	+3.17	1.41	-1.76
ALBU	Serum albumin	+8.9	3.21	-5.69
ENOA	Alpha enolase	+2.2	3.62	1.42
FLNC	Filamin C	+8.13	12.45	4.32

IGHG4	Ig gamma-4 chain C region	+14.7	8.77	-5.93
PRDX6	Peroxiredoxin-6	+2.12	6.07	3.95
PROF1	Profilin-1	+3.73	1.22	-2.51
TNNC2	Troponin C	+5.29	1.52	-3.77
TRFE	Serotransferrin	+10.56	7.25	-3.31

(iii) Significant decrease in abundance but no significant difference in synthesis

AT2A1	Sarcoplasmic/endoplasmic reticulum Ca ²⁺ ATPase	-1.45	6.90	8.35
G3P	Glyceraldehyde-3 phosphate dehydrogenase	-2.74	0.24	2.98
PEBP1	Phosphatidylethanolamine-binding protein 1	-2.21	0.42	2.63
PGAM2	Phosphoglycerate mutase 2	-3.56	5.35	8.91
TPIS	Triosephosphate isomerase	-2.12	0.72	2.84

(iv) Significant increase in abundance and significantly greater synthesis rate

HSPB1	Heat shock protein beta-1	+4.72	6.22	1.5
ANKR2	Ankyrin repeat domain-containing protein-2	+4.43	6.17	1.74

(v) Significant decrease in abundance and significantly greater synthesis rate

ENOB	Beta-enolase	-1.84	6.62	8.46
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(vi) No change in abundance but a significantly lesser synthesis rate

ALDOA	Fructose-bisphosphate aldolase A	-1.36	4.63	5.99
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824 **References**

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1023

1024

Figure 1

Resistance exercise on days 1, 4 and 7

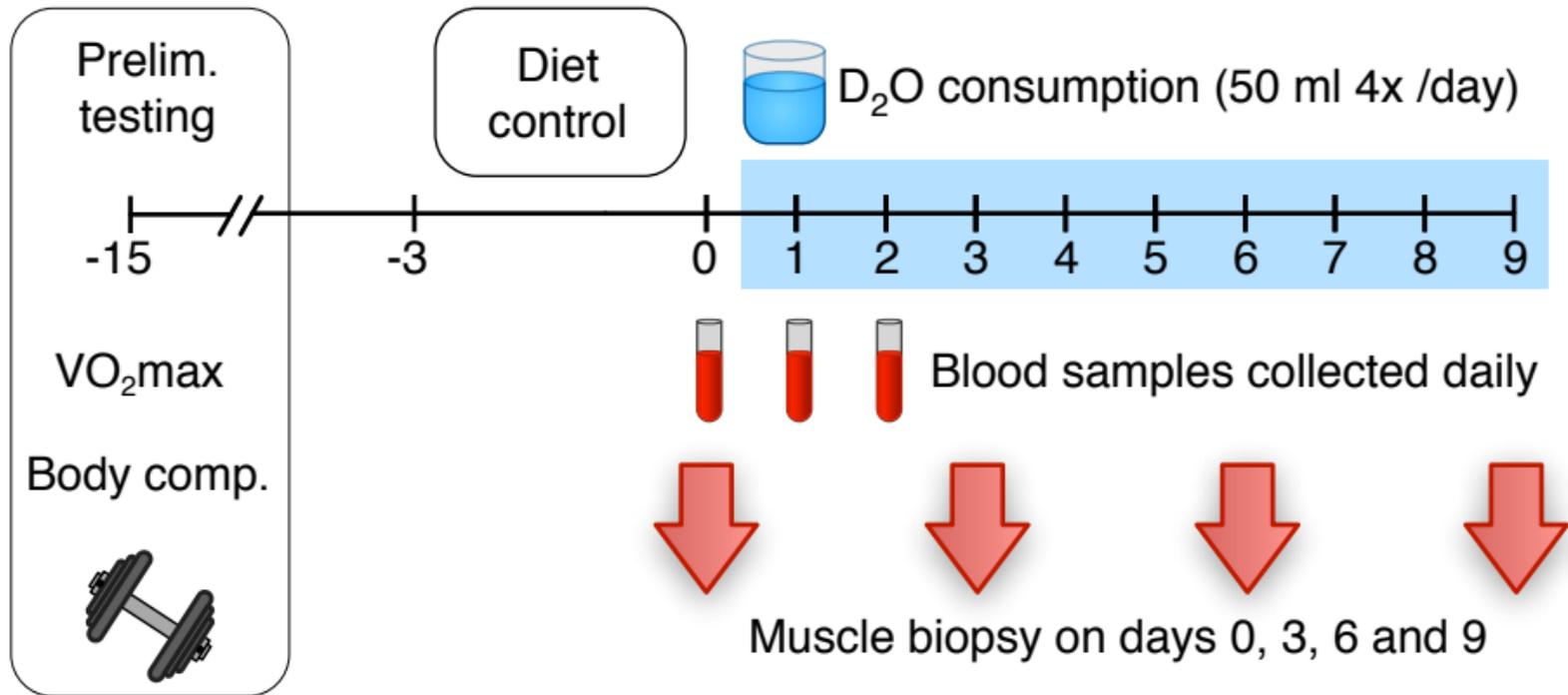


Figure 2

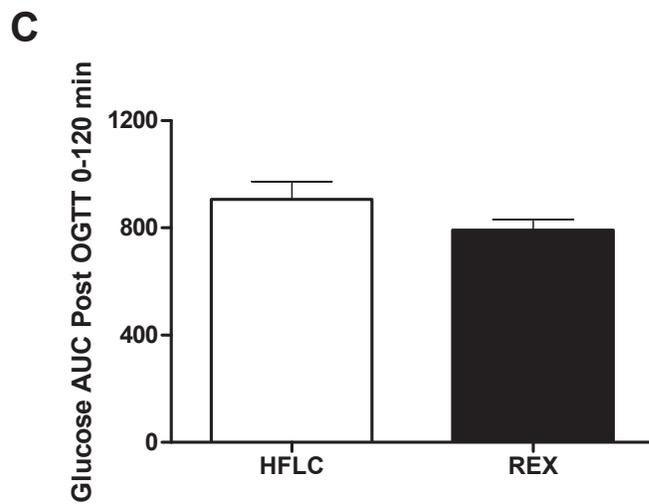
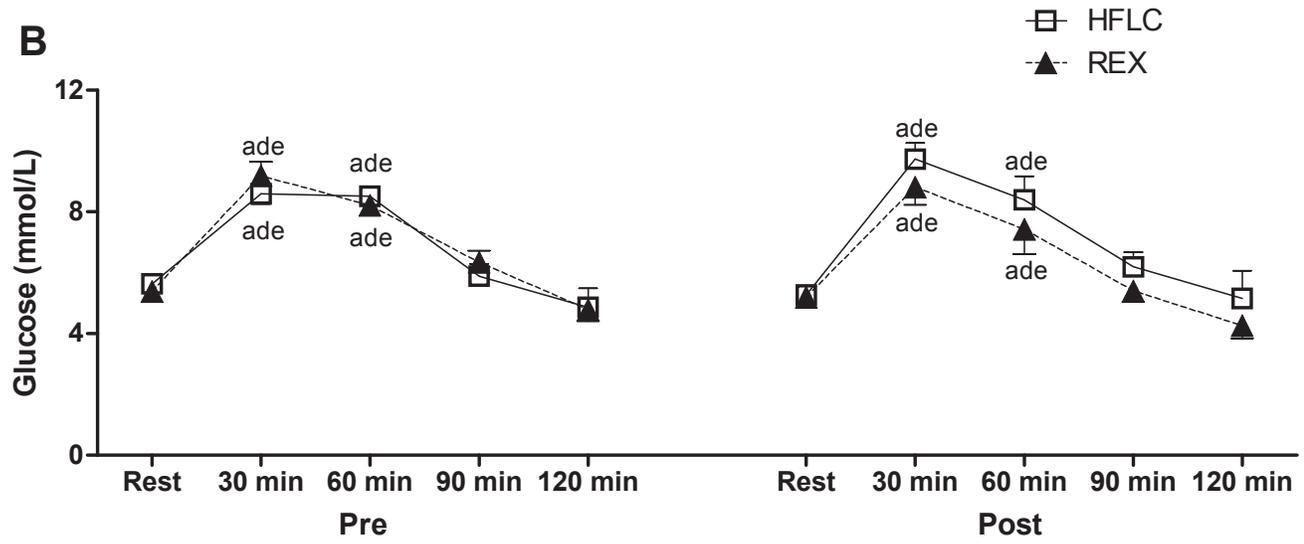
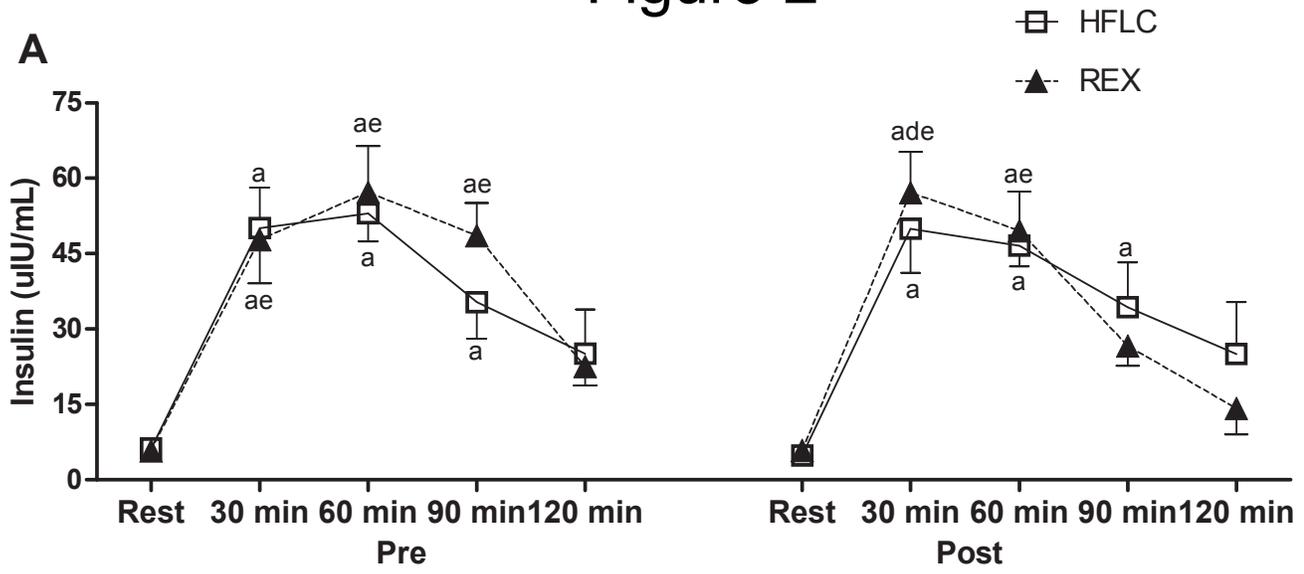


Figure 3

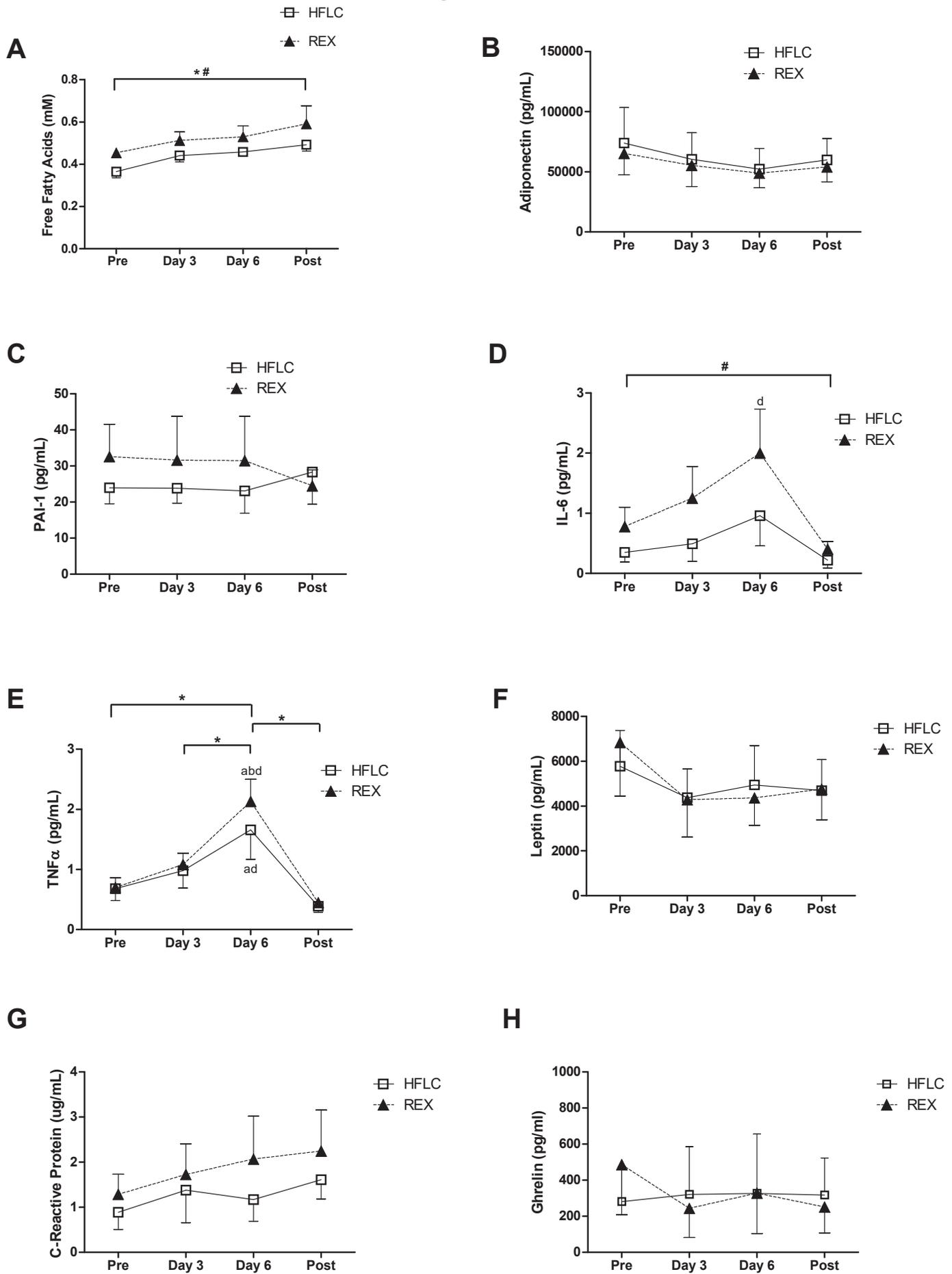
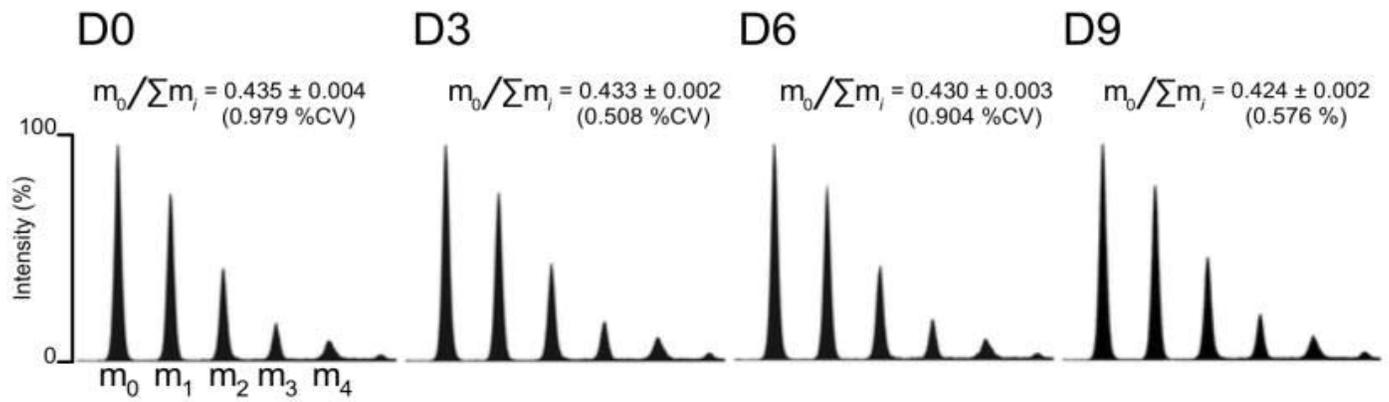


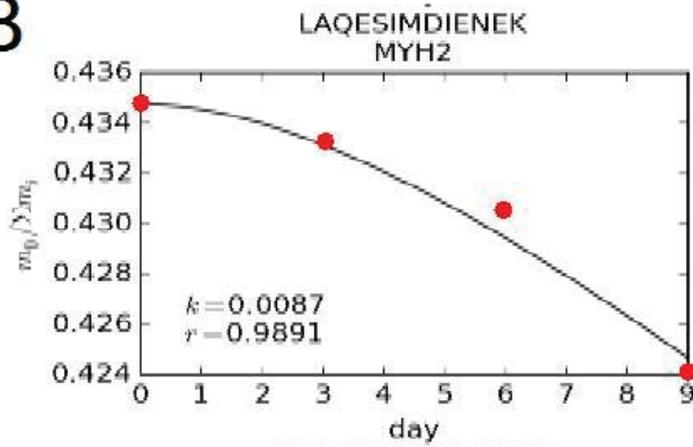
Figure 4

A

MYH2 peptide LAQESIMDIENEK [2+], mass = 1518.7675



B



C

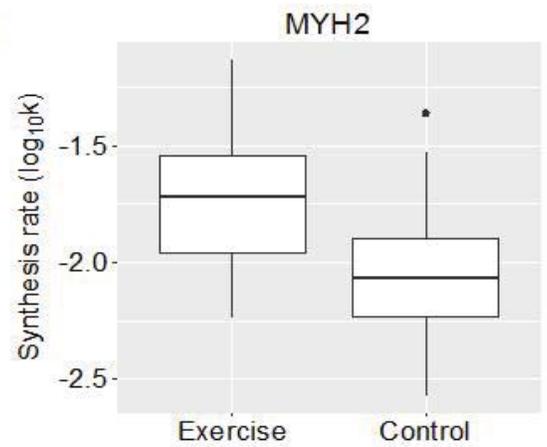
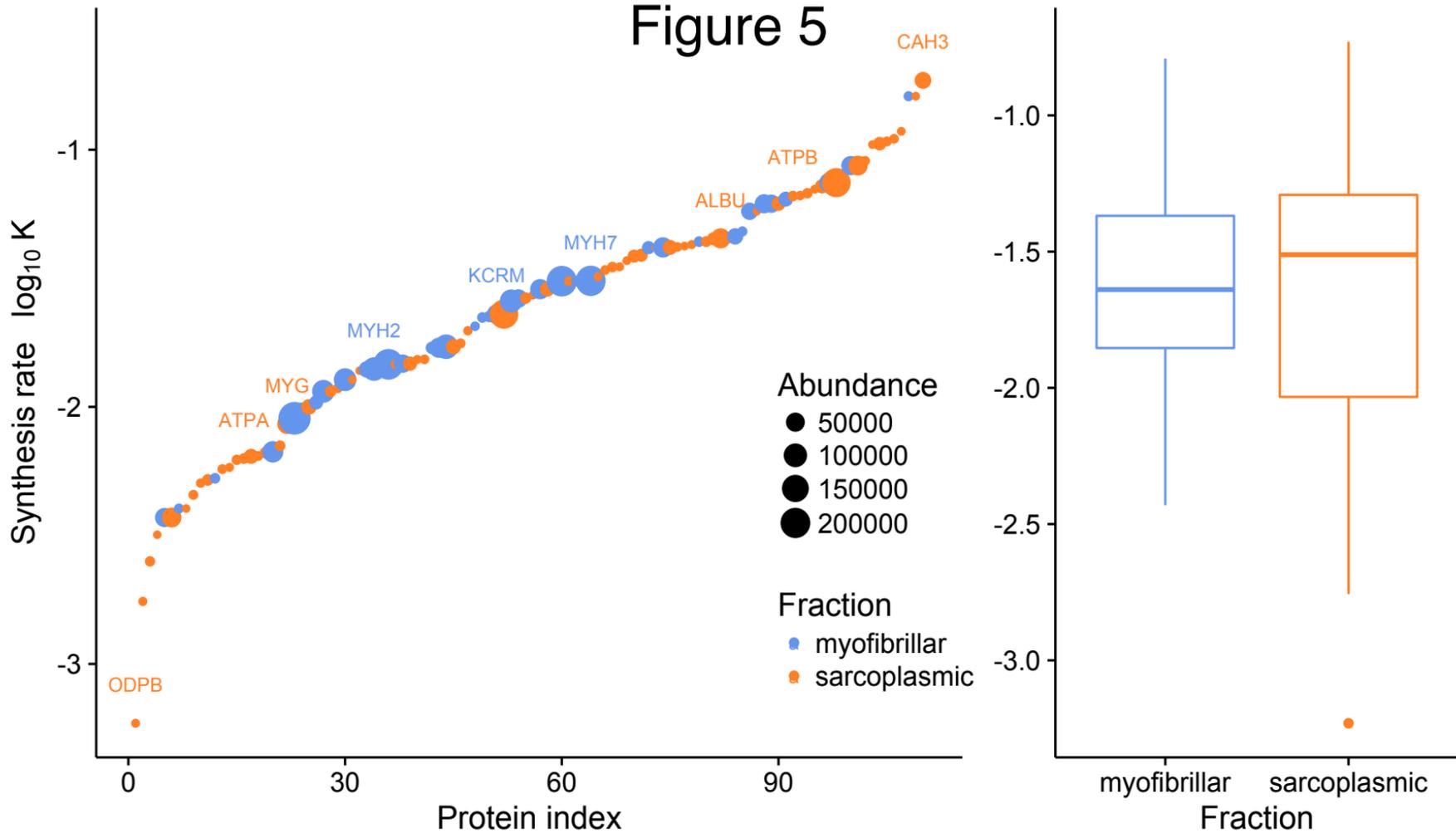


Figure 5



Volcano Plot
of protein abundance

Figure 6

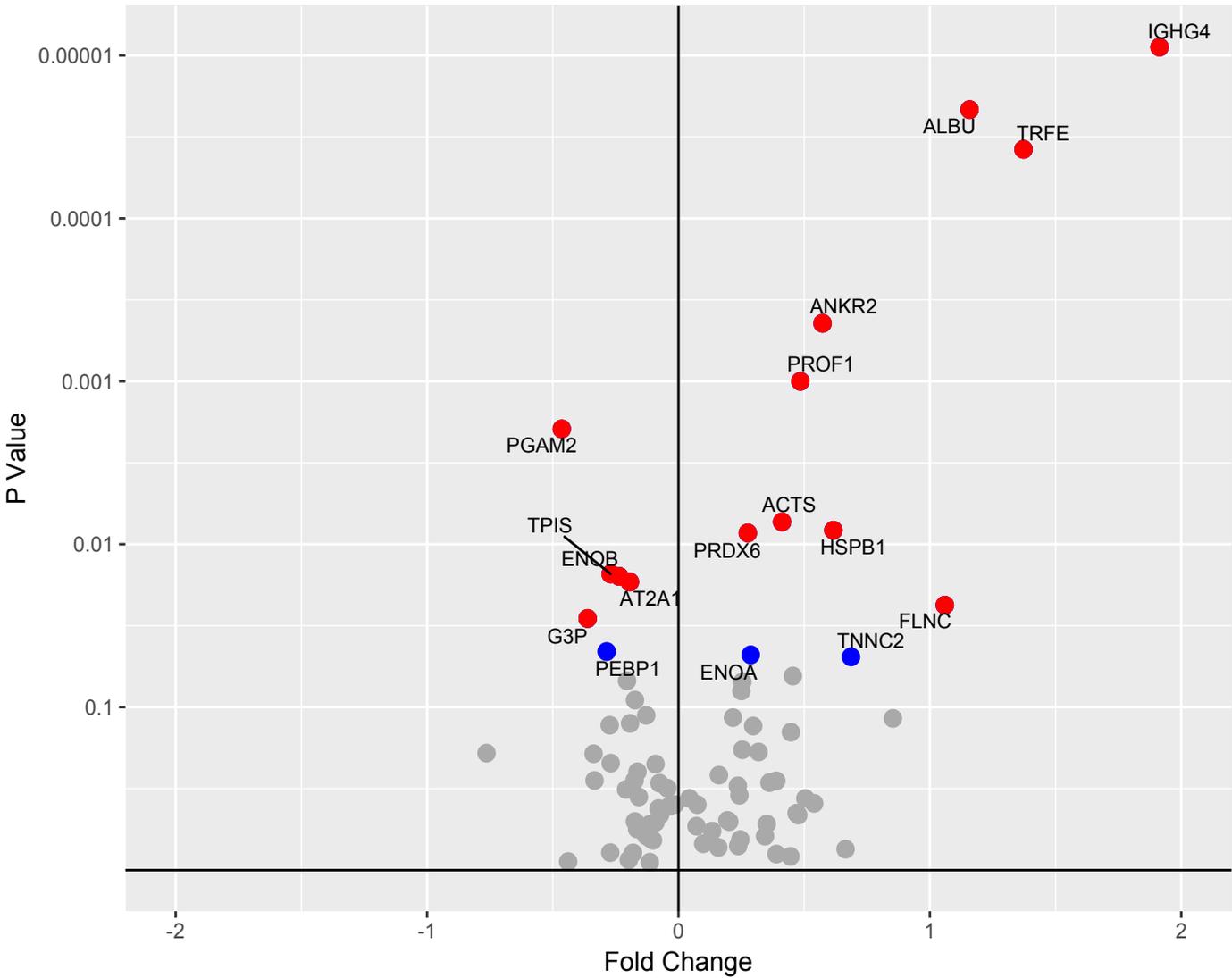


Figure 7

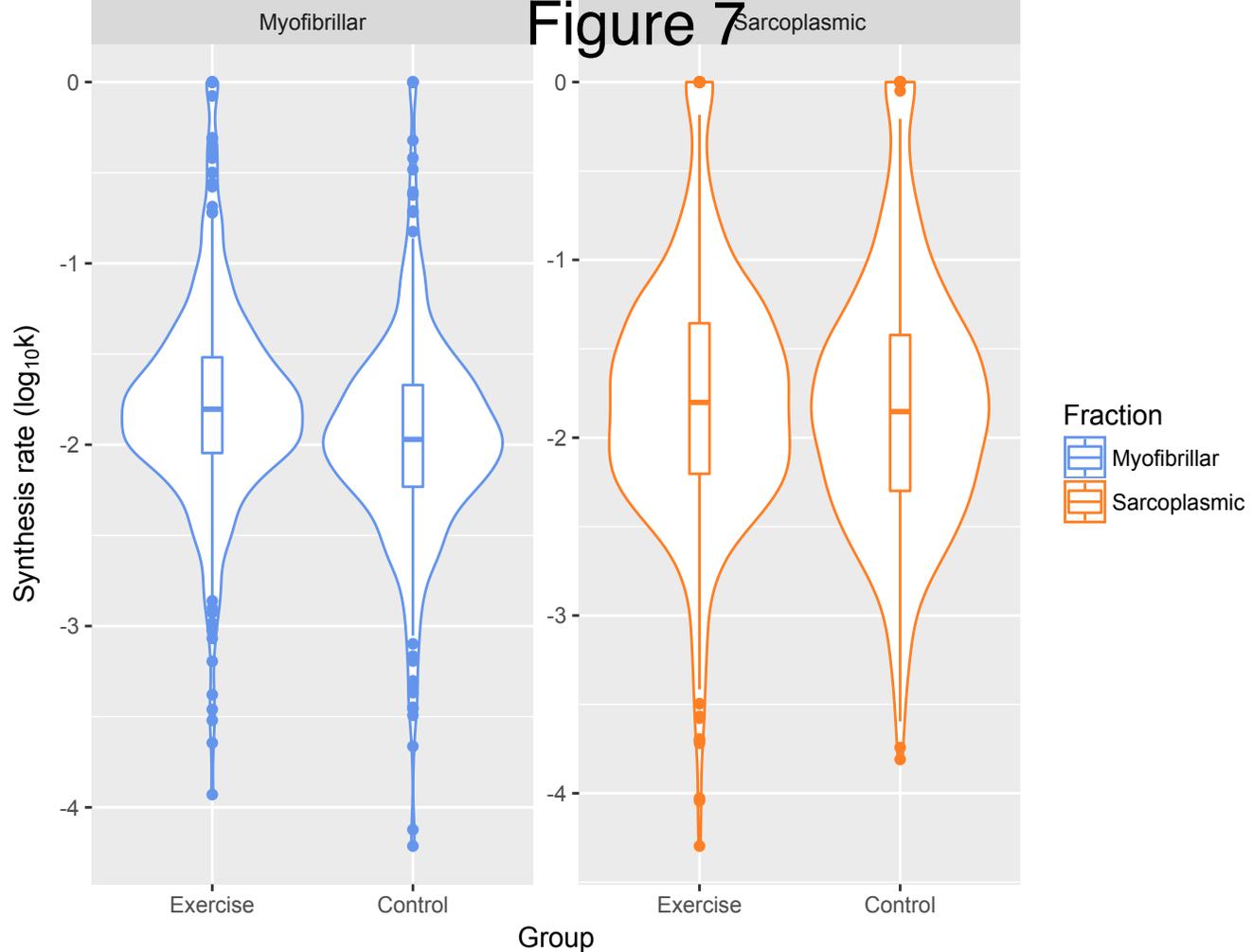
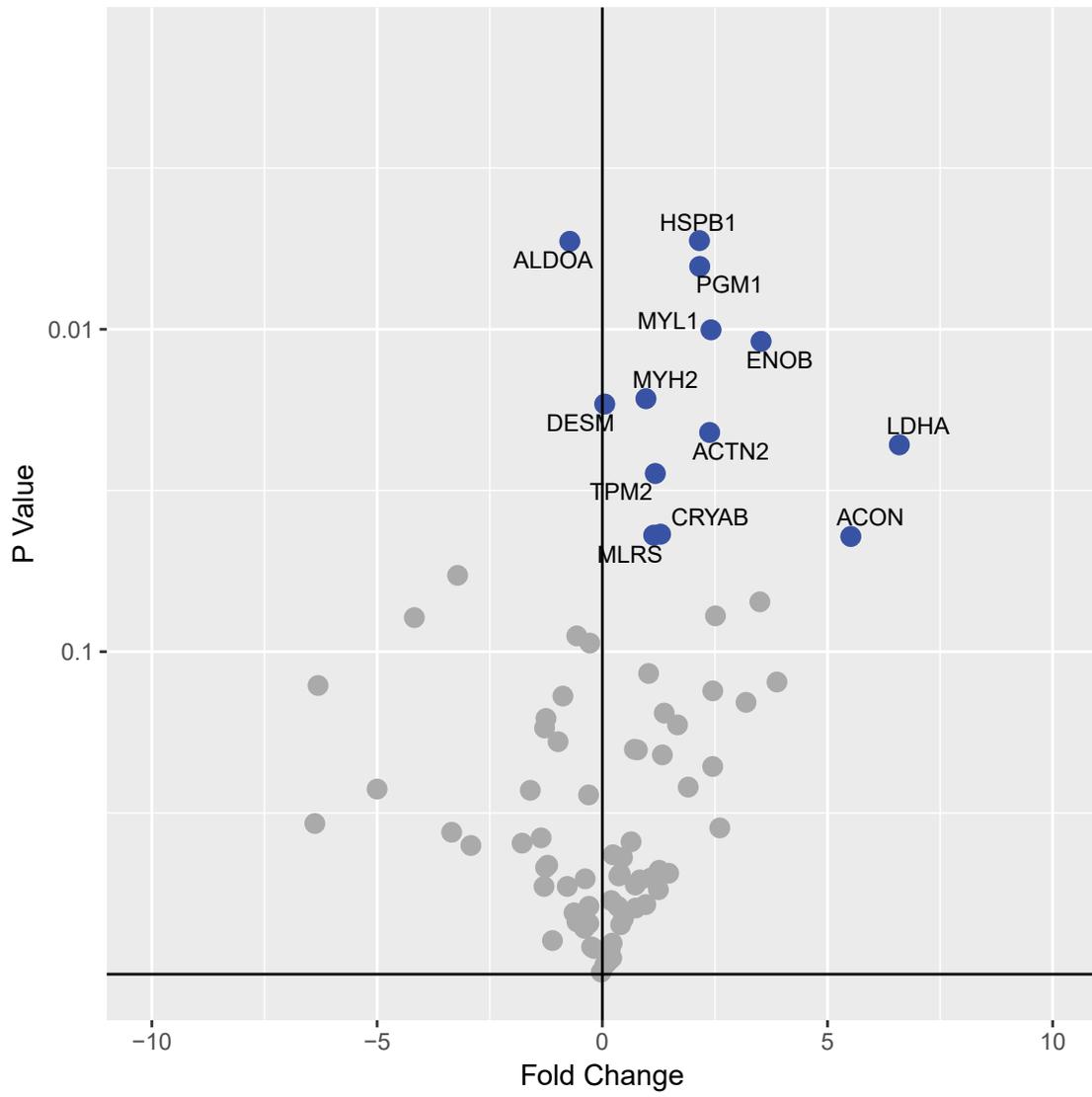


Figure 8



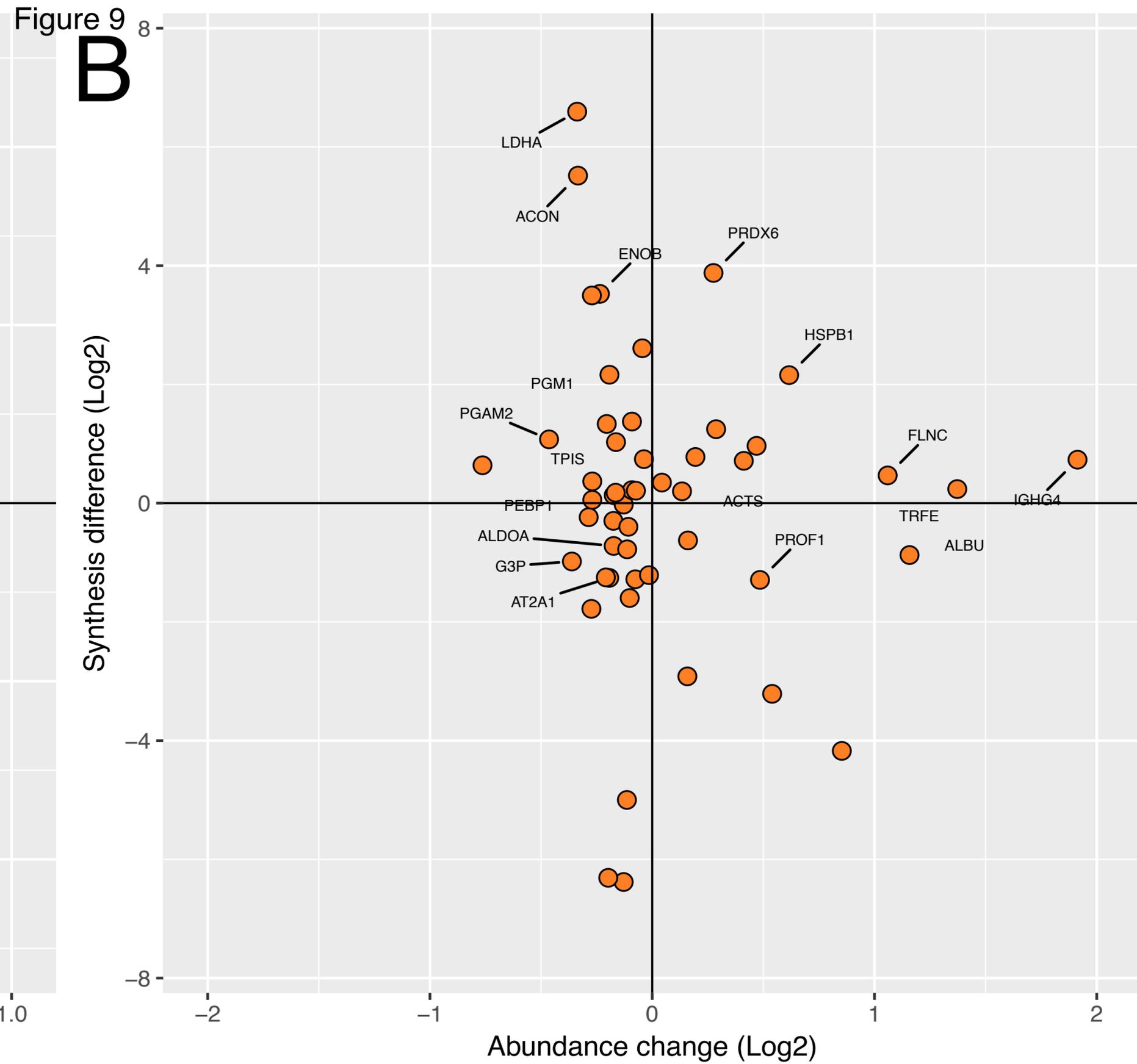
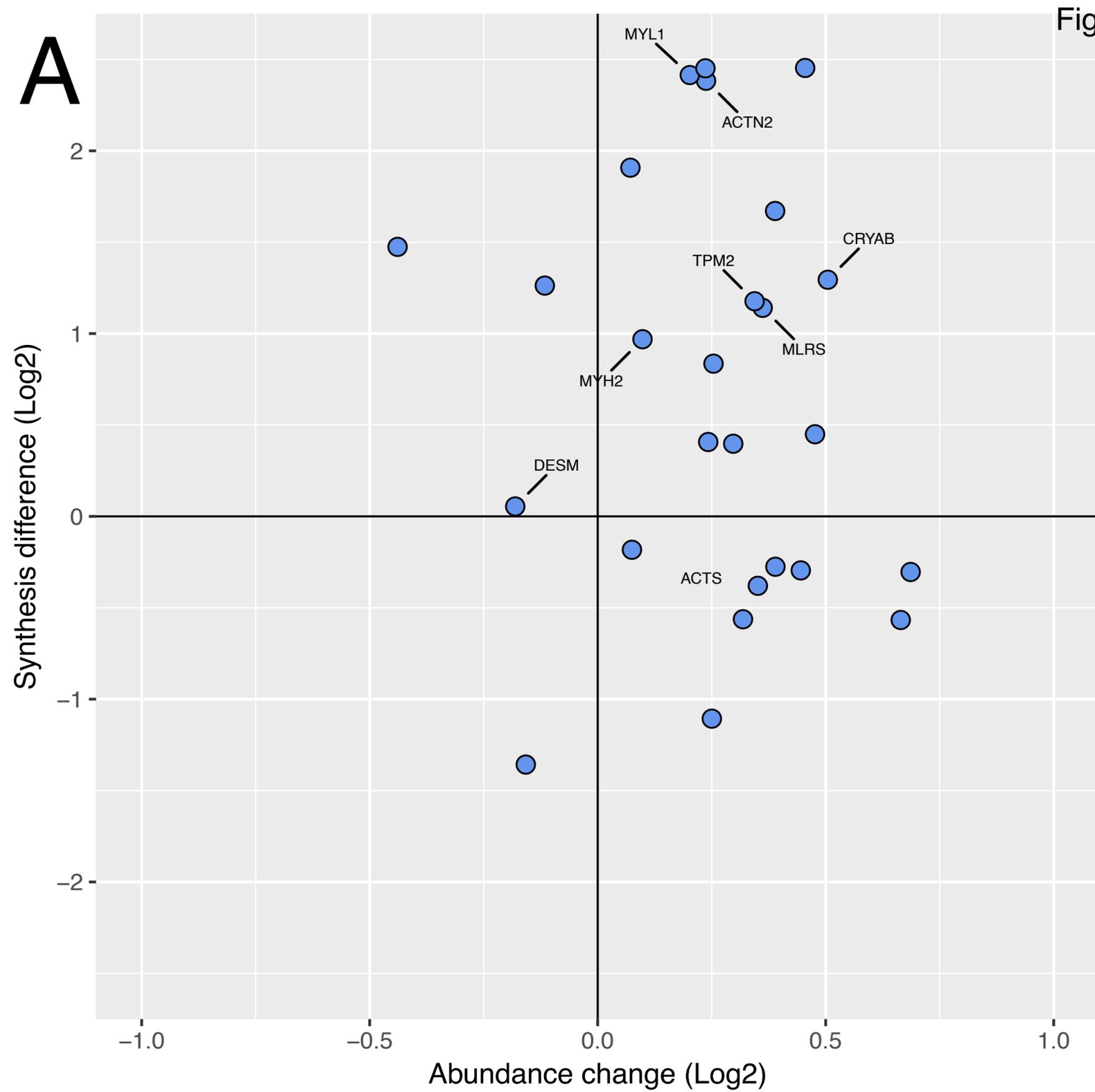


Figure 10

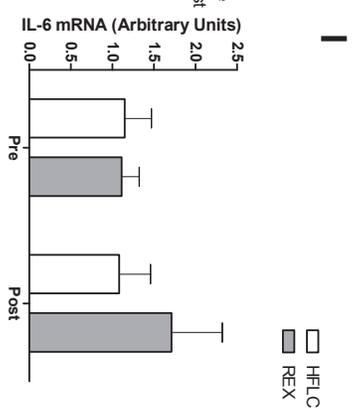
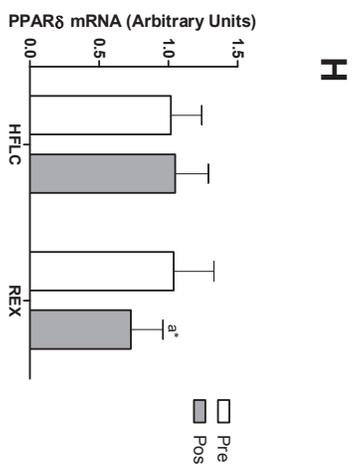
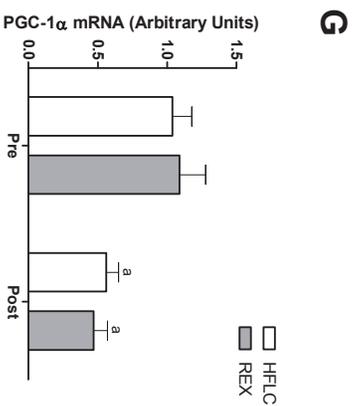
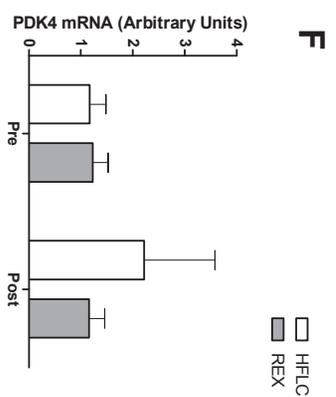
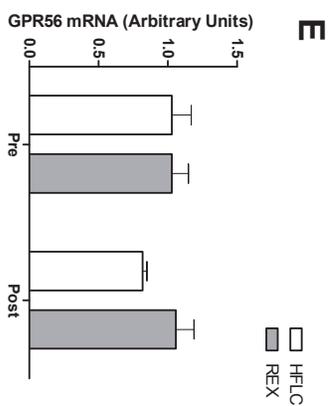
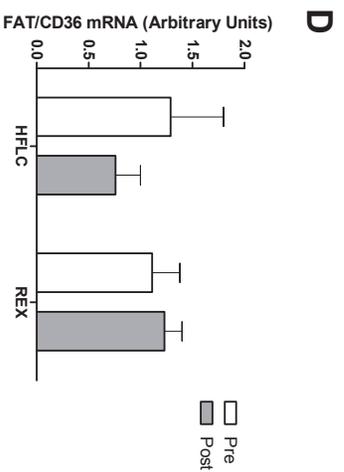
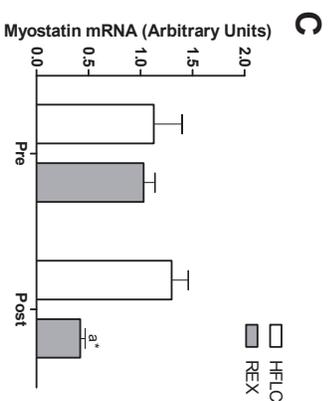
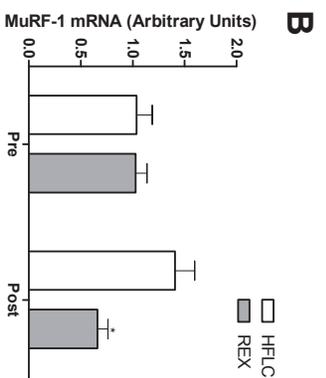
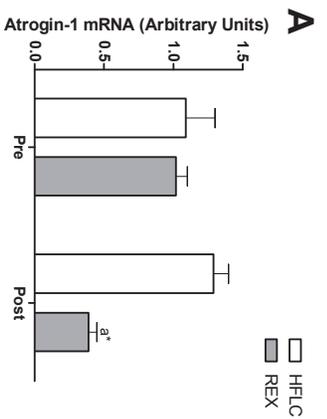


Figure 11

