1 **Title (150 characters):**

2 Regulation of Na+,K+-ATPase isoforms and phospholemman (FXYD1) in skeletal muscle fibre types

3 by exercise training and cold-water immersion in men

4

5 **Authors:**

6 Danny Christiansen [1], Robyn M. Murphy [2], James R. Broatch [1], Jens Bangsbo[3], Michael J.

7 McKenna[1], David J. Bishop [1,4]

8

9 **Affiliations:**

10 [1] Institute of Sport, Exercise and Active Living (ISEAL), Victoria University, Melbourne, Victoria,

11 3011, Australia. [2] Department of Biochemistry and Genetics, La Trobe Institute for Molecular

12 Science, La Trobe University, Melbourne, Victoria, 3086, Australia. [3] Department of Nutrition,

13 Exercise and Sports (NEXS), University of Copenhagen, 2200 Copenhagen N, Denmark. [4] School

14 of Medical and Health Sciences, Edith Cowan University, Perth, Australia

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18

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21 **Corresponding Author:**

22 Robyn M. Murphy, Department of Biochemistry and Genetics, La Trobe Institute for Molecular

23 Science, La Trobe University, Melbourne, Victoria, 3086, Australia.

24 Email: R.Murphy@latrobe.edu.au

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26

27 **Summary in key points (150 words):**

28 • It is unclear how Na+,K+-ATPase (NKA) isoforms are regulated in different skeletal muscle

29 fibre types by exercise training in humans, and the effect on phospholemman (FXYD1)

30 protein abundance in different fibre types remains to be elucidated. We investigated the

31 impact of six weeks of training on NKA-isoform protein abundance (α_{1-3}, β_{1-3} and FXYD1) in

32 type I and II muscle fibres in men.

33 • We show that intense interval training selectively increases the protein content of NKA α_1

34 and β_3 in both fibre types, β_1 in type II fibres, and decreases FXYD1 in type I fibres.

- These results suggest the favourable impact of intense training on human muscle Na$^+$/K$^+$ regulation could be attributable, in part, to fibre type-dependent modulation of NKA-isoform abundance.
- Given that cold exposure has been shown to modulate cellular redox state, which has been linked to increased NKA expression, we also investigated the effect of exercise training plus cold-water immersion (CWI) on the fibre type-specific responses of NKA isoforms and FXYD1. We found that CWI was without effect on the responses to training.

Abstract (250 words)

Little is understood about the fibre type-dependent regulation of Na$^+$,K$^+$-ATPase (NKA) isoforms by exercise training in humans. The main aim of this study was therefore to assess the impact of a period of repeated exercise sessions on NKA-isoform protein abundance in different skeletal muscle fibre types in men. Post-exercise cold-water immersion (CWI) has been reported to increase oxidative stress, which may be one mechanism underlying increases in NKA-isoform expression. Thus, a second aim was to evaluate the effect of CWI on training-induced modulation of NKA-isoform abundance. Vastus lateralis muscle biopsies were obtained from nineteen men at rest before (Pre) and after (Post) six weeks of intense interval cycling, with training sessions followed by passive rest (CON, n=7) or CWI (10°C; COLD, n=5). Training increased (p<0.05) the abundance of NKAα$_1$ and NKAβ$_3$ in both type I and type II fibres, NKAβ$_1$ in type II fibres, but was without effect on NKAα$_2$ and NKAα$_3$ (p>0.05). Furthermore, training decreased FXYD1 protein content in type I fibres, which abolished its fibre type-specific expression detected at Pre (p<0.05). CWI was without impact on the responses to training (p>0.05). These results highlight that NKA isoforms are regulated in a fibre type-dependent fashion in response to intense training in humans. This may partly explain the improvement in muscle Na$^+$/K$^+$ handling after a period of intense training. CWI may be performed without adversely or favourably affecting training-induced changes in NKA-isoform abundance.

Abbreviations

AMPKβ2, 5' AMP-activated protein kinase subunit β$_2$; CaMKII, Ca2+-calmodulin-dependent protein kinase isoform 2; COLD, cold-water immersion group; CON, control group; C$_t$, cycle threshold; CV, coefficient of variation; CWI, cold-water immersion; EDL, extensor digitorum longus; FXYD1, phospholemman isoform 1; HSP70, heat-shock protein 70; GXT, graded exercise test; K$^+$, potassium; K$_m$, Michaelis–Menten constant; MHC, myosin heavy chain; Na$^+$, sodium; NF-1, neurofibromatosis type 1; NKA, Na$^+$,K$^+$-ATPase; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SERCA1, sarco/endoplasmic reticulum Ca^{2+}-ATPase isoform 1; VO$_{2peak}$, maximum oxygen uptake.

Introduction

Skeletal muscle contractions invoke a cellular loss of potassium (K^+) and a gain in sodium (Na^+) ions, which has been associated with impaired muscle force development (McKenna *et al.*, 2008). These Na^+ and K^+ fluxes are counterbalanced, primarily, by one single, active transport system - the Na^+,K^+-ATPase (NKA) (Clausen, 2003). In skeletal muscle, the active NKA complex is composed of a catalytic α, a structural and regulatory β, and an accessory (phospholemman; FXYD) subunit (Clausen, 2013). Each of these subunits is expressed as different isoforms in both type I and II human skeletal muscle fibres ($α_{1-3}$, $β_{1-3}$ and FXYD1) (Thomassen *et al.*, 2013; Wyckelsma *et al.*, 2015). It has been shown in rats that differences in NKA-isoform abundance between these fibre types could be responsible for the discrepancy between muscles in their capacity for Na^+ and K^+ transport (Kristensen & Juel, 2010a). Thus, fibre-type specific study of NKA adaptation is fundamentally important to understand the regulation of Na^+/K^+ homeostasis and muscle function.

One study has investigated training-induced effects on NKA-isoform abundance in isolated type I and II muscle fibres from humans. In that study, Wyckelsma *et al.* (2015) found no effect of four weeks of repeated-sprint training (12 sessions of three sets of 5 × 4-s running sprints with 20 s of rest between sprints and 4.5 min of rest between sets) on α-isoform abundance ($α_1$, $α_2$ and $α_3$). In contrast, the abundance of these isoforms has been reported to change in whole-muscle samples in response to a substantially (~12-18 times) greater training volume (Mohr *et al.*, 2007; Iaia *et al.*, 2008; Bangsbo *et al.*, 2009). Thus, the chosen volume and duration of training may have reduced the likelihood of detecting changes in NKA with training in the study by Wyckelsma *et al.* (2015). It is by no means clear how the NKA isoforms are regulated by exercise training in the different fibre types in humans, and the effect of exercise training on FXYD1 abundance in different muscle fibre types remains to be elucidated.

There is great interest in the use of cold-water immersion (CWI) to optimise muscle recovery, and how it may affect adaptations to exercise training (Versey *et al.*, 2013; Broatch *et al.*, 2014; Roberts *et al.*, 2015). But no study has investigated the effect of post-exercise CWI on muscle NKA protein content. In humans, cold exposure has been shown to increase the systemic level of norepinephrine (Gregson *et al.*, 2013), which has been linked to greater oxidative stress (Juel *et al.*, 2015). In many cell types, cold exposure has also been reported to perturb redox homeostasis and increase the production of reactive oxygen species (ROS) (Selman *et al.*, 2002). Given these cellular stressors could be important for the isoform-dependent modulation of NKA-isoform expression in response to exercise in humans (Murphy *et al.*, 2008), post-exercise CWI may be a potent stimulus to affect NKA-isoform content in humans. However, this hypothesis remains currently untested.

105   The aims of the present study were to evaluate the effect of exercise training on NKA-isoform protein

106   abundance (α_{1-3}, β_{1-3} and FXYD1) in type I and type II skeletal muscle fibres in humans and to

107   examine if these adaptations could be modified by post-exercise CWI. In addition, as limited

108   information is available on the reliability of western blotting for NKA isoforms, and to support our

109   interpretation of data, we determined the technical error of this method for each of the NKA isoforms.

110

111

112 **Methods**

113 *Ethical Approval*

114 This study was approved by the Human Research Ethics Committee of Victoria University, Australia

115 (HRE12-335) and conformed to the Declaration of Helsinki. The participants received a detailed oral

116 and written, plain-language explanation of the procedures, potential risks, and benefits associated

117 with the study before providing oral and written consent.

118

119 *Participants*

120 Nineteen healthy males volunteered to participate in this study. Their age, body mass, height and

121 peak oxygen uptake ($\dot{V}O_{2peak}$) were (mean ± SD) 24 ± 6 y, 79.5 ± 10.8 kg, 180.5 ± 10.0 cm and 44.6

122 ± 5.8 mL·kg^{-1}·min^{-1}, respectively. The participants were non-smokers and engaged in physical

123 activity several days per week, but were neither specifically nor highly trained.

124

125 *Experimental design*

126 Before the first biopsy session, participants reported to the laboratory on two separate occasions to

127 be accustomed to the exercise protocol and recovery treatments. During this period, they also

128 performed a graded exercise test (GXT) to volitional exhaustion on an electromagnetically-braked

129 cycle ergometer (Lode, Groningen, The Netherlands) and their $\dot{V}O_{2peak}$ was assessed in accordance

130 with published methods (Broatch *et al.*, 2014). These visits were concluded at least 3 days prior to

131 the first biopsy session and separated by a minimum of 24 h. We utilised a parallel, two-group,

132 longitudinal study design (Fig. 1). Participants were matched on their pre-determined $\dot{V}O_{2peak}$ and

133 randomly assigned by a random-number generator (Microsoft Excel, MS Office 2013), in a counter-

134 balanced fashion, to one of two recovery treatments: Cold-water immersion (COLD, $n = 9$) or non-

135 immersion rest at room temperature (CON, $n = 10$). The assigned recovery protocol was performed

136 upon completion of every intense, sprint interval exercise session during the six weeks of training. A

137 muscle biopsy was obtained at rest before the first, (Pre) and approximately 48-72 h after the last

138 (Post), training session. These samples were used to determine protein abundance in type I and II

139 muscle fibres. This study was part of a larger research project investigating the effects of post-

140 exercise CWI on muscle adaptation in humans. Due to insufficient size of some muscle biopsies

141 allocated to this part of the study, protein abundance was determined in twelve of these participants

142 (CON, $n = 7$; COLD, $n = 5$).

143

144 *Exercise protocol and training*

145 All experimental and training sessions took place in the Exercise Physiology Laboratory at the

146 Institute of Sport, Exercise and Active Living (ISEAL), Victoria University (Melbourne, Australia). The

147 participants completed the first training session on the same electrically-braked cycle ergometer as
148 used during the familiarisation. After a 5-min warm-up at a constant absolute intensity (75 W), the
149 participants performed four 30-s maximal-intensity ('all-out') sprint efforts at a constant relative
150 flywheel resistance of 7.5 % of body mass, interspersed by 4 min of passive recovery in which they
151 remained seated with their legs resting in the pedals. Each effort was commenced from a flying start
152 at ~120 rpm, brought about by the investigators' manual acceleration of the flywheel before each
153 effort. The participants remained seated in the saddle throughout the entire session. Augmented
154 verbal feedback was provided to the participants by one investigator in a consistent manner
155 throughout each effort. This protocol was repeated in every training session and was performed
156 under standard laboratory conditions (~23°C, ~35% relative humidity). To ensure a progressive
157 physiological stimulus, participants performed four sprint repetitions in weeks 1-2, five in weeks 3-4,
158 and six in weeks 5-6. Pedal resistance was modified (7.5-9.5% of body mass) during training to
159 ensure a minimum fatigue-induced decline in mean power output of 20 W$\cdot$s^{-1}.

160

161 *Recovery treatments*
162 Five minutes after termination of the sprint interval exercise, participants commenced their
163 designated 15-min recovery treatment, consisting of either rest in a seated posture with the legs fully
164 extended on a laboratory bed at room temperature (~23°C, CON) or 10°C water immersion up to the
165 umbilicus in the same position in an inflatable bath (COLD; iBody, iCool Sport, Miami QLD,
166 Australia). The water temperature was held constant by a cooling unit (Dual Temp Unit, iCool Sport,
167 Miami QLD, Australia) with constant agitation.

168

169 *Muscle sampling*
170 Muscle was sampled from the *vastus lateralis* muscle of the participants' right leg using a 5-mm
171 Bergström needle with suction. In preparation, a small incision was made at the muscle belly through
172 the skin, subcutaneous tissue and fascia under local anaesthesia (5 ml, 1% Xylocaine). Separate
173 incisions were made for each biopsy and separated by approximately 1-2 cm to help minimise
174 interference of prior muscle sampling on the physiological response. The participants rested on a
175 laboratory bed during each sampling procedure and the biopsies were obtained after ~30 min of rest
176 in the supine position. Immediately after sampling, samples were rapidly blotted on filter paper to
177 remove excessive blood, and instantly frozen in liquid nitrogen. The samples were then stored at -
178 80°C until subsequent analyses. The incisions were covered with sterile Band-Aid strips and a
179 waterproof Tegaderm film dressing (3M, North Ryde, Australia).

180
181

182 *Dissection of single-fibre segments*

183 Skeletal muscle single-fibre segments were collected and prepared for western blotting as previously

184 described (Murphy, 2011). Approximately 105 (range: 17-218) mg w.w. of muscle was freeze dried

185 for 28 h, providing 24 (3.4-49.0) mg d.w. muscle for dissection of individual fibres. By use of fine

186 jeweller's forceps, a minimum of 20 single-fibre segments were separated from each biopsy sample

187 in a petri dish under a light microscope at room temperature (~45-60 min per biopsy). A camera

188 (Moticam 2500, Motic Microscopes) attached to a monitor was connected to the microscope to

189 manually measure, by use of a ruler, the length of each fibre segment. The mean (range) segment

190 length was 1.5 (1.0-3.2) mm and a total of 520 fibre segments were collected. After dissection,

191 segments were placed in individual microfuge tubes with the use of forceps and incubated for 1 h at

192 room temperature in 10 µL SDS buffer (0.125 M Tris-HCl, 10% glycerol, 4% SDS, 4 M urea, 10%

193 mercaptoethanol, and 0.001% bromophenol blue, pH 6.8), after which they were stored at -20 ºC

194 until analysed.

195

196 *Preparation of muscle fibre pools*

197 One half of each solubilised fibre segment was used to qualitatively determine fibre type by western

198 blotting with antibodies against MHC I and II (see section on *Immunoblotting*). The other half of each

199 fibre segment was grouped with other fibre segments from the same biopsy according to MHC

200 expression, to form samples of type I or II fibres from each biopsy, similar to the procedure described

201 previously (Kristensen *et al.*, 2015). The mean ± SD number of fibre segments analysed per

202 participant was 36 ± 3 (427 segments in total). The number of fibre segments included in each pool

203 of fibres per biopsy before and after training, respectively, was 8 (range 4-14) and 5 (range 2-10) for

204 pools of type I, and 10 (range 6-13) and 12 (range 7-16) for pools of type II, fibres. Hybrid fibres (i.e.,

205 expressing multiple MHCI and IIa isoforms) were excluded from analysis ($n = 22$, ~4 %). Type IIx

206 fibres, classified by a lack of MHC I and II content despite protein present in the sample, were also

207 excluded ($n = 5$, < 1%). Some lanes on the Stain Free gel were empty, indicating that no fibre was

208 successfully transferred into the microfuge tube ($n = 8$, ~1.5 %). Based on agreement between two

209 independent researchers, who conducted visual inspections of the blots, fibre segments and points

210 on the calibration curves were excluded from analysis if their band was unable to be validly quantified

211 due to noise on the image caused by artefacts or if they were too faint or were saturated.

212 *Calibration curves*

213 To be able to compare across gels, and to ensure that blot density was within a linear range of

214 detection (Murphy & Lamb, 2013), a four-point calibration curve of whole-muscle crude homogenate

215 with a known amount of protein was loaded onto every gel. The homogenate was prepared from an

216 equal number ($n = 5$) of pre- and post-training freeze-dried, fibre-type heterogeneous muscle

217   samples. The samples were manually powdered using a Teflon pestle in an Eppendorf tube and

218   then incubated for 1 h at room temperature in SDS buffer (0.125 M Tris-HCl, 10% glycerol, 4% SDS,

219   4 M urea, 10% mercaptoethanol, and 0.001% bromophenol blue, pH 6.8). The protein concentration

220   of the homogenate was estimated by Stain Free gel electrophoresis (Bio-Rad, Hercules, CA). The

221   intensity of the protein bands was compared to a standard curve of mixed human muscle

222   homogenate with a known protein concentration.

223   *Immunoblotting*

224   To determine MHC isoform abundance in each fibre segment, half the solubilised segment (5 μL)

225   was loaded onto 26-wells, 4-15% or 4-20% Criterion TGX Stain Free gels (Bio-Rad Laboratories,

226   Hercules, CA). Each gel was loaded with 20 segments from the same participant (n = 10 for pre and

227   post training), two protein ladders (PageRuler, Thermo Fisher Scientific) and a calibration curve.

228   NKA α_{1-3}, β_{1-3}, and FXYD1 protein in the muscle fibre pools was quantified by loading 15 μg w.w.

229   muscle per sample, along with a calibration curve, onto the same gel using the same gel type as per

230   above. Pools of type I and II fibres from Pre and Post from the same participant were loaded onto

231   the same gel. Gel electrophoresis was performed at 200 V for 45 min. After UV activation for 5 min

232   on a Criterion Stain Free Imager (Bio-Rad), proteins in gels were wet-transferred to 0.45 μm

233   nitrocellulose membrane for 30 min at 100 V in a circulating, ice-cooled bath using transfer buffer

234   (25 mM Tris, 190 mM glycine and 20% methanol). The current was on average ~0.50-0.75 mA and

235   did not exceed 0.95 mA. After transfer, membranes were incubated for 10 min in Pierce Miser

236   solution (Pierce, Rockford, IL, USA), washed five times in double-distilled H_2O, and blocked for 1.5

237   h in blocking buffer (5% non-fat milk in Tris-buffered saline Tween, TBST) at room temperature with

238   rocking. Membranes containing fibre pools were cut horizontally at 170 kDa, 70 kDa and 25 kDa to

239   re-determine fibre type (MHC isoforms, ~200 kDa) and to quantify one NKA α isoform (~100 kDa),

240   along with one β isoform (~50 kDa) and FXYD1 (~12 kDa) on the same membrane. Membranes

241   were incubated with rocking overnight at 4°C (preceded or followed by 2 h at room temperature) in

242   primary antibody diluted in 1% bovine serum albumin (BSA) in phosphate-buffered saline with

243   0.025% Tween (PBST) and 0.02% NaN_3 at concentrations detailed in Table 3. To improve the

244   visualisation of fibre type, the top portions of each membrane (>170 kDa) were stripped between

245   MHCIIa (1st) and MHCI (2nd) probes for 30 min at 37 °C in western blot stripping buffer (#21059,

246   Thermo Fisher Scientific, MA USA). NKA isoforms were quantified as first probes, except for α_2 and

247   β_3, which were quantified as second probes following quantification of α_1 and β_1, respectively. Use

248   of different host species for α_1 (mouse) and α_2 (rabbit), and the distinct molecular bands of β_1 and β_3

249   allowed their quantitative assessment on the same membrane. No stripping of these membrane

250   portions were performed. After incubation in primary antibody, membranes were washed three times

251   in TBST and incubated for 1 h at room temperature in horseradish peroxidase (HRP)-conjugated

252 secondary antibody (goat anti-mouse immunoglobulins or goat anti-rabbit immunoglobulins; Pierce,
253 Rockford, IL, USA) diluted 1:20.000 with 5 % non-fat milk. After another three membrane washes in
254 TBST, protein bands were visualised using enhanced chemiluminescence (SuperSignal West
255 Femto, Rockford, Pierce, IL, USA) on a ChemiDoc MP imaging system (Bio-Rad). Quantification of
256 bands was performed in Image Lab 5.2.1 (Bio-Rad). Linearity between blot signal (density) and
257 tissue loaded for calibration curves was established on every membrane. The same researcher was
258 responsible for performing all western blots included in this study.

259

260 *Antibodies for immunoblotting*
261 Full details for the primary antibodies are shown in Table 1. Validation of antibodies is shown in Fig.
262 2 and was performed with positive and negative controls using mouse (EDL, soleus and brain), rat
263 (EDL, soleus, brain, kidney and cardiac muscles) and human (breast cancer cell lines, embryonic
264 kidney cells and skeletal muscle) tissues. The rats (Sprague Dawley, six months old) and mice were
265 sedentary and healthy. Rat and mouse tissue was obtained from animals used under La Trobe
266 University Animal Ethics Committee (approval AEC 14-33). Antibodies used to detect myosin heavy
267 chain slow- (type I, #A4.840) and fast-type (type IIa, #A4.74) isoforms were produced using the entire
268 immunogen sequence (MYH7 and MYH2, respectively). The former antibody recognises a C-
269 terminus epitope, whilst the latter remains to be mapped (DSHB).

270

271 *Reliability of western blotting*
272 The reproducibility of western blotting for each of the NKA isoforms and FXYD1 was determined
273 from triplicate western blots for each of the proteins and is expressed as the coefficient of variation
274 (CV; Fig. 3C). The calibration curves from whole-muscle, crude homogenate, which were loaded on
275 every gel, were used for the analysis. Protein abundance and total protein for each amount of protein
276 loaded was determined by normalising the density for a given loading amount to that of the slope of
277 the calibration curves.

278

279 *Statistical analysis*
280 Statistical analyses were performed in Sigma Plot (Version 11.0, Systat Software, CA). Data was
281 assessed for normality using the Shapiro-Wilk test. An appropriate transformation was used, if
282 required, to ensure a normal distribution of data before subsequent analysis. A two-way repeated-
283 measures (RM) ANOVA was used to evaluate the effects of time (Pre, Post) and fibre type (I and II)
284 within group and using data from both groups (pooled data). A one-way ANOVA was used to assess
285 the effect of group (COLD vs. CON) on Pre to Post change in protein content within fibre type (I and
286 II). Data normalised to total protein, and not relative changes, were used for these analyses. Where

287 applicable, multiple pairwise, *post hoc*, comparisons used the Tukey test. Cohen's conventions were

288 adopted for interpretation of effect size (*d*), where <0.2, $0.2-0.5$, $>0.5-0.8$ and >0.8 were considered

289 as trivial, small, moderate and large, respectively (Cohen, 1988). Data are reported as geometric

290 mean ± 95% confidence intervals (CI95) in figures. Note that the protein expression at Pre is not

291 equal to 1.0 due to the nature of using geometric means. F statistic (F), and *d* +/- CI95 are shown

292 for time and fibre-type effects, as well as group interactions. The α-level was set at $p \leq 0.05$.

293

294 **Results**

295 In figures, individual data are displayed on the left with each symbol representing the same

296 participant for all isoforms. On the right, geometric means ± CI95 are shown. Fold-changes are

297 reported relative to participants' geometric mean at Pre in type I fibres in CON.

298

299 *Validation of antibodies for immunoblotting*

300 The results from our antibody validation are shown in Fig. 2.

301 The isoform specificity of the NKA α_1 monoclonal antibody (#a6F) was verified using rat kidney and

302 brain as positive controls (Fig. 2A). Our results support previous findings in similar tissues

303 (Arystarkhova & Sweadner, 1996). We were also able to replicate the muscle type-specific

304 distribution of α_1 observed previously in rat skeletal muscles using other α_1 antibodies (Lucchesi &

305 Sweadner, 1991; Fowles *et al.*, 2004).

306 Specificity of the NKA α_2 polyclonal antibody (#07-674) was verified by a clear band at the predicted

307 molecular weight (105 kDa) in human and rat skeletal muscles, rat cardiac muscle and brain (Fig.

308 2B). These findings support previous results in the same tissues (Lucchesi & Sweadner, 1991;

309 Thompson & McDonough, 1996; Wyckelsma *et al.*, 2015). We found that α_2 was absent in rat kidney,

310 which also corroborates with the literature (Hundal *et al.*, 1994; Lavoie *et al.*, 1996; Crambert *et al.*,

311 2002). BLAST analysis of the peptide sequence specific to the α_2 antibody (#07-674, lot #2444088;

312 aa sequence 432-445, human) revealed no cross reactivity with other NKA isoforms.

313 NKA α_3 protein is highly expressed in rat brain (Lavoie *et al.*, 1997), but absent in rat cardiac muscle

314 (Lucchesi & Sweadner, 1991; Sweadner *et al.*, 1994) and kidney (Hundal *et al.*, 1994). One study

315 has shown that α_3 may be absent also in rat skeletal muscle (Lavoie *et al.*, 1997). By use of the

316 monoclonal NKA α_3 antibody (#MA3-915), we support these previous findings (Fig. 2C). The multiple

317 bands in human skeletal muscle could indicate multiple splice variants, as observed previously

318 (Tumlin *et al.*, 1994).

319 The monoclonal NKA β_1 antibody (#MA3-930) has been used previously in this journal to quantify β_1

320 protein in human muscle samples despite no clear evaluation of specificity of the lot used (Murphy

321 *et al.*, 2004). As β_1 protein is heavily glycosylated (Vagin *et al.*, 2006), and highly expressed in human

322 cancer cell lines (Salyer *et al.*, 2013), we tested the specificity of our β_1 antibody lot using

323 deglycosylated (PNGase treated for 3 h) and control human crude skeletal muscle samples and two

324 human breast cancer (MDA-MB-231 and MCF10.Ca1d) and control (HEKs, human embryonic

325 kidney) cell lines (Fig. 2D). Our findings of a substantial downshift of the predicted β_1 band in

326 deglycosylated samples and the markedly higher density of the same band in cancer cell lines vs.

327 HEKs strongly support the specificity of our antibody lot for β_1 protein. BLAST analysis of the peptide

328 sequence specific to the β_1 antibody (#MA3-930; aa sequence 195-199, sheep) confirmed absence

329 of cross reactivity with other NKA isoforms.

330 Specificity of the polyclonal NKA β_2 antibody (#22338-1-AP) was supported by detection of

331 recombinant β_2 protein (#Ag17818, ProteinTech; Fig. 2E, bottom panel). In further support, our

332 antibody was able to detect a band at the predicted molecular weight in our positive (rat brain), but

333 not in our negative (rat cardiac muscle), control sample. It also revealed a muscle type-specific

334 expression of β_2 in rat (Fig. 2E), in line with what has been found previously (Fowles *et al.*, 2004;

335 Kristensen & Juel, 2010b). BLAST analysis of the peptide sequence specific to the β_2 antibody lot

336 used in the present study revealed that it did not cross react with other NKA isoforms.

337 Using the monoclonal NKA β_3 antibody (#610992), we detected a band at the predicted molecular

338 weight in human muscle (albeit weak in our whole-muscle, crude homogenate), rat soleus and

339 cardiac muscle and kidney (Fig. 2F). Presence of β_3 protein in rat kidney is in coherence with

340 presence of β_3 gene transcripts in the same tissue (Malik *et al.*, 1996). We found that β_3 protein was

341 absent in rat EDL muscle and brain, suggesting tissue-specific expression at the protein level.

342 Specificity of the polyclonal FXYD1 antibody (#13721-1-AP) was supported by detection of a band

343 at the predicted molecular weight for our positive controls (human and rat skeletal muscles, rat

344 cardiac muscle and kidney; Fig. 2G). FXYD1 is either not expressed or very lowly expressed in

345 central nervous tissue (Crambert *et al.*, 2002). In accordance, we did not detect it in rat brain. The

346 higher density of the band in rat soleus vs. EDL muscle supports previous findings (Rasmussen *et*

347 *al.*, 2008). The longer migration of FXYD1 in the human vs. rat tissues could indicate specie- or case-

348 specific differences in post-translational modification, such as phosphorylation (Fuller *et al.*, 2009;

349 Thomassen *et al.*, 2016). BLAST analysis of the peptide sequence specific to the FXYD1 antibody

350 (sequence 1-92 encoded by BC032800) showed no signs of cross reactivity with NKA isoforms.

351

352

353 *Western blotting technical error*

354 The points constituting calibration curves on gels were strongly correlated ($r^2 \geq 0.98$, n = 22 gels,

355 Fig. 3A). Representative blots for these curves are shown in Fig. 3B. The reproducibility of western

356 blotting for each of the NKA isoforms and FXYD1 is shown in Fig. 3C. The technical error for these

357 isoforms were ~10-30 % for the protein loading amount used (1.5 fibre worth of protein), isoform-

358 dependent, and for most isoforms inversely related to the amount of protein loaded on each gel (Fig.

359 3C).

360

361 *Representative blots and verification of fibre type of fibre pools*

362 Representative blots for training-induced effects on protein abundance, and verification of fibre type

363 of fibre pools are shown in Fig. 4. It is clear from these results that fibres were grouped correctly,

364 confirmed by the clear difference in MHC-isoform expression between type I and II fibre pools.

365

366 *Na⁺,K⁺-ATPase α_1, α_2, and α_3*

367 In CON, α_1 protein increased with training (main effect for time: F = 19.78; p = 0.004; d = 1.41$\pm$0.77;

368 n = 7) in both type I (p = 0.008; d = 1.20$\pm$0.80) and II fibres (p = 0.002; d = 1.56$\pm$0.78). Similarly,

369 in COLD, α_1 protein increased with training (main effect for time: F = 8.66; p = 0.042; d = 1.55$\pm$0.81;

370 n = 4) in both type I (p = 0.053; d = 1.40$\pm$0.85) and II fibres (p = 0.029; d = 1.61$\pm$0.82). The increase

371 in α_1 protein was not different between groups in both fibre types (type I: p = 0.607; d = 0.07$\pm$0.04;

372 type II: p = 0.348; d = 0.36$\pm$0.21; Fig. 5A). In both groups, α_2 protein remained unchanged with

373 training (main effect for time in CON: F = 3.65; p = 0.105; d = 0.34$\pm$0.33; n = 7; and in COLD: F =

374 0.69; p = 0.452; d = 0.48$\pm$0.34; n = 5; Fig. 5B). Based on the pooled data (both groups), α_2 protein

375 remained unchanged with training in both fibre types (type I: p = 0.161, n = 12; type II: p = 0.112, n

376 = 12). Based on the same data, α_2 protein was 17 ± 46 % more abundant in type II, compared to

377 type I, fibres (main effect for fibre type: F = 9.63; p = 0.010; d = 0.38$\pm$0.26; n = 12). The α_3 protein

378 remained unchanged in both groups with training (main effect for time in CON: F = 3.71; p = 0.103;

379 d = 0.53$\pm$0.52; n = 7; and in COLD: F = 0.55; p = 0.501; d = 0.03$\pm$1.02; n = 5; Fig. 5C).

380

381 *Na⁺,K⁺-ATPase β_1, β_2, and β_3*

382 β_1 protein remained unchanged with training in both groups (main effect for time in CON: F = 1.25;

383 p = 0.306; d = 0.37$\pm$0.45; n = 7; and in COLD: F = 0.01; p = 0.960; d = 0.10$\pm$0.58; n = 5; Fig. 6A).

384 Based on the pooled data (both groups), β_1 protein increased by 44 ± 75 % in type II fibres with

385 training (p = 0.038; d = 0.53$\pm$0.35), but remained unchanged in type I fibres (p = 0.149; d =

386 0.29$\pm$0.24). Using the same data, β_1 protein was 62 ± 80 % more abundant in type II than in type I

387 fibres at Post (p = 0.003; d = 0.77±0.45, n = 12). As our western blots did not allow for quantitative

388 assessment of training-induced effects on NKA β_2 protein abundance, these data were excluded

389 from analysis and are not presented. A representative blot is shown in Fig. 4E. Based on the pooled

390 data (both groups), β_2 protein was 54 ± 95 % more abundant in type II, compared to type I, fibres

391 (main effect for fibre type: F = 14.84; p = 0.003; d = 0.56±0.56; n = 11). In CON, β_3 protein increased

392 with training (main effect for time: F = 38.62; p < 0.001; d = 1.23±1.05; n = 7) in both type I (p =

393 0.003; d = 1.18±0.97) and II fibres (p < 0.001; d = 1.24±1.15). Similarly, in COLD, β_3 protein

394 increased with training (main effect for time: F = 22.40; p = 0.009; d = 1.18±2.93; n = 5) in both type

395 I (p = 0.006; d = 1.18±2.93) and II fibres (p = 0.012; d = 1.21±1.92). The increase in β_3 protein was

396 not different between groups in either fibre type (group × time interaction: F = 0.59, p = 0.459 for

397 type I; and F = 0.04, p = 0.854 for type II). Based on the pooled data (both groups), β_3 protein

398 increased with training (main effect for time: F = 64.94; p < 0.001; d = 1.13±1.19; n = 12) in both

399 type I (2.55 ± 2.39 fold; p < 0.001; d = 1.07±1.35) and II fibres (2.22 ± 1.81 fold; p < 0.001; d =

400 1.22±1.03; Fig. 6B).

401

402 *Phospholemman (FXYD1)*

403 In both groups, FXYD1 protein also remained unchanged with training (main effect for time in CON:

404 F = 1.56, p = 0.258, d = 0.66±0.34, n = 7; and in COLD: F = 0.23, p = 0.654, d = 1.17±0.27, n =

405 5). Based on the pooled data (both groups), FXYD1 protein decreased by 33 ± 40 % in type I (p =

406 0.012; d = 0.82±0.22), but remained unchanged in type II fibres (p = 0.535; d = 0.51±0.17). Based

407 on the same data, FXYD1 protein was 35 ± 35 % more abundant in type I, compared to type II, fibres

408 at Pre (main effect for fibre type: F = 6.31; p = 0.020; d = 1.01±0.57; n = 12), but not at Post (F =

409 0.79; p = 0.384; d = 0.31±0.17; n = 12; Fig. 6C).

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417   **Discussion**

418   The main novel findings, which are summarised in Fig. 7, were that six weeks of intense exercise

419   training increased NKA α_1 and β_3 protein abundance in both fibre types and β_1 protein in type II

420   fibres, but had no impact on α_2 and α_3 abundance in either fibre type. Furthermore, training

421   decreased FXYD1 protein content in type I fibres and abolished its fibre type-dependent expression

422   detected before training. These results support that improvements in muscle K$^+$ regulation after a

423   period with repeated, intense exercise sessions in humans (McKenna, 1995; Nielsen *et al.*, 2004)

424   may, in part, be attributable to fibre type-specific modulation of NKA-isoform protein abundance. This

425   fibre type-dependent regulation of NKA could be one explanation for the dissociation between

426   muscle NKA activity and whole-muscle NKA-isoform protein content previously reported after a

427   period of intense training in humans (Aughey *et al.*, 2007). Furthermore, CWI neither adversely nor

428   favourably affects training-induced adaptations in NKA-isoform abundance in different muscle fibre

429   types. The technical error of western blotting for the NKA isoforms was ~10-30 % and isoform-

430   dependent.

431   *Regulation of NKA α isoforms in type I and II human muscle fibres by repeated-intense training*

432   A novel finding of the present study was that NKA α_1 protein abundance increased in both fibre types

433   with training (>2 fold, Fig. 5A). This indicates that α_1 protein content may be regulated similarly in

434   type I and II muscle fibres in response to sprint interval training in humans. In contrast, training was

435   without effect on α_2 and α_3 protein content. No effect of sprint training on α-isoform (α_1, α_2 and α_3)

436   abundance has previously been detected in isolated type I and II human skeletal muscle fibres

437   (Wyckelsma *et al.*, 2015), although the duration of both the sprints (4 s) and the training period (4

438   weeks) was shorter. However, other human studies have reported increases in [^{3}H]-ouabain binding

439   site content, reflecting increased α-isoform content, with sprint interval training (McKenna *et al.*,

440   1993; Harmer *et al.*, 2006). Our present results suggest these increases in binding may, in part, be

441   related to elevated α_1 protein content in both fibre types. While lack of sufficient muscle precluded

442   measurement of [^{3}H]-ouabain binding site content, our results support that increases in α_1 protein

443   content may also be important for improvements in a muscle's capacity for K$^+$ regulation during

444   repeated, intense exercise in humans (McKenna *et al.*, 1993).

445   Although there was not a significant increase in NKA α_2 protein content after training (pooled data:

446    $p<0.07$, $d = 0.42$), α_2 protein content was quantitatively higher in around three quarters of individual

447   type I and II fibre pools. This suggests a possible upregulation in α_2 that was not detected due to

448   inter-individual variability. Biological variability could perhaps also have influenced this result given

449   the low number of fibre segments (range: 2-16) contained in each fibre pool. However, this

450   explanation seems less likely, since AMPKβ2, SERCA1 and actin can be validly quantified in pools

451 consisting of 2-20 fibre segments, and pools of few segments ($n = 4$) are reproducible for quantitative

452 protein analysis (unpublished observation). In addition, consistent with a previous observation in

453 recreationally active men (37 %; Thomassen *et al.*, 2013), we found higher α_2 protein content in type

454 II vs. type I fibres (~17 %; Fig. 8B). This raises the possibility that the ion transport function of NKA

455 may be fibre type-dependent in humans, in line with what has been shown in rats (Kristensen & Juel,

456 2010a). No fibre-type difference for α_2 protein content was detected in subjects with a substantially

457 lower VO$_{2peak}$ relative to our participants (Wyckelsma *et al.*, 2015). Thus, in humans, higher training

458 status may be associated with greater type-II muscle fibre α_2 protein content.

459 Little is known about the relevance of NKA α_3 in skeletal muscles. Consistent with our current finding,

460 the α_3 protein content remained unchanged at the whole-muscle level after 3 weeks of cycling (8 ^{x}

461 5-min at 85% VO$_{2max}$) in well-trained men (Aughey *et al.*, 2007). Albeit not quantified in absolute

462 terms, our validation blots for α_3 revealed that this isoform is lowly expressed at the protein level in

463 human skeletal muscle (Fig. 2). This, along with the low α_3 mRNA expression detected previously in

464 the same tissue (Aughey *et al.*, 2007), downplays the functional importance of α_3 for the muscle's

465 contractile function in humans.

466 *Regulation of NKA β isoforms in type I and II human muscle fibres by repeated-intense training*

467 In a previous human study, four weeks of repeated-sprint training selectively increased the protein

468 content of NKA β_1 in type II muscle fibres (identified in individual fibre segments) (Wyckelsma *et al.*,

469 2015). In agreement, in the present study, β_1 protein abundance was higher (44%) after training in

470 type II fibres only. These observations, along with the increase in α_1 abundance in the present study,

471 could indicate a need for improved NKA activity in this fibre type with intense training. In support, in

472 rat gastrocnemius muscle, higher NKA hydrolytic activity was reported in membrane vesicles with a

473 reduced (50 %) molar α_2/β_1 ratio caused by higher β_1 content, relative to vesicles with a greater ratio

474 (1.0) (Lavoie *et al.*, 1997). Although our data did not allow for quantification of the possible impact of

475 training on β_2 protein content, we were able to confidently show that β_2 protein abundance is higher

476 in type II, relative to type I, fibres (Fig. 4E). This is in accordance with a previous finding in individual

477 fibre segments (27 % higher in type II fibres) (Wyckelsma *et al.*, 2015). As the K$_m$ for Na$^+$ of α/β_2

478 heterodimers (7.5-13 mM) is higher than the corresponding K$_m$ for α/β_1 complexes (4-5.5 mM) in rat

479 skeletal muscles (Kristensen & Juel, 2010a), our findings raise the possibility that human muscle

480 NKA activity may also be fibre type-dependent. Further research is needed to determine if NKA

481 activity is fibre type-dependent in human muscle. It has been reported in humans that NKA β_3 protein

482 content is substantially elevated with age in whole muscle (2.5 fold), and in type I (1 fold) and II (3

483 fold) skeletal muscle fibres (McKenna *et al.*, 2012; Wyckelsma *et al.*, 2016). In rat skeletal muscles,

484 a similar age-associated rise in β_3 content was reversed by ~14 weeks of endurance training (Ng *et*

485   *al.*, 2003), indicating that regular, continuous muscle activity potently affects β_3 content with age. We

486   demonstrate here that brief, repeated-intense training substantially increases NKA β_3 protein content

487   (increase > 2 fold) in type I and II human muscle fibres. Thus, the type of muscle activity seems

488   critical for β_3 content of both muscle fibre types in humans. The rise in β_3 content occurred in parallel

489   with elevated α_1 protein abundance (Fig. 5A and 6B), suggesting an enhanced potential for α_1/β_3

490   complex assembly in both fibre types post training. This supports that the β_3 isoform could take part

491   in the maintenance of resting membrane potential in both contracting and non-contracting muscle

492   fibres, in line with the ion transport function of α_1 (Radzyukevich *et al.*, 2013), although it could exert

493   other yet unidentified functions in skeletal muscles.

494   *Regulation of phospholemman (FXYD1) in human muscle fibre types by repeated, intense training*

495   Previous human studies using whole-muscle samples reported no alterations in FXYD1 protein

496   abundance after 10 days to 8 weeks of intense training (Thomassen *et al.*, 2010; Benziane *et al.*,

497   2011; Nordsborg *et al.*, 2012; Skovgaard *et al.*, 2014). Conversely, we found that six weeks of intense

498   training decreased FXYD1 abundance by 33 % in type I fibres (Fig. 6C). This decrease is reinforced

499   by the large effect size (0.82), small confidence interval (22 %), and good reproducibility (CV < 22

500   %; Fig. 3C). However, FXYD1 abundance was unchanged in type II fibres (Fig. 6C), highlighting for

501   the first time that FXYD1 is regulated in a fibre type-dependent manner by intense training in human

502   muscle. These results underline that physiologically relevant adaptations may have been overlooked

503   in the previous human studies due to their fibre type heterogeneous samples. But their use of sample

504   fractionation, i.e. removal of an indefinite amount of protein (Murphy & Lamb, 2013), may also have

505   influenced these previous outcomes. Co-localisation of unphosphorylated FXYD1 with α/β

506   heterodimers may inhibit their activation by increasing K_m (i.e. decreased affinity) for Na^+ and K^+

507   (Crambert *et al.*, 2002), whereas the interaction of FXYD1 with α_1 or α_2 isoforms remains unaffected

508   by exercise (Benziane *et al.*, 2011). This suggests that the decline in type-I fibre FXYD1 abundance

509   in the current study, independent of contraction-stimulated effects on its co-localisation with NKA α/β

510   complexes *per se,* may have been functionally important. Future training studies should combine

511   coimmunoprecipitation analyses and measures of NKA function (e.g. maximal *in vitro* activity or

512   muscle K^+ release during exercise), with fibre type-specific protein analyses to evaluate this

513   possibility.

514   *No effect of CWI on the effect of training on NKA-isoform protein abundance in different human*

515   *muscle fibre types*

516   In the present study, post-exercise CWI was without effect on the fibre type-specific changes in NKA-

517   isoform protein abundance with training. This underlines that CWI can be performed regularly after

518   intense training sessions (or match play) by athletes, to utilise its potent placebo effect (Broatch *et*

519   *al.*, 2014), without adversely affecting training-induced changes in NKA-isoform abundance, and

520   possibly, NKA function. Whether an absence of a CWI effect relates to an insufficiency to activate

521   the molecular signalling events prerequisite to transcriptional and/or post-transcriptional modification

522   cannot be resolved from the present study. However, our unpublished data reveal that a single

523   session of post-exercise CWI transiently increases NKA α_2 mRNA content in skeletal muscle of

524   healthy men, indicating a bout of CWI may augment the transcription, stabilisation, or both, of this

525   NKA mRNA transcript in human muscle. The present results highlight that this transient effect of CWI

526   does not translate into increased protein abundance after weeks of training. This raises the possibility

527   that α_2 protein content is regulated, in part, by factors other than an increased potential for mRNA

528   translation induced by higher mRNA content in response to repeated exercise sessions in human

529   muscle.

530   *Reliability of western blotting for NKA isoforms*

531   In many human studies, inferences about training-induced effects on the capacity of skeletal muscle

532   for ion regulation were based, in part or fully, on modest changes (9-39 %) in protein abundance

533   quantified using western blotting (Green *et al.*, 2004; Nielsen *et al.*, 2004; Iaia *et al.*, 2008;

534   Thomassen *et al.*, 2010; Gunnarsson *et al.*, 2012). This is surprising considering these studies were

535   limited by the use of fibre-type heterogeneous, fractionated samples (Murphy & Lamb, 2013), and,

536   for some, no consideration of blot linearity (Mollica *et al.*, 2009) nor antibody lot validation. With the

537   use of Stain Free imaging technology, wet transfer, and highly-sensitive chemiluminescence, we

538   report that in our hands the technical error of western blotting using protein from a fibre segment of

539   ~1-3 mm in length, amounting to ~15 μg wet weight tissue, is of a similar magnitude (~10-30 %) as

540   some of these previously reported changes (Fig. 3C). Our results highlight the importance of taking

541   into account the reliability of western blotting when interpreting changes in muscle NKA-isoform

542   protein abundance.

543   *Conclusions and perspectives*

544   The effectiveness of intense intermittent training to improve the capacity of skeletal muscle for

545   transmembrane Na⁺/K⁺ transport in humans is well-documented. The present study revealed that

546   these improvements may be ascribed, in part, to selective modulation of the protein abundance of

547   NKA β_1 in type II fibres, FXYD1 in type I fibres, and NKA α_1 and β_3 in both fibre types. The insights

548   gained from this study improve our understanding of how NKA function may regulated in different

549   muscle fibre types to accommodate the need for Na⁺ and K⁺ transport during intense, intermittent

550   exercise in human skeletal muscle. Furthermore, our data highlight that CWI can be performed on a

551   regular basis without adversely affecting training-induced modulation of NKA-isoform protein

552   abundance. Future work should assess protein responses in different muscle fibre types in

553 combination with measurements of muscle K+ handling to elucidate the functional relevance of these

554 fibre type-dependent protein responses to training.

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752 **Additional information**

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754 **Competing interests**

755 The authors have no conflict of interest that relates to the content of this article.

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757 **Author contributions**

758 Exercise testing and training were performed at Institute of Sport, Exercise and Active Living

759 (ISEAL), Victoria University, Melbourne, VIC 3011. Antibody validation, reproducibility, and protein

760 analyses were performed at the Department of Biochemistry and Genetics, La Trobe University,

761 Melbourne, VIC 3086, Australia. All authors contributed to drafting and critically revising of this

762 manuscript. DC, RMM, JRB and DJB contributed to the conception, design of experiments, and

763 collection and analysis of data. DC RMM, JRB, MJM and DJB contributed to data interpretation. All

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781 **Legends**

782 **Figure 1**. *A time-aligned schematic representation of the experimental setup.* Muscle was

783 sampled at rest before exercise (Pre), 2 min post (+0h) and 3 h after (+3h) 15-min of passive rest at

784 room temperature (CON group) or cold-water immersion up to the umbilicus (~10°C; COLD group)

785 that followed the first intermittent, sprint training session. A final biopsy was sampled at rest 48-72 h

786 following the last training session (Post) of six weeks of intense interval training either without (CON)

787 or with cold-water immersion (COLD) after each training session. This study was part of a larger

788 study. Muscle sampled at Pre and Post only was used for the current analyses.

789

790 **Figure 2.** *Validation of antibodies used to quantify Na+,K+-ATPase (NKA) isoforms and FXYD1.*

791 Crude samples of human vastus lateralis (Hu) and rat skeletal muscles (EDL, extensor digitorum

792 longus; Sol, soleus), rat cardac muscle/heart (Hrt), kidney (Kid) and brain (B), breast cancer cell

793 lines (M1, MDA-MB-231; M2, MCF10.Ca1d) and a control cell line (HEK, human embryonic kidney

794 293) were loaded onto 4-15% gradient, Criterion Stain Free gels. After SDS-PAGE, proteins were

795 wet-transferred onto 0.45 μm nitrocellulose membrane. Membranes were incubated with antibodies

796 raised against each of the NKA isoforms (α_{1-3} in A, B and C; β_{1-3} in D, E and F, respectively) or

797 FXYD1 (G), post-treated with specific secondary antibodies and imaged using chemiluminescence.

798 Isoform bands and molecular weight markers (lad) are shown in each image. In D, deglycosylation

799 of human skeletal muscle samples were performed using PNGase incubation (3 h) at concentrations

800 indicated in units (U). Recombinant β_2 protein (#Ag17818, Protein Tech) identical to the 64-171 aa

801 derived from E.coli, PGEX-4T with N-terminal GST was used to verify the specificity of the β_2

802 antibody (E, *bottom panel*). See *Methods* section for additional details.

803

804 **Figure 3**. *Representative calibration curves (A), blots (B), and western blotting reproducibility*

805 *(C) for Na+,K+-ATPase isoforms and FXYD1.* For every calibration curve, 5, 20, 30 and 60 μg w.w.

806 of human vastus lateralis muscle was loaded onto the gels. To enable visualisation of all symbols,

807 some data series in (A) and points in (B) were shifted by 1.70 units on the horizontal axis.

808 Reproducibility was expressed as the coefficient of variation (CV), and calculated by normalising the

809 density for a given loading amount to that of the slopes of the calibration curves for isoform blots and

810 total protein on Stain Free gel. In B), total protein on Stain Free gels (*top panel*) and representative

811 blots for the four-point calibration curves (*bottom panels*). Note that CV could not be calculated for

812 the α_3 and β_2 isoforms for 60 μg w.w. muscle, as this point was excluded for some of the calibration

813 curves due to saturation (cf. *Methods*).

814

Figure 4. *Representative blots for the effect of training without (CON) or with (CWI) cold-water immersion on NKA-isoform protein abundance.* Total protein on Stain Free gels used for analysis (*top panel*), myosin heavy chain isoform expression of fibre pools (*middle panel*), and representative blots for NKA isoforms from the same run (*bottom panel*). Blots for NKA α_1, α_2 and α_3 are shown in A, B and C, and NKA β_1, β_2, β_3 and FXYD1 in D, E, F and G, respectively.

Figure 5. *Effect of six weeks of repeated, intense training with (COLD) or without (CON) post-exercise cold-water immersion on Na+,K+-ATPase α-isoform protein abundance in type I and II human skeletal muscle fibres.* A) α_1, B) α_2, and C) α_3 protein abundance. Individual values (left) and geometric mean ± 95% confidence intervals (right) are displayed on each graph for CON (● closed symbols) and COLD (○ open symbols). Each symbol represents one participant (left) and is the same for protein and gene data (Fig. 2). The horizontal, dotted line represents the geometric mean expression at Pre in CON. Muscle was sampled at rest before (Pre) and after 6 weeks of training (Post). *$p < 0.05$, different from Pre within group; #$p < 0.05$, different from type I fibres based on pooled group data from both time points (please note the underscore of # for pooled group data).

Figure 6. *Effect of six weeks of repeated, intense training with (COLD) or without (CON) post-exercise cold-water immersion on Na+,K+-ATPase β-isoform and FXYD1 protein abundance in type I and II human skeletal muscle fibres.* A) β_1, B) β_3, and C) FXYD1 protein abundance. Individual values (left) and geometric mean ± 95% confidence intervals (right) on each graph for CON (● closed symbols) and COLD (○ open symbols). Each symbol represents one participant (left) and is the same for protein and gene data (Fig. 3). The horizontal, dotted line represents the geometric mean expression at Pre in CON. Muscle was sampled at rest before (Pre) and after 6 weeks of training (Post). Note the different scale on the secondary axis and that NKA β_2 protein data were excluded from analysis. *$p < 0.05$, different from Pre within group; *$p < 0.05$, different from pooled group data at Pre; #$p < 0.05$, Fibre type difference within time point; #$p < 0.05$, Fibre type difference within time point for pooled group data (please note the underscore of # and * for pooled group data).

Figure 7. *Summary of key findings.* A) Effect of repeated, intense exercise training on the protein abundance of Na+,K+-ATPase isoforms (α_{1-3} and β_{1-3}) and phospholemman (FXYD1) in different muscle fibre types (type I and type II). Bold vertical lines without arrow indicate NKA isoforms that remained unchanged with the given intervention. B) Protein distribution of Na+,K+-ATPase isoforms (α_{1-3} and β_{1-3}) and phospholemman (FXYD1) in type I and type II fibres, and the effect of the training period on this distribution (Post).
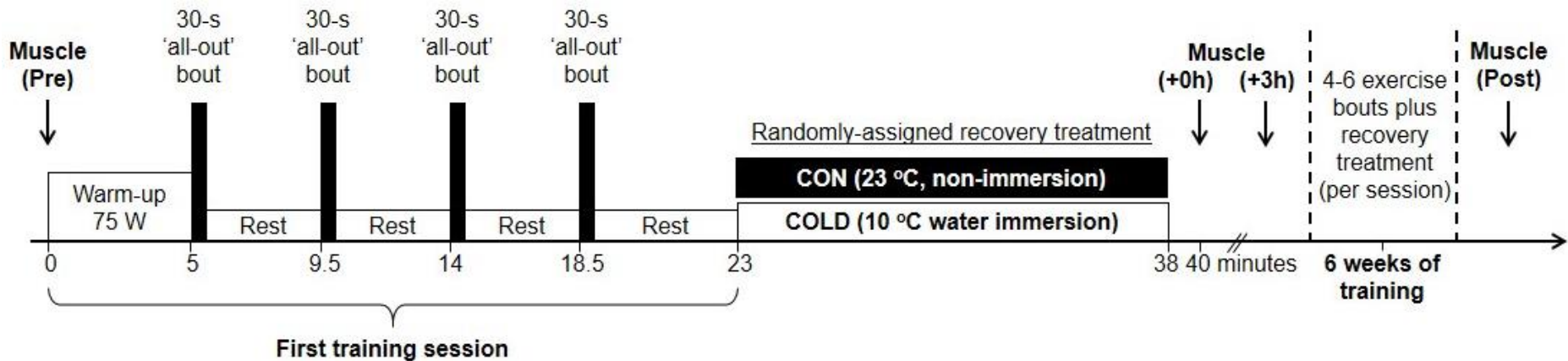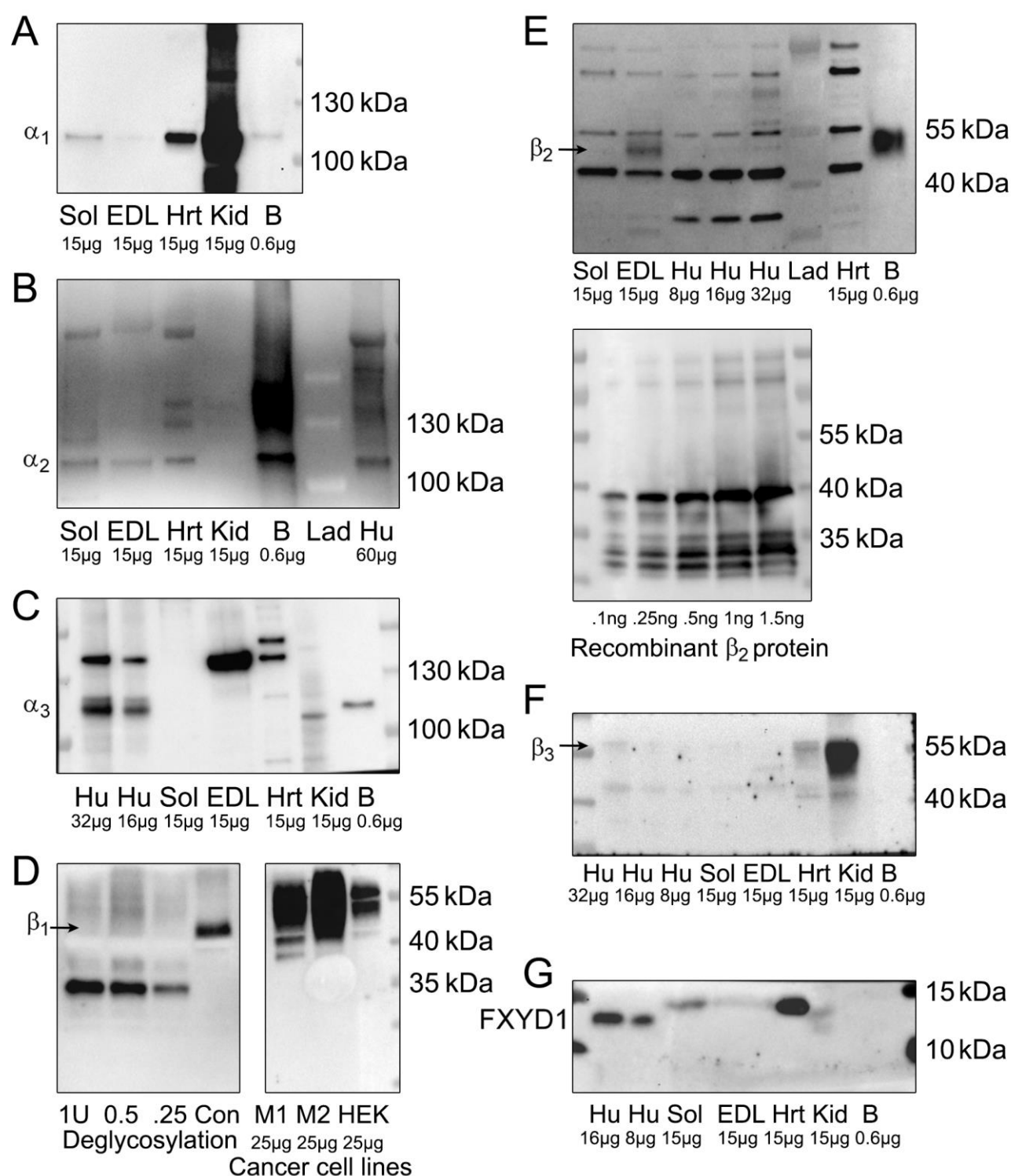
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Figures

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Figure 1

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861   **Figure 2**

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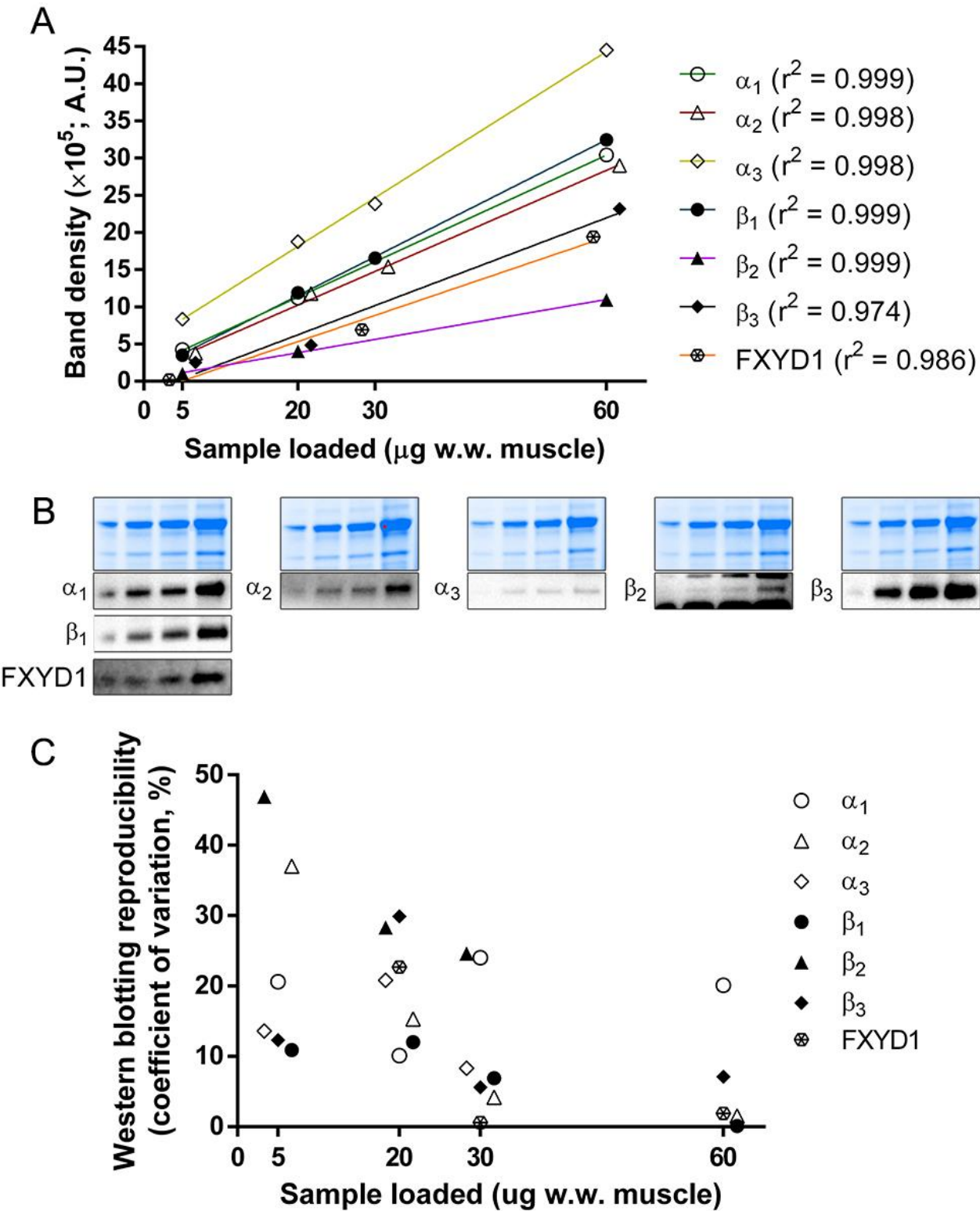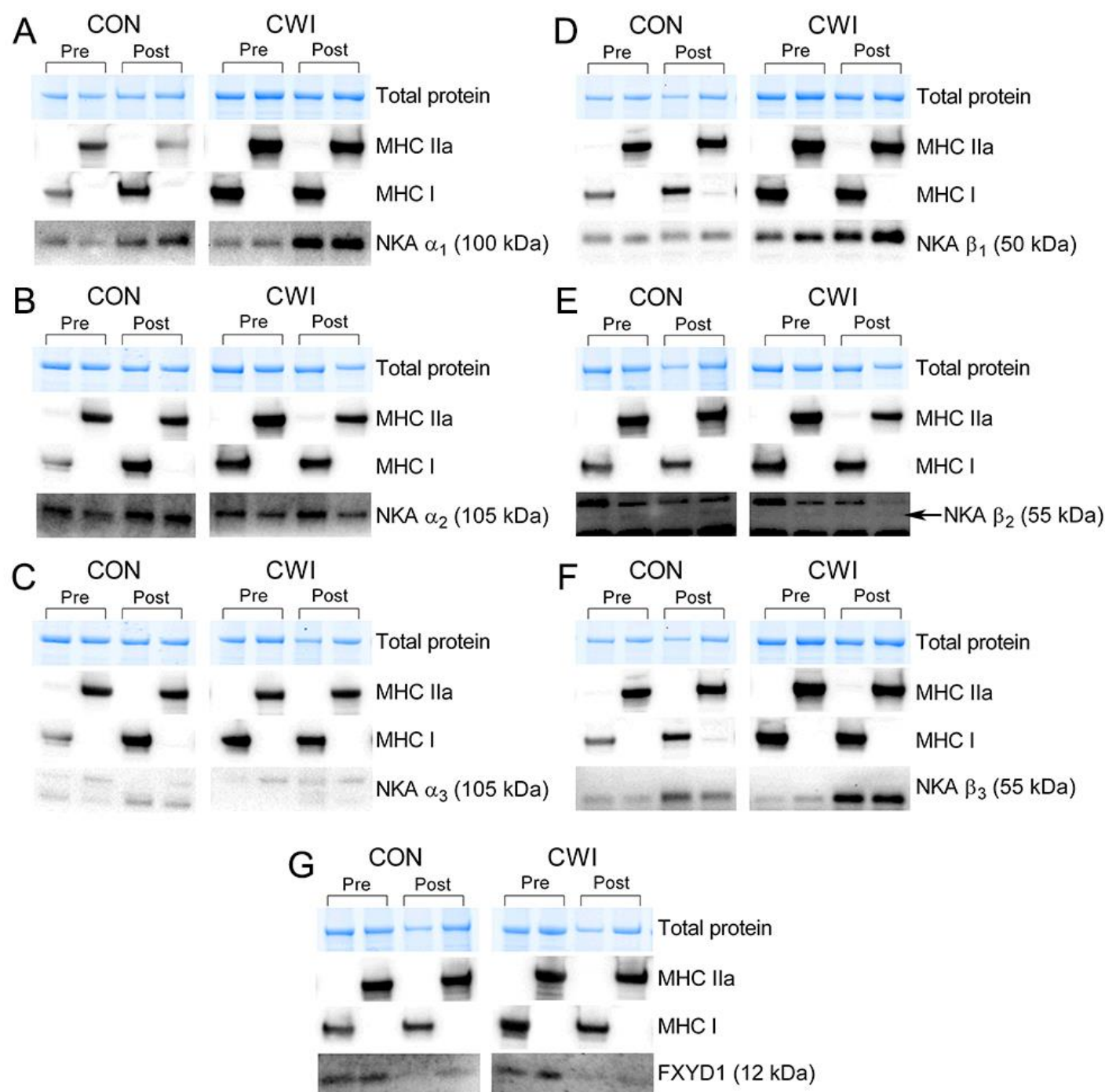
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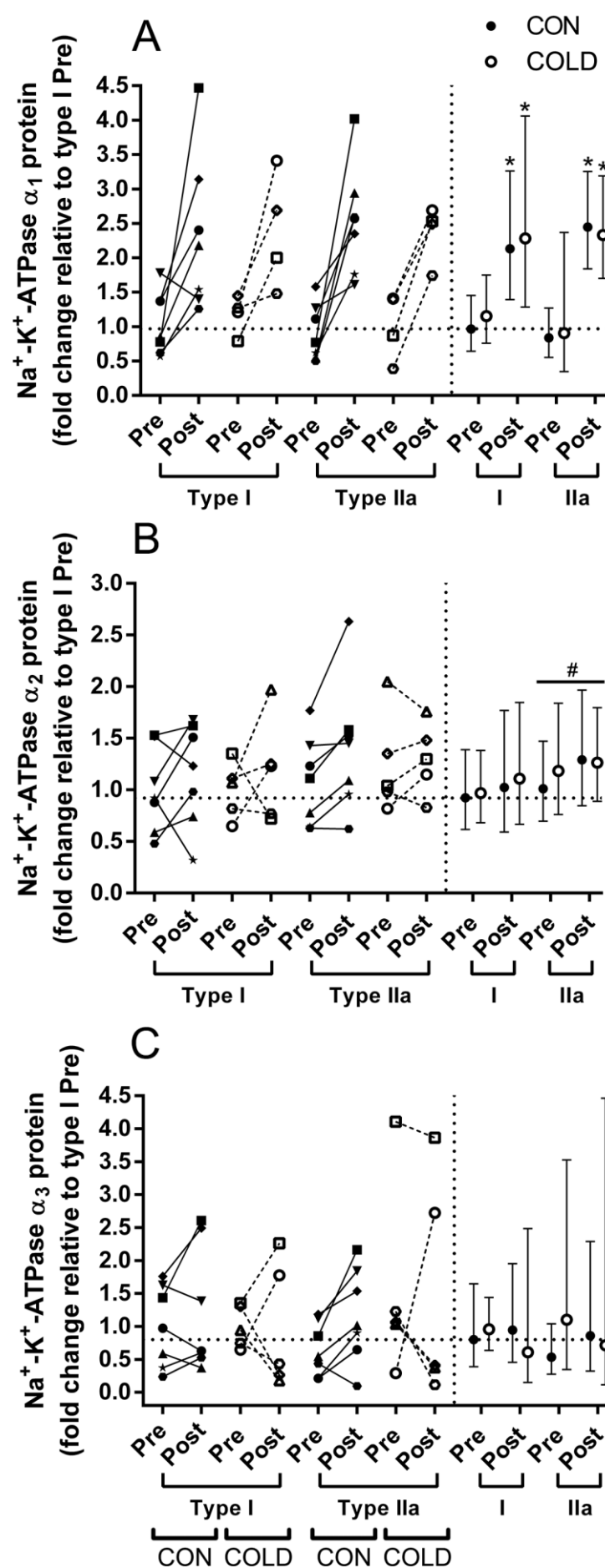
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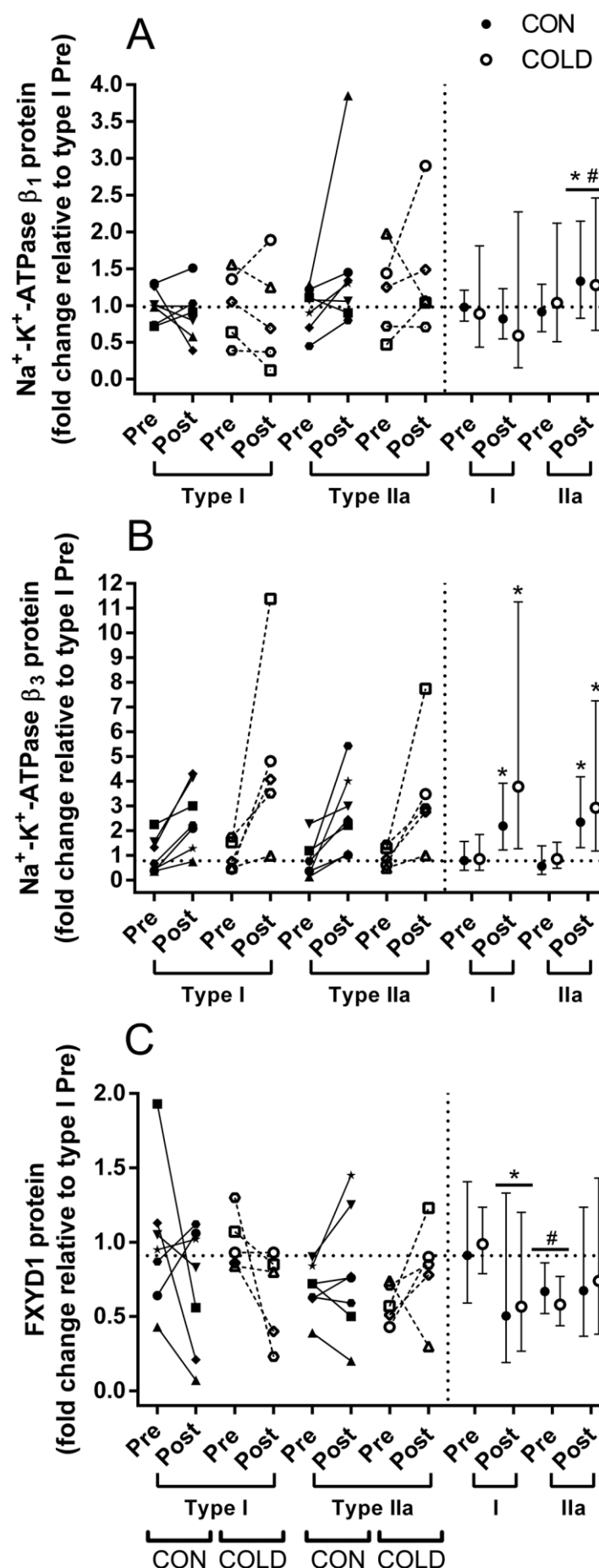
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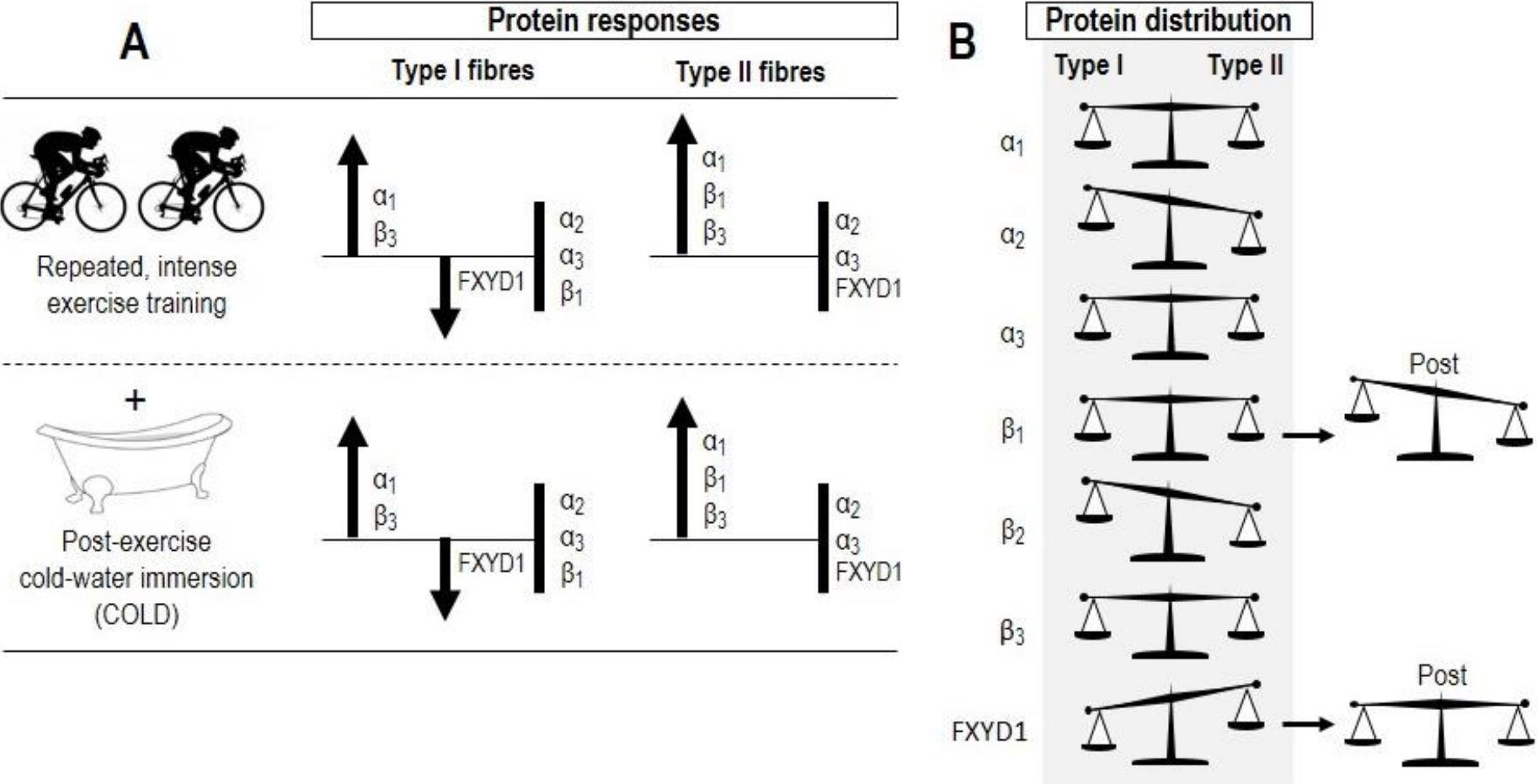
879 **Tables**

Table 1. Primary antibodies used for quantification of protein abundance of Na^+,K^+-ATPase isoforms, phospholemman (FXYD1) and myosin heavy chain isoforms in groups of type I or type II human skeletal muscle fibres

Protein	Primary antibody and supplier	Host species and isotype (antibody type)	Concentration	Molecular mass (kDa)
Na^+,K^+-ATPase α_1	Developmental Studies Hybridoma Bank (DSHB), University of Iowa (#a6F-s, lot from 8/15/13)	Mouse IgG, (monoclonal)	1:100	~100
Na^+,K^+-ATPase α_2	Merck Millipore (#07-674, lot #2444088)	Rabbit, IgG (polyclonal)	1:5000	~105
Na^+,K^+-ATPase α_3	Thermo Fisher (#MA3-915, lot #NJ175778)	Mouse, IgG (monoclonal)	1:1.000	~105
Na^+,K^+-ATPase β_1	Thermo Fisher (#MA3-930, lot #OH178971)	Mouse, IgG (monoclonal)	1:10.000	~50
Na^+,K^+-ATPase β_2	United BioResearch, Proteintech (#22338-1-AP, lot #00022128)	Rabbit, IgG (polyclonal)	1:500	~55
Na^+,K^+-ATPase β_3	BD Biosciences (#610992, lot #20600)	Mouse, IgG (monoclonal)	1:500	~55
FXYD1	United BioResearch (Proteintech #13721-1-AP, lot #00015622)	Rabbit, IgG (polyclonal)	1:5000	~12
MHC I	Developmental Studies Hybridoma Bank (DSHB), University of Iowa (#A4.840)	Mouse, IgM (monoclonal)	1:200	~200
MHC IIa	Developmental Studies Hybridoma Bank (DSHB), University of Iowa (#A4.74)	Mouse, IgG (monoclonal)	1:200	~200

MHC, myosin heavy chain; FXYD1, phospholemman. Antibodies were diluted in 1% bovine serum albumin in 1 × phosphate-buffered saline with 0.02% sodium azide and 0.025% Tween.

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