

1 *For submission to "PLOS ONE*

2 *Our file: Reid2021-Vf"*

3

4 **Regulation of skeletal muscle metabolism and contraction**
5 **performance via teneurin-latrophilin action.**

6

7 **Andrea L. Reid¹, David W. Hogg¹, Thomas L. Dodsworth¹, Yani Chen¹, Ross M. Reid², Mei**
8 **Xu¹, Mia Husic¹, Peggy R. Biga², Andrew Slee³, Leslie T. Buck¹ Dalia Barsyte-Lovejoy⁴,**
9 **Marius Locke⁵, David A. Lovejoy^{1*}**

10

11 1. Department of Cell and Systems Biology, University of Toronto, Toronto, ON Canada, 2.
12 Department of Biology, University of Alabama, Birmingham AL USA, 3. Protagenic
13 Therapeutics, Inc, Lexington MA USA, 4. Structural Genomics Consortium, University of
14 Toronto, Toronto, ON Canada 5. Department of Kinesiology, University of Toronto, Toronto, ON
15 Canada.

16

17 ***Corresponding Author:** David A. Lovejoy, Ph.D
18 Department of Cell and Systems Biology
19 University of Toronto,
20 Toronto, Ontario
21 M5G 3G5, Canada
22 Email: david.lovejoy@utoronto.ca
23 Tel: +1 (647) 999 2977
24

25 **Number of Words:** 13,243

26 **Number of Figures:** 11

27 **Number of Tables:** 3

28 **Number of References:** 72

29 **Keywords:** TCAP, GPCR, peptides, diabetes, calcium, CRISPR, siRNA, mitochondria

30

31

32 **Abstract**

33 Skeletal muscle regulation is responsible for voluntary muscular movement in vertebrates. The
34 genes of two essential proteins, teneurins and latrophilins (LPHN), evolving in ancestors of
35 multicellular animals, form a ligand-receptor pair, and are now shown to be required for skeletal
36 muscle function. Teneurins possess a bioactive peptide, termed the teneurin C-terminal associated
37 peptide (TCAP) that interacts with the LPHNs to regulate skeletal muscle contractility strength
38 and fatigue by an insulin-independent glucose importation mechanism. CRISPR-based knockouts
39 and siRNA-associated knockdowns of LPHN-1 and-3 shows that TCAP stimulates an LPHN-
40 mediated cytosolic Ca^{2+} signal transduction cascade to increase energy metabolism and enhance
41 skeletal muscle function via increases in type-1 oxidative fiber formation and reduce the fatigue
42 response. Thus, the teneurin/TCAP-LPHN system is presented as a novel mechanism likely to
43 regulate the energy requirements and performance of skeletal muscle.

44

45 **Introduction**

46 Skeletal muscle is critical for all voluntary behaviours and is derived from the earliest contractile
47 proteins present in the ancestral single-celled heterotrophs. Enhanced contractile strength and
48 efficient energy metabolism among these primitive skeletal muscle cells were critical for both
49 locomotion and feeding [1,2]. Because of these integrated requirements for the evolutionary
50 success of early metazoans, we have postulated that essential intercellular signaling systems
51 originating phylogenetically early, conferred a selective advantage upon these basal heterotrophs
52 by linking sensory and motor functions with cell metabolism [3]. Numerous studies have indicated
53 that the teneurins and their receptors, the latrophilins (LPHN) are part of an ancient regulatory
54 system that modulates cell adhesion and metabolism. The introduction of the teneurin and LPHN
55 genes into multicellular animals occurred via lateral gene transfer from prokaryotes into a single-
56 celled ancestor of metazoans [4-9]. Thus, the teneurins and LPHNs were evolutionarily poised to
57 play a seminal role in the development and coordination of cell-to-cell communication, adhesion
58 and metabolic activities.

59 Teneurins are essential for development and maintenance of the central nervous system (CNS)
60 [10-18]. Comprising a family of four paralogous proteins in vertebrates, the teneurins possess
61 several functional domains that confer specialized actions on their extracellular and intracellular
62 regions [19-21]. As type-II proteins, their carboxyl terminus is displaced extracellularly. The most
63 distal region contains a β -barrel structure unique to metazoans, but is similar to that found in
64 prokaryotic Tc-toxins [9,14,22-25]. Associated with this structure lies an extended amino-acid
65 chain termed the ‘teneurin C-terminal associated peptide’ (TCAP) [22-24]. The TCAPs possess
66 primary structure similarity to the Secretin superfamily of peptides that include, not only secretin
67 paralogues such as vasoactive intestinal peptide (VIP), growth hormone-releasing hormone

68 (GHRH), glucagon and pituitary adenylate cyclase activating peptide (PACAP), but also the
69 calcitonin and corticotropin-releasing factor (CRF) families. One of the distinguishing aspects of
70 this peptide superfamily are their roles in the coordination of sensory, motor and energy
71 metabolism [3,26,27].

72 The LPHNs are G-protein-coupled receptors (GPCR) belonging to the Adhesion GPCR family
73 (ADGRL) and are cognate receptors of the teneurins in vertebrates [28-30]. LPHNs have three
74 distinct paralogous forms (LPHN1-3) and can bind to the C-terminal region of the teneurins, which
75 include the TCAP region. For example, Teneurin-2 and LPHN-1 binds with nanomolar affinity at
76 the lectin-domain of LPHN-1[31]. A splice variant of C-terminal domain of teneurin-2, also termed
77 ‘LPHN1-associated synaptic surface organizer’ (Lasso), binds to LPHN with, likewise, high
78 affinity in neurons. Moreover, transgenic over-expression of both TCAP-1 and the hormone-
79 binding domain (HBD) of LPHN-1 results in co-precipitation of both transgenic proteins
80 indicating an interaction between TCAP-1 and LPHN-1 [32]. Recent structural studies of the
81 teneurins indicate that the TCAP region may be auto-catalytically cleaved from the teneurins after
82 interaction with the LPHNs [14,22], or could be the result of a distinct teneurin splice variant
83 resulting in the mRNA expression of the terminal exon that includes the TCAP sequence [33,34].

84 Regardless of the mechanism of TCAP release, the expected TCAP mature peptide, based on its
85 genomic sequence, has distinct biological properties. Synthetic TCAP-1 regulates cytoskeletal
86 elements and energy metabolism in neurons critical for neuroplastic modulation in the central
87 nervous system (CNS). In rats, TCAP-1 modifies dendritic arborization and spine density in the
88 hippocampus [35,36], findings that were corroborated in primary cultures of rat embryonic
89 hippocampal tissues that exhibited increased filopodia, neurite and axon development [33,37,38].
90 Thus, these actions indicate a role of TCAP-1 in CNS energy metabolism. Moreover, subcutaneous

91 administration of TCAP-1 into rats increases brain glucose uptake as assessed by functional
92 positron emission tomography (fPET). These observations were corroborated by the expected
93 decreased serum glucose and insulin levels in rats, and in cell culture studies showing that TCAP-1
94 stimulates glucose uptake by increased glucose transporter transit to the membrane and
95 subsequently increases in ATP turnover providing increased energy for the neurons [39].

96 However, given the evolutionary history of the teneurins, it is plausible that the teneurins, LPHNs,
97 and TCAP could also play a role in the regulation of skeletal muscle. Skeletal muscle is one of the
98 most important sites of glucose metabolism and is responsible for 40% of glucose-associated
99 energy requirements [39] and 80% of glucose uptake under insulin-stimulated conditions [40].
100 Muscle function and metabolism are intrinsically linked, as evidenced by metabolic syndromes
101 that result in poor muscle function and degradation. A key example of this is demonstrated in
102 patients with type-II diabetes where patients have reduced skeletal muscle function in the grip
103 force test compared to non-diabetic patients [41,42].

104 Based on these previous findings, we investigated the role of TCAP-1 on skeletal muscle function
105 for the first time. We demonstrate that skeletal muscle possesses the critical elements of teneurin-
106 LPHN interaction, and show that TCAP-1 regulates skeletal muscle contractile kinetics *in vivo* in
107 rats. These studies are supplemented by *in vitro* studies, using the mouse skeletal cell line, C2C12,
108 to show that TCAP regulates intracellular skeletal Ca^{2+} flux similar to that shown in neurons [3,39].
109 Moreover, like neurons, the TCAP-mediated Ca^{+2} response leads to increased glucose metabolism
110 and mitochondrial activation, but results in skeletal muscle fiber regulation. We posit that the
111 teneurin-LPHN interaction is essential for skeletal muscle physiology and regulates skeletal
112 muscle performance.

113 **Materials and Methods**

114 **Peptide Synthesis and Solubilization**

115 Both peptides; rat/mouse TCAP-1 and scrambled TCAP-1 (Fig. 1), were synthesized commercially
116 by AmbioPharm, Inc. and prepared as an acetylated salt at 95% purity. Peptides were solubilized
117 in saline after alkalization as previously described [37] then diluted into the required media for *in*
118 *vitro* or *in vivo* studies (see below). The primary structure of all four rat and mouse TCAPs are
119 identical to each other (Fig.1A) and possess a 73-83% sequence identity among the overall
120 sequences, although most of these changes reflect homologous and conservative substitutions. For
121 this reason, TCAP-1 was used in both rat and mouse preparations. Synthetic rat/mouse TCAP was
122 prepared with an initial N-terminal pyroglutamic acid to inhibit N-terminal-directed peptidases,
123 and a C-terminal amidated-residue as expected based on the genomic sequence [24,37]. As a
124 control peptide, we have utilized a scrambled (sc) amino acid sequence version of rat/mouse
125 TCAP-1 where each residue, with the exception of the initial pyroglutamyl residue (pE), was
126 randomized in its placement within the peptide (Fig. 1B). This sc-TCAP-1 has been used in
127 previous studies to establish an additional level of controls to ensure that TCAP-1 is not affecting
128 non-specific (e.g. oligopeptide transporters; non-target receptors) actions. The vehicle included sc-
129 TCAP solubilized in 0.9% saline or cell culture medium, unless otherwise stated.

130

131 **Figure 1. Primary structures of rat and mouse TCAP peptides. A.** Comparison of the amino
132 acid sequences of mouse and rat TCAPs. **B.** Primary structure of the peptides used in this study.
133 Grey boxed regions indicates regions of identity relative to the rat/mouse TCAP isoforms.

134 **Animals**

135 Male adult Sprague-Dawley (SD) rats (Charles River, Canada) were used for the short-term and
136 long-term muscle function studies. The metabolic and endocrine studies of TCAP-1 on rats were
137 approved by the University of Toronto Animal Care Committee (UACC) under the auspices of the
138 Canadian Council of Animal Care. Male adult Wistar rats (~250 g) (Charles River, USA) were
139 used for the functional positron emission tomography (fPET) studies performed by Molecular
140 Imaging, Inc. (Ann Arbor, MI, USA) and approved by the American Association of Animal
141 Laboratory Care (Hogg et al., 2019). In both sets of studies, animals were weighed weekly and
142 monitored for any signs of distress or illness (e.g. loss of hair, extreme weight loss, abnormal
143 behaviours). However, no animals showed overt indications of stress and all were utilized for these
144 studies.

145

146 **In Vivo Studies**

147

148 The short-term application of TCAP-1 utilized 16 male adult SD rats (250g) that were acclimated
149 for 1 week (w) on a 12:12 light-dark (LD) cycle. For 5 days (d) daily, the animals were treated
150 with either vehicle or TCAP-1 (10 nmoles/kg) by subcutaneous (SC) injection in the intrascapular
151 region. Animals were tested for muscle function by electrical muscle stimulation (see below) 3d
152 after the last treatment. Animals were immediately euthanized afterward. For the long-term study,
153 20 adult male SD rats (350g), acclimated for 1w on a 12:12 LD cycle and were treated with either
154 vehicle or intrascapular TCAP-1 (SC; 25 nmoles/kg) for 3 months (1 injection/week). Muscle

155 function by electrical muscle stimulation was tested 2w after the last treatment. Animals were
156 immediately euthanized after electrical muscle stimulation studies (see below).

157

158 **PCR Expression of teneurin and LPHN**

159 RNA was extracted from tibialis anterior (TA) muscles using TRIzol (Thermo Scientific,
160 Waltham, MA, USA) following the manufacturer's instructions. The PCR reaction mix included
161 5 μ L cDNA, 2 μ L forward primer and 2 μ L reverse primer (Invitrogen; Table 1), 14.2 μ L water
162 (Sigma, Oakville, ON), 3 μ L 10x Taq Buffer with KCl (Thermo Scientific), 1.8 μ L MgCl₂ (Thermo
163 Scientific), 1 μ L deoxynucleotide Solution Mix and 0.5 μ L Taq DNA Polymerase (New England
164 Biolabs). The reactions were incubated in an Eppendorf Mastercycler Gradient Thermal Cycler
165 for 7m at 95°C; followed by 35 cycles of 60s at 95°C, 90s at 67°C, and 35s at 72°C; and then held
166 at 4°C. cDNA samples were resolved on a 3% agarose gel at 100 V for 1.5h and visualized using
167 a Bio-Rad ChemiDoc MP System with 0.5s exposure.

168

169

170

171

172

173

174

175

176

177

178 **Table 1. Primers used for in vitro and in vitro RT-PCR analyses.** Forward and reverse primer pairs for
179 the four teneurins, TCAPs, three ADGRLs, and β -actin control are indicated.

180

181	Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Expected
182				band size
183				
184	Teneurin-1	CACAGTCAGCGGCGTTACATCTTTGAG	GAATCCGTCATGCATCAGGTGTATTGT	342 bp
185	Teneurin-2	ATCCTGAACTCGCCGTCCTCCTTA	CTCCAGGTTCTGAGTGGACACGGC	405 bp
186	Teneurin-3	GTGAGTACCGTTGATGTCAAAGATG	AGTGGAAATACCCGGTGGGGAAGCAC	427 bp
187	Teneurin-4	ATCGACCAATTCCTGCTGAGCAAG	CCAGAGAGGCATCCCGGTAGAGTC	567 bp
188				
189	TCAP-1	ACGTCAGTGTTGAATGGGAGGACTA	CCTCCTGCCTATTTCACTCTGTCTCAT	351 bp
190	TCAP-2	GACAAGATGCACTACAGCATCGAG	CCATCTCATTCTGTCTTAAGAACTGG	496 bp
191	TCAP-3	CAACAACGCCTTCTACCTGGAGAAC	CGATCTCACTTTGTCGCAAGAACT	506 bp
192	TCAP-4	TTGCCTCCAGTGGTCCATCTT	TGGATATTGTTGGCGCTGTCTGAC	602 bp
193				
194	ADGRL1	AGCCAGAGGACTTGACTCA	TTCTAGGCCTCAGAGCTACAT	249 bp
195	ADGRL2	TGGAGCAAAAAGTC	TTCAAAACAGC	203 bp
196	ADGRL3	TGAGCAACTGTGTGCAAATT	TAACCACCAGCCACACCAT	327 bp
197				
198	β -actin	CAGCCATGTACGTAGCCATCCA	ATGTCACGCACGATTTCCCTCT	247 bp
199				

200

201

202 **Histological Studies**

203 TA muscle was excised then flash-frozen in liquid nitrogen cooled-isopentane. The tissue was

204 cryo-sectioned at 10 μ m at -20°C and transferred to coverslips and fixed using ice-cold methanol.

205 Following blocking for 1h with 10% normal goat serum (NGS: Cell Signaling, Inc.), the primary

206 antibody (Table 2), diluted in 1% NGS, was added and incubated overnight (ON) at 4°C.

207 Subsequently, after 3 phosphate-buffered saline (PBS) washes, the secondary antibody was added

208 and incubated for 1h at room temperature (RT) in the dark. Coverslips were attached, and the tissue

209 imaged using confocal microscopy (Leica TCS-SP8) at 400x magnification. For fluorescence

210 analyzes of protein expression, Image J software was used to measure arbitrary fluorescent units

211 (AFU), where changes of AFU are proportional to protein expression changes. A total of 5 slides

212 were quantified with 8 regions of interest (ROI) investigated. ROI was defined as regions with
213 multiple cell interactions free of artifacts.

214

215 **Table 2.** Primary and secondary antibodies used in western blot analyzes.

216

217	Name	Primary Antibody	Secondary Antibody	Expected band size
218				
219				
220	LPHN-1	Goat polyclonal IgG	Donkey anti-goat HRP-linked	130 kDa
221	LPHN-2	Goat polyclonal IgG	Donkey anti-goat HRP-linked	163 kDa
222	LPHN-3	Rabbit polyclonal IgG	Donkey anti-rabbit HRP-linked	162 kDa
223	SDH-A	Mouse monoclonal IgG	Rabbit anti-mouse HRP linked	70 kDa
224	β -actin	Mouse monoclonal IgG	Rabbit anti-mouse HRP linked	42kDa
225				

226

227

228 **Functional Positron Emission Tomography (fPET)**

229 Male Wistar rats were treated with either vehicle or TCAP-1 (10 nmoles/kg) by intrascapular SC
230 injection. 3d after treatment, 1 mCi of [^{18}F]-2-deoxyglucose (^{18}F -DG) radiotracer (IBA Molecular)
231 was administered intravenously (IV) under anesthesia. fPET scans were performed using a
232 Siemens Inveon microPET small animal PET scanner using the protocol as previously described
233 [39]. Briefly, body temperature was maintained with a thermostat-regulated recirculating water-
234 heated pad. Static emission data was acquired for 20m. The PET list mode data was converted to
235 2-dimensional (2D) sinograms, corrected for random coincidences, and normalized for scanner
236 uniformity. The PET image analysis was performed using the Amira 5.5.0 analysis software
237 package. For whole body ROIs (regions of high ^{18}F -DG uptake), a low threshold was set to

238 delineate specific signals in the whole body while eliminating background. The total PET counts
239 were calculated from all voxels within the segmented volumes of interest. These images were then
240 compiled into 3-dimensional (3D) projections, thus allowing for accurate analyzes of muscle
241 tissue. Fluorescence of the mean pixel was calibrated to volume of muscle being analyzed (mean
242 pixel fluorescence/mm³).

243

244 **NADH staining and analysis**

245 TA muscles from the treated SD rats were cryo-sectioned at 10 µm thickness as described above.
246 Cryo-sections were washed 2x with PBS, then 0.2% NBT solution in PBS (containing 0.1%
247 NADH; Sigma, Oakville, ON) was added and incubated for 30m at 37°C. Slides were washed 2x
248 in PBS before mounted, imaged at 100x magnification and analyzed on Image J software for pixel
249 density, where darker pixels represent higher levels of NADH. Expression of NADH was analyzed
250 based on a minimum of 100 fibres per tissue, with a minimum of 3-4 tissues analyzed for each
251 group.

252 **Muscle function and integrity testing by electrical muscle stimulation**

253 The electrical muscle stimulation protocol was followed as described by Holwerda and Locke
254 [43] with minor modifications. Briefly, animals were anesthetized with 5% isoflurane in 1L/min
255 O₂, and subsequently positioned into the testing apparatus. A 25g needle was inserted through the
256 soft tissue of the knee in order to ensure a stable position. The foot was placed on a lever attached
257 to a servomotor and taped in position. Electrodes were placed below the skin but adjacent to the
258 TA muscle. Dynamic Muscle Control (DMC; Version 5; Aurora Scientific) software was used for
259 electrical stimulation and analyzes. The correct voltages for peak tetanic tension was established
260 by increasing voltage by 1V increments until optimal tetanus twitch was achieved. The test began

261 with a single tetanus and single twitch protocol to establish the baseline followed by a 6-min
262 fatigue protocol (8V, 200 Hz, 300 ms). After the termination of the protocol, tetanic and twitch
263 tensions were recorded at 0, 1, and 5 mins. Animals were immediately euthanized after recovery
264 measurements were recorded.

265 **Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR):**

266 TA muscle mRNA and cDNA were prepared as previously described (see above). The cDNA
267 from all samples was used to prepare pools to establish standard curves of each gene. The cDNA
268 pool or cDNA samples were mixed with MasterMix containing SYBR select. The reactions were
269 loaded in a 384-well PCR plate and run in a BioRAD qRT thermal cycler for 2m at 50°C, 7m at
270 95°C; followed by 39 cycles of 60s at 95°C, 90s at 67°C, and 35s at 72°C. Melting curves were
271 established by a step-wise gradient from 60-90°C. The myosin heavy chain (MHC) isoforms,
272 MHCI, MHCIIa, MHCIIx and MHCIIb were analyzed by real-time PCR using the mRNA and
273 cDNA prepared above (see Table 3).

274

275

276

277

278

279

280

281 **Table 3.** Rat MHC isoforms and β -actin control primers used for qRT-PCR. Forward and reverse
282 primer pairs are indicated for the four MHC isoforms.

283

284

285 Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
287 MHC I	GAATGGCAAGACGGTGACTGT	GGAAGCGTACCTCTCCTTGAGA
288 MHC IIa	ATGACAACCTCTCTCGCTTTG	TTAAGCTGGAAAGTGACCCGG
289 MHC IIb	GAACACGAAGCGTGTCATCCA	AGGTTTCGATATCTGCGGAGG
290 MHC IIx	CCAATGAGACTAAGACGCCTGG	GCTATCGATGAATTGTCCCTCG
291 β -actin	AGCCATGTACGTAGCCA	CTCTCAGCTGTGGTGGTGAA

292

293

294

295

296 **In Vitro Studies**

297

298 **Culture and cDNA analyses of C2C12 cell line**

299 The immortalized murine skeletal muscle cell line, C2C12, was used for all *in vitro* studies. Cells
300 were maintained at 60-70% confluency with Dulbecco's Modified Eagle Medium (DMEM)
301 supplemented with 20% fetal bovine serum (FBS) and a penicillin/streptomycin antibiotic
302 combination (Invitrogen, Burlington, ON, Canada). To induce differentiation, the media was
303 changed to DMEM supplemented with 10% horse serum (HS) with the penicillin/streptomycin
304 antibiotic combination, and the cells were allowed to differentiate for 6d (media replaced every 24
305 hrs). For treatment, cells were serum-starved for 3h and then treated with either vehicle or TCAP-1
306 (100 nM). The identification of teneurin, TCAP and LPHN cDNAs in C2C12 cells was performed

307 with mRNA extracted from differentiated mouse C2C12 cells using the method described above
308 using the primer sequences indicated in Table 1.

309

310 **Live-cell calcium imaging in C2C12 myotubules**

311 The C2C12 skeletal cells were grown and differentiated on poly-D-lysine-coated 25 mm round
312 No. 1 glass coverslips (Warner Instruments, Hamden, CT, USA). Changes in intracellular Ca^{2+}
313 were assessed using the membrane-permeable fluorescent indicator fluo-4, AM (Invitrogen,
314 Burlington, ON, Canada). Cells were loaded with fluo-4 by incubating coverslips in DMEM
315 containing 10 μM fluo-4 for 30m (37°C) followed by a 15m wash in Locke's Buffer (305-310
316 mOsmol at 22°C). In experiments assessing changes in intracellular Ca^{2+} , coverslips were placed
317 in a flow-through bath chamber (RC-40HP, Warner Instruments, Hamden, CT, USA) of an
318 inverted microscope (Axio Observer Z1, Zeiss, Toronto, ON, Canada) equipped with a 40 \times oil
319 immersion objective. Cells were continuously bulk-perfused with Locke's buffer via a gravity drip
320 perfusion system at a rate of 2–3 ml/min at RT. Changes in fluo-4 fluorescence were imaged using
321 a green fluorescent protein (GFP) filter set (Semrock, Rochester, NY, USA) and a X-Cite 120
322 fluorescence illumination system (Excelitas Technologies, Mississauga, ON, Canada), controlled
323 by Volocity 4.0 imaging software (Quorum Technologies Inc., Guelph, ON, Canada).
324 Fluorescence emissions were detected with an Orca-ER Hamamatsu B/W CCD digital camera
325 (Hamamatsu, Middlesex, NJ, USA). Fluo-4 was excited with a wavelength of 480 nm for 100ms
326 every 3-5s and fluorescence emission was measured at wavelength of 516 nm.

327

328

329

330 **Caffeine stimulation experiments**

331 Caffeine action on the cells was used to establish that the cells were viable. Caffeine (4 mM;
332 Sigma-Aldrich, Oakville, ON) was applied to C2C12 myotubes to stimulate Ca²⁺ release from the
333 sarcoplasmic reticulum (SR). Cells were either pre-treated with TCAP-1 (100 nM) or vehicle for
334 1h before stimulation with caffeine. Using Velocity 4.0 imaging software, ROIs were taken from
335 cytosolic regions within the myotubules (n= 4 coverslips, 4-5 ROIs per coverslip).

336

337 **GLUT4 Immunocytochemical studies**

338 C2C12 cells were differentiated, as described above. After 3h of serum starvation, myotubules
339 were treated either with vehicle, TCAP-1 (100 nM) or insulin (100 nM) for 15 or 30m. Cells were
340 then fixed using 4% paraformaldehyde and subsequently blocked with 10% NGS for 1h at RT.
341 The GLUT4 primary antibody, diluted in 1% NGS, was added to the cells and incubated at 4°C
342 overnight (OT). Following 4 PBS washes, the secondary antibody (diluted in 1% NGS) was added
343 and incubated 1h at RT. The coverslips were mounted using DAPI-containing Vectashield. Slides
344 were imaged on a confocal microscope with a 40x oil objective. The images were analyzed using
345 Image J, where myotubules were selected as ROIs and were analyzed for red pixel intensity values,
346 representing GLUT4 levels, and normalized to area size (n= 3-4 coverslips per treatment, 7-8
347 myotubules per coverslip). For the IP3R inhibitor, 2-aminoethoxydiphenyl borate (2-APB; Sigma
348 Aldrich, Oakville, ON) experiments, 2-APB (100 µM) was applied for 4m before the start of
349 treatment with either sham (Locke's buffer) or TCAP-1 (100 nM), containing 2-APB for
350 continuous blocking of IP3R.

351

352

353 **Radioactive glucose uptake**

354 The ³H-2-deoxyglucose uptake protocol was followed as previously described with minor
355 modifications [44,45]. At day-6 post-plating, C2C12 myotubules were washed 2x with Locke's
356 without serum and glucose. The culture was incubated in the Locke's buffer for 1h at 37°C
357 followed by exposure to 100 nM insulin, 100 nM TCAP-1, 100 nM SC-TCAP-1, or saline. ³H-2-
358 deoxyglucose (0.5 µCi/ml) was added to the culture 5m before termination of treatment exposure.
359 Uptake of ³H-2-deoxyglucose was stopped immediately after 5m with 3x washes of ice-cold 0.9%
360 saline solution. The cells were digested with 1 mL of 0.05 M NaOH at 0, 30, 45, and 60 min after
361 treatment. Radioactivity of the cell lysates were measured using a beta liquid scintillation counter
362 (Beckman Coulter), and recorded in counts per minute (CPM).

363

364 **Intracellular ATP and NADH assays**

365 ATP assays were conducted using Promega ATP Assay kits (Wisconsin, USA) following the
366 manufacturer's instructions. Briefly, C2C12 cells were seeded at 10,000 cells/well in 96-well
367 plates. The following day, cells were treated with either vehicle or TCAP-1 (100 nM) and lysed at
368 0, 15, 30 and 60m after treatment. Ultra-Glo recombinant luciferase (Promega, Wisconsin, USA)
369 was added to the media to determine ATP levels. Fluorescence from blank wells was subtracted
370 from all samples to account for background signal noise. As the fluorescence signal naturally
371 decays over the course of the experiment, TCAP-1-treated cells were compared relatively to the
372 vehicle-treated cells for each time point (n=8). For the resazurin NADH assay, the C2C12 cells
373 were seeded at 10,000 cells /well in 96-well plates. The resazurin assay was started the following
374 day by adding the resazurin solution (525 nM, Sigma) to all wells. Cells were treated with either
375 vehicle or TCAP-1 (100 nM). Fluorescent readings were measured every 5m over 1h, with

376 excitation at 530 nm and emission read at 590 nm. The measurements of blank wells not containing
377 cells were subtracted from all readings.

378

379 **Western blot of succinate dehydrogenase in C2C12 cells**

380 Following TCAP-1 treatments, proteins were extracted from C2C12 cells and lysed with
381 radioimmunoprecipitation assay (RIPA) buffer supplemented with the protease inhibitor,
382 phenylmethylsulfonic fluoride (PMSF) (Cell Signaling Technology, MA USA) and measured
383 using a bicinchonic acid (BCA) protein assay (Pierce BCA Protein Assay; Thermo-Fisher
384 Scientific, Toronto, Canada). All protein extracts were re-suspended in sample buffer and size-
385 fractioned by SDS-PAGE, electro-transferred to nitrocellulose membranes. Afterwards,
386 membranes were incubated with the succinate dehydrogenase (SDH) primary antibody and
387 subsequently treated with secondary antibody conjugated with chemiluminescent tags (Table 2).
388 Following TCAP-1 treatments, C2C12 cells were lysed with 500 μ L of RIPA buffer supplemented
389 with PMSF. Cells were harvested and centrifuged at 20,000 rcf for 20m at 4°C. Protein
390 concentrations (as described above) were measured to standardize the dilutions of respective
391 supernatant samples. Samples (15 μ g) were re-suspended in sample buffer and size-fractioned by
392 SDS-PAGE (10%) at 100V for 1h. The protein extract was electro-transferred to Hybond-ECL
393 nitrocellulose membranes (Amersham) for 2h at 100V. Membranes were washed 3x with PBS and
394 blocked in 5% milk-PBST (5% w/v non-fat milk powder in PBS with 0.2% Tween20; PBST) at
395 RT for 1h, then incubated with primary antibodies in 1% milk-PBST OT at 4°C. The membranes
396 were washed 3x in PBST at RT and incubated with a horseradish peroxidase (HRP)-conjugated
397 secondary antibody (VWR, Amersham; diluted to 1:7500 in 1% milk-PBST) for 1h at RT then

398 washed 3x in PBST at RT. The proteins were detected by adding chemiluminescence detection
399 reagent (ECL Amersham) to the membranes and exposing onto ECL Hyperfilm (VWR) for 30m.

400 **Diacylglycerol (DAG) and inositol triphosphate (IP3) assays**

401 The protocols provided by commercial DAG and IP3 assays (MyBiosource, San Diego,
402 California, USA) were followed. To determine the downstream Ca^{2+} response, 6 replicates of
403 C2C12 cells were prepared using the TCAP-1 treatment protocol described above then treated with
404 either vehicle, the IP3R antagonist, 2-APB, or the phospholipase C inhibitor, U73122. Cell lysates
405 were added to a microELISA plate coated with purified mouse DAG or IP3 antibodies.
406 Subsequently, 3,3',5,5'-tetramethylbenzidine (TMB) solution was added to detect the HRP-
407 conjugates as colour changes. Finally, sulphuric acid (0.01N) was added to terminate the reaction.
408 The absorbance change was measured at 450 nm by spectrophotometry (SpectraMax Plus, NH,
409 USA). For the IP3R inhibitor, 2-APB (100 μ M) was applied before the start of treatment with
410 either sham (Locke's buffer with scTCAP-1) or TCAP-1 (100 nM). For live-cell fluorescence
411 experiments, C2C12 cells were differentiated and intracellular Ca^{2+} flux was assessed via fluo-4
412 via a flow-through bath chamber of an inverted microscope. Cells were quantified with a GFP
413 filter set at 480 nm with the fluorescence emission measured at 516 nm.

414

415 **Mitochondrial Ca^{2+} accumulation and membrane potential measurement in C2C12** 416 **myotubules**

417 Changes in mitochondrial Ca^{2+} levels were assessed using fluorescent indicator, Rhodamine-2-
418 AM (Rhod-2). C2C12 myotubules were loaded with Rhod-2 by incubating coverslips in DMEM
419 containing 4 μ M Rhod-2 (from a 1mM stock solution in DMSO with 20% pluronic; Invitrogen-
420 Pluronic™ F-127) for 30m at 22°C. Cells were washed once for 30m at 37°C in Locke's Buffer

421 Cells then acclimated for 15m at 22°C. To assess changes in mitochondrial Ca²⁺ levels, cells were
422 continuously perfused in a flow-through chamber as indicated previously. Changes in Rhod-2
423 fluorescence was imaged using a TRITC filter set (Semrock, Rochester, NY, USA) and an X-Cite
424 120 fluorescence illumination system (Quorum Technologies, Inc. Guelph ON, Canada).
425 Emissions were detected using an Orca-ER Hamamatsu BW CCD digital camera as described
426 above. Rhod-2 was excited at 552nm every 100 ms and measured at 577nm. Multiple ROI were
427 taken from the nuclear regions of the myotubules (n=5, 5-7 ROIs per coverslip). Changes in
428 mitochondrial membrane potential were assessed using R123-based fluorescence. C2C12
429 myotubules were prepared with by incubating a coverslips in DMEM containing 5 µM R123 for
430 30m (37°C) followed by a 15m wash in Locke's Buffer. Changes in R123 fluorescence was imaged
431 using the green GFP filter set using the same experimental configuration as previously described.
432 R123 was excited with a wavelength of 480 nm for 100 ms every 5s and fluorescence emission
433 was measured at 516 nm.

434

435 **SiRNA knockdowns (KD) and CRISPR Knockouts (KO) of LPHN-1 and -3**

436 For the siRNA KD studies, transfection with siRNA oligonucleotides was performed after 4d of
437 C2C12 differentiation using Dharmacon SmartPOOL (Horizon Inc. Canada) siRNA for LPHN1
438 (L-061299-00-0005), LPHN3 (L-040779-00-0005) and a non-targeting control (D-001810-10-05).
439 Dharmacon SmartPOOL siRNAs targeted against LPHN-1 and-3, glyceraldehyde-3-phosphate
440 (GAPD) and a non-targeting control were re-suspended in 1x siRNA buffer from 20 µM stocks.
441 The stocks were diluted in serum-free and antibiotic-free DMEM to 250 nM. A 7.5µL aliquot of
442 Mirus TransIT-X2 (Mirus Bio LLC) transfection reagent was diluted in 200 µL serum- and
443 antibiotic-free DMEM and incubated at RT for 30m. The mixture was added to C2C12 cells (see

444 above) with a final siRNA concentration of 25 nM. The cells were differentiated in siRNA-
445 containing media for 2d for a total of 6d of differentiation before use in the experiments. For
446 CRISPR studies, single-guided RNA (sgRNA) constructs were designed to target the mouse
447 LPHN-1 and -3 gene at 3 locations (see Fig. 2). C2C12 cells were transfected with sgRNA
448 constructs (Fig. 2C) and a Cas9 plasmid, generating heterogenous pools of transfected cells. The
449 CRISPR/Cas-transfected C2C12 cells (either heterogenous pools or clones) were trypsinized and
450 pelleted for DNA extraction. Genomic DNA was extracted using Lucigen QuickExtract DNA
451 extraction solution (Biosearch Technologies, Inc) according to the manufacturer's direction. The
452 LPHN-1 and -3 genes were amplified by PCR and digested by T7 endonuclease using the EnGen
453 Mutation Detection Kit (New England Biolabs) according to directions in combination with the
454 custom primers that flank the appropriate CRISPR-targeting regions (Fig. 2C). The fragments were
455 identified as previously described above. Clones that showed low or no WT PCR amplicon were
456 screened for LPHN1 expression by qRT-PCR. Selected clones showing significantly reduced
457 LPHN1 mRNA expression by qRT-PCR and western were termed 'LPHN1 E5U KO' and 'LPHN1
458 E5D KO' based on the exon position of mutated site and were used for further study (Fig. 2). The
459 activity of the clones were determined by TCAP-1-induced cytosolic Ca²⁺ flux in the manner
460 described above. Peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) expression
461 in C2C12 cells was determined using the qRT-PCR methods described above.

462

463 **Figure 2. CRISPR-based targets for the mouse LPHN-1 and -3 genomic sequences. A.**

464 Schematics of LPHN-1 and -3 genomic organization. The oligonucleotides targeting these regions
465 are indicated as arrows. **B.** Sequence of the oligonucleotides used in the CRISPR knockdowns as
466 indicated in 'A' above.

467

468 **Statistical Analyses**

469 All data on graphs are represented as mean \pm SEM. All data were analyzed by Student's t-test or
470 one or two-way ANOVA, as described within each figure caption. Tukey's post-hoc test and
471 Sidak's post-hoc test were used to determine significance in one-way and two-way ANOVA
472 analyzes, respectively. An *a priori* hypothesis of $p < 0.05$ was used as a threshold for statistical
473 significance. GraphPad Prism 7-8 was used to analyze each statistical test.

474

475 **Results**

476

477 The primary structure of rat and mouse TCAP-1 possesses a high degree of homology among the
478 other three paralogues (Fig 1A). Because the primary structure of rat and mouse TCAP-1 is
479 identical, it was used for all studies, as well as a proxy for the other TCAP isoforms. The first
480 studies completed established that the teneurins, TCAP and LPHNs could be expressed in rat
481 skeletal muscle. Secondly, the physiological role of TCAP-1 in rat skeletal muscle was
482 examined.

483 **In Vivo Rat Studies**

484 In rat TA muscle mRNA extracts, all 4 teneurin mRNAs were identified based on the PCR primers
485 indicated in Table 1. Teneurins-3 and-4 showed the strongest response, although both teneurins-1
486 and -2 were present, albeit weakly expressed. In contrast, TCAP-1 and -2 showed a strong signal
487 relative to that indicated by teneurins-1 and -2 whereas TCAP-4 showed a signal consistent with
488 teneurin-4. Although these studies were not quantitative, they do establish that both teneurins and

489 TCAP paralogues are present in rat skeletal muscle. However, importantly, both LPHN-1 and -3
490 cDNA bands were also strongly expressed, although there was no evidence of LPHN-2 in this
491 preparation (Fig 3A).

492

493 **Figure 3. Expression of the teneurin/TCAP-LPHN immunoreactivity (ir) in rat skeletal**
494 **muscle: A.** PCR expression of teneurins, TCAP and LPHNs in rat TA muscle. **B.** Immunological
495 expression of β -dystroglycan, teneurin and LPHN in rat skeletal muscle. Arrows indicate nodes of
496 aggregation (Scale bar: 100 μ M). **C.** Enhanced examination of ir-LPHN regions in TA muscle
497 cells. Left panel scale bar indicates 100 μ M, whereas the right panel scale bar indicates 50 μ M.
498 Black arrows indicate cells with high LPHN-1 labelling, whereas white arrows indicate cells of
499 low LPHN-1 labelling. **D.** Quantification of ir-LPHN-1 as a function of muscle cell diameter (size)
500 as shown in 'C' (Student's t-Test $p < 0.0001$; ****). **E.** Changes in fiber type in short-term TCAP-1
501 administration (t-test indicated for each pair). **F.** Changes in fiber-type over long term TCAP-1
502 administration (t-test indicated for each pair).

503

504 To corroborate these cDNA studies, immunohistochemical (IHC) studies were performed in rat
505 TA muscle tissue. Initially, β -dystroglycan (DG) labelling was used to establish the sarcolemmic
506 boundary of the cells, as previous studies indicated a relationship between DG and TCAP signaling
507 [33]. Further, IHC co-localization labeling of Teneurin-1,-3 -and LPHN-1 was utilized to establish
508 the cellular anatomical relationship between the teneurins and LPHNs. Immunoreactive (ir)
509 teneurin-1 did not show a strong signal (data not shown), consistent with the RT-PCR data
510 indicated above, however the ir-teneurin-3 showed a clear response indicating specific nodes of
511 aggregation in the sarcolemma (Fig. 3B). Importantly, ir-LPHN-1 labelling of these tissues showed
512 co-localization with the ir-DG along with ir-teneurin and ir-TCAP labelling consistent with the

513 PCR studies indicated in Fig. 3A. The variation among the PCR and ir-teneurin -1 and -3
514 expression was expected due to affinity differences among the antibodies and primers (see
515 discussion). Moreover, these studies establish a clear relationship between TCAP, teneurins and
516 LPHNs in rat skeletal muscle (Fig. 3B). Morphological differences, with respect to cross-section
517 diameter between the vehicle- and TCAP-1-treated animals could be established. Thus, TCAP-1
518 administration induced a 25% increase ($p < 0.001$) between the number of small and intermediate
519 cells relative to the untreated vehicle rats (Fig. 3C, D). Because small and intermediate fibers are
520 typically oxidative muscle fibres, these observations suggested that TCAP could stimulate glucose
521 uptake in skeletal muscle. To corroborate these findings, the expression of myosin heavy chain
522 (MHC) was evaluated in the TA muscle of both short-term and long term TCAP-treated animals.
523 In both cases, there was a significant ($p < 0.01$) 3- to 3.5-fold increase in the expression of the MHCI
524 fibres in the TCAP-1-treated animals compared to the non-treated vehicle, although significant
525 ($p < 0.05$) differences were also observed among MHCIIa, MHCIIx and MHCIIb expression among
526 the treated and untreated animals (Fig. 3E,F).

527 Taken together, these studies indicated that TCAP-1 may increase glucose transport into skeletal
528 muscle. Therefore, TCAP-1-induced glucose uptake into the hind-limb was measured directly by
529 fPET. Thus, using ^{18}F -deoxyglucose (FDG), a single dose of SC TCAP-1-treatment-induced FDG
530 uptake in the hind-limb muscle by 2-fold ($p < 0.05$) (Fig. 4.A,B) after 3d of treatment relative to
531 vehicle treatment. These data corroborated our supposition that TCAP-1 acted, in part, to increase
532 glucose importation into skeletal muscle. If this was the case, then this increase in glucose
533 importation should increase skeletal muscle NADH production as a result of 2-glyceraldehyde-3-
534 phosphate conversion to 2-1,3 diphosphoglycerate and secondarily through elements of the tri-
535 carboxylic (TCA) acid cycle in the conversion to pyruvate. TCAP-1-treated muscle significant

536 (p<0.05) increased NADH-staining compared to vehicle (Fig. 4C, D) supporting increased TCAP-
537 1-mediated glucose transport.

538

539 **Figure 4. Glucose uptake and metabolism in rat hind-limb and TA muscle.** **A.** Function
540 positronic emission tomography (fPET) of rat hind limb showing increase of ¹⁸F-deoxyglucose
541 (¹⁸F-DG) uptake after 3 days from TCAP-1 administration. **B.** Quantification of the ¹⁸F-DG uptake
542 in hindlimb after 3 days. (n=5; student's t-test; p<0.05). **C.** Stimulation of NADH in TA muscle
543 after administration of TCAP-1. Above panel, (hematoxylin and eosin stain), bottom panel, NADH
544 activity (shown as black regions). Scale bar indicates 100 μM. **D.** Quantification of the NADH-
545 labelled cells shown in 'C'. * p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Mean ± SEM
546 indicated n=4.

547

548 Muscle performance is related to the amount of energetic substrates available, therefore, we
549 examined the role of TCAP-1 on muscle activity *in vivo* in rats by determining the efficacy of
550 TCAP-1-mediated contractility using electrical stimulation of the TA muscle. After a 5-d daily
551 treatment of either vehicle or TCAP-1, following a 3-d washout period, muscle contractility was
552 assessed. TCAP-1-treated animals showed improved muscle dynamics. TCAP-1-treated animals
553 exhibited enhanced baseline contraction kinetics with respect to increased peak twitch force
554 (p<0.05) (Fig. 5 A,B), slower contraction velocity (p<0.05) (Fig 5C), and potentially higher faster
555 relaxation rates (Fig. 5D) compared to vehicle-treated animals. Following baseline measurements,
556 a 6-m fatigue protocol was induced in the muscle where contractile kinetics were recorded at 0, 1,
557 and 5m after the fatigue protocol. TCAP-1 enhanced recovery from the twitch stimulation (Fig.
558 5E-G). Although TCAP-1 did not influence peak twitch force (Fig. 5E), it significantly (p<0.05)
559 maintained twitch max dx/dt (Fig. 5F) and 1/2RT (Fig. 5G) over the course of the fatigue protocol

560 which was diminished in vehicle-treated animals. All data was normalized to muscle mass. The
561 treatment did not affect muscle mass (Fig. 5H), tetanic force (Fig. 5I) or the fatigue force curve
562 (Fig. 5J). Thus, TCAP-1 enhanced the efficiency of the existing muscle morphology, rather than
563 increasing muscle mass, and maintained contraction cycling efficiency during fatigue. To assess
564 the effects of a long-term (LT) treatment, rats were administered either vehicle or TCAP-1, for 3
565 months (1 injection/week). Two weeks post-treatment, the TCAP-1-treated animals elicited a
566 comparable peak twitch force to vehicle-treated animals (Fig. 5K-J), however had significantly
567 ($p<0.05$) slower contraction velocity and faster ($p<0.05$) relaxation rate (Fig. 5L,M).

568

569 **Figure 5. *In vivo* actions of TCAP-1 on rat TA muscle kinetics. A-J.** Muscle twitch kinetics
570 after animals treated with TCAP-1 once a day for 5 days before testing **A.** Representative twitch
571 traces (black, vehicle; gray, TCAP-1). **B.** Baseline contraction kinetics (t-test). **C.** Contraction
572 velocity (t-test). **D.** Relaxation rate (t-test). **E.** Peak twitch force (2-way ANOVA). **F.** Twitch max
573 dx/dt (2-way ANOVA). **G.** 1/2RT analysis (2-way ANOVA). TCAP-1 treatment did not affect
574 muscle weight (t-test) (**H**), tetanic force (t-test) (**I**) or fatigue force over time (**J**) ($n=7-8$). **K-M.**
575 Long term treatment of TCAP-1 on rat hind-limb twitch kinetics. **K.** contraction max dx/dt. **L.**
576 1/2RT rate. **M.** Relaxation rate.

577

578 **In Vitro Mouse Cell Studies**

579 The initial PCR screen of C2C12 cells indicated that, although only teneurin-3 was highly
580 expressed (Fig.6A), all 4 TCAP transcripts could be discerned (Fig. 6B). In both undifferentiated
581 C2C12 myoblasts, and 6-d myotubules, the transcripts for LPHN-1 and -3 were present (Fig. 6C).
582 IHC expression of TCAP-1 showed a similar punctate expression in the cytosol of the C2C12

583 myoblasts (Fig. 6D) as we have previously shown for neurons [38] where FITC-labelled TCAP-1
584 was present at several sarcolemmic regions consistent with the expected expression of the receptor
585 [32,33,38]. Moreover, because TCAP-1 regulates actin organization and polymerization in
586 neurons [38], the C2C12 cells were treated with TCAP-1 and examined using the phalloidin stain
587 to highlight actin fibers (Fig. 6E). This resulted in a major increase in actin polymerization in the
588 TCAP-1-treated cells at both 30m ($p<0.01$) and 2d ($p<0.001$).

589

590 **Figure 6. Expression of teneurin, TCAP and LPHN in C2C12 myoblasts.** A. PCR-based
591 teneurin expression; B. PCR-based TCAP expression; C. PCR-based LPHN expression. D. C2C12
592 cells labelled with TCAP-1 antisera showing the difference between the endogenous ir-TCAP and
593 the presence of FITC-TCAP-1 localization. ir-TCAP-1 is indicated in red, whereas the DNA-
594 associated DAPI labelling is indicated in blue. FITC-labeled TCAP-1 is shown in green. Arrows
595 indicate regions of FITC-TCAP-1 uptake. Scale bars indicate 50 μ M E. Actions of TCAP-1 on the
596 proliferation of the C2C12 myoblasts when treated with TCAP-1 at 30 minutes and 2 days. Actin
597 is indicated in red, whereas the nuclei are indicated in the DAPI-based blue. Scale bars indicate
598 100 μ M.

599

600 Having established that TCAP-1 behaved in a similar manner as previously shown in neurons, the
601 viability of the Ca^{2+} response in the differentiated myotubules was evaluated to determine their
602 efficacy before proceeding to further studies. Initially, caffeine was used to determine the limits
603 of the Ca^{2+} response in the differentiated myotubules relative to the TCAP-1 response (Fig. 7A-
604 D). These studies indicated that the myotubules were active and viable, and with respect to the
605 Ca^{2+} response, did not show an appreciable decrease in cytosolic Ca^{2+} concentrations (Fig. 7B),
606 although it did attenuate the rate of cytosolic Ca^{2+} concentrations ($p<0.01$). Taken together, these

607 studies indicated that the myotubules were viable with respect to our preparation, and that the
608 attenuating TCAP-1 response indicated that additional regulating factors were likely present. Thus,
609 given these observations, the direct action of TCAP-1 on Ca²⁺ flux in myotubules was examined
610 (Fig. 7E-H). TCAP-1 increased Ca²⁺ concentrations by almost 4-fold relative to the control-treated
611 cells (p<0.001).

612

613 **Figure 7. Caffeine- and TCAP-1- mediated Ca²⁺ response in differentiated C2C12 myocytes.**
614 **A.** Heat-map images showing the Ca²⁺ response induced by caffeine, TCAP-1 or vehicle. Mean
615 and SEM is indicated. **B.** Dynamic concentration changes over the period of analysis shown in
616 ‘A’. Mean ± SEM is indicated. **C.** Total concentration changes of the manipulations of the study
617 period indicated in ‘A’. **D.** Rate of Ca²⁺ release between caffeine and TCAP-1. **E.** TCAP-1
618 mediated Ca²⁺ actions show normal morphology in cells. **F.** Rate of increase in Ca²⁺-associated
619 fluorescence after administration of TCAP-1. **G.** Quantification of the change in TCAP-1 mediated
620 intracellular Ca²⁺ concentrations indicated in ‘F’. Significance was determined by a Students t-
621 test. (* p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Mean ± SEM indicated n=4).

622

623 In skeletal muscle, the predominant glucose transporter protein (GLUT) isoform is the insulin-
624 sensitive GLUT4 protein. Using insulin as a control, a significant (p<0.001) increase in the
625 expression of the ir-GLUT4 transporters was observed for both TCAP-1 and insulin treatments
626 over 30m (Fig. 8 A,B) in C2C12 cells. To determine whether this GLUT4 response was dependent
627 on the TCAP-1-mediated Ca²⁺ release, the IP3R antagonist, 2-APB, that abolishes the TCAP-1
628 Ca²⁺ response, was investigated. In the presence of the inhibitor, both TCAP-1 (p<0.01) and insulin
629 (p<0.001) inhibited the ir-GLUT4 expression (Fig. 8A,C). This importation of glucose by TCAP-1
630 was further corroborated in C2C12 cells showing that TCAP-1 significantly (p<0.0001) induced

631 ^3H -2-deoxyglucose increase into the cytosol over 30m, similar to that of insulin (Fig. 8D).
632 However, both peptides show distinct glucose-uptake profiles, where insulin induces a significant
633 increase at 30m ($p<0.001$), 45m ($p<0.001$) and 60m ($p<0.01$), whereas TCAP-1 induces an
634 increase at 30m ($p<0.001$) but was attenuated by 45m ($p<0.01$) and returns to baseline at 60m. The
635 sc-TCAP-1 treatment, used separately as a negative peptide control in this study, showed no
636 significant change from the saline vehicle.

637

638 **Figure 8: TCAP-mediated glucose metabolism in C2C12 cells.** **A.** Regulation of ir-GLUT4 by
639 TCAP-1 and insulin myotubules. Red indicates ir-GLUT4 whereas blue shows DAPI staining of
640 the nuclei. Arrows indicated regions of high immunoreactivity. Scale bar=100 μM . **B.**
641 Quantification of ir-GLUT4 expression over 30 minutes. **C.** Effect of the IP3R inhibitor, 2-APB
642 on TCAP-1- and insulin-mediated ir-GLUT4 labelling. **D.** Uptake of ^3H -2-deoxyglucose in C2C12
643 myoblasts by TCAP-1 and insulin. **E.** Changes in static ATP concentrations following treatment
644 by TCAP-1. **F.** NADH production increase as determined by a resesourin assay following TCAP-1
645 treatment relative to the vehicle. **G.** Increased ir-succinate dehydrogenase expression after 1 hour
646 following TCAP-1 treatment as determined by western blot. **H.** Quantification of the data indicated
647 in 'G'. Significance was determined by a t-test as indicated in C, F and H or one-way ANOVA
648 shown in B D and E. (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Mean \pm SEM indicated
649 $n=6$).

650

651 Increased glucose importation increases ATP and NADH turnover in cells due to glycolytic and
652 tricylic acid (TCA) cycle activity. Therefore, this was examined with respect to TCAP-1 treatment.
653 As a result, both ATP ($p<0.001$) (Fig. 8E) and NADH ($p<0.001$) (Fig. 8F) turnover were
654 significantly increased after 30m of TCAP-1 treatment, although NADH levels remained about
655 60% higher ($p<0.001$) than vehicle levels after 60m. Moreover, succinate dehydrogenase (SDH; a

656 rate-limiting step of the TCA cycle) protein expression was increased by over 2-fold after TCAP-
657 1 treatment corroborating the previous experiments of increased ATP and NADH production
658 ($p < 0.05$; Fig. 8G,H).

659 As we have previously established that the IP3-DAG pathway is important for TCAP-1-mediated
660 intracellular Ca^{2+} flux in neurons, this pathway was examined in C2C12 cells. Relative to the
661 vehicle, TCAP-1 induced a significant increase at 5 and 15 min ($p < 0.001$; Fig. 9A) in intracellular
662 DAG concentrations and a major increase between 1 and 15 min ($p < 0.0001$; Fig. 9B) in IP3
663 concentrations. To confirm this action, TCAP-1-treated C2C12 cells were blocked with either the
664 IP3R antagonist, 2-APB, or the phospholase C inhibitor, U73122. The 2-APB and U73122
665 treatment reduced TCAP-1-mediated Fluo-4 concentrations to about 30% ($p < 0.01$) of their original
666 values indicating that the IP3-DAG pathway plays an active role in increase of TCAP-1-mediated
667 intracellular Ca^{2+} flux (Fig. 8C,D). Because this TCAP-1-mediated rise in intracellular Ca^{2+}
668 concentrations can target the mitochondria [39], the mitochondrial Ca^{2+} dye, rhodamine 1-AM
669 (Rhod-2) was utilized to determine the concentration of Ca^{2+} sequestration in mitochondria. There
670 was a 5-fold increase ($p < 0.001$) in Rhod-2-associated Ca^{2+} labelling over 200s (Fig. 9E,F). Related
671 to this, Rhod-123, was used to determine the level of mitochondrial polarization. TCAP-1
672 treatment significantly decreased Rhod-123 fluorescence ($p < 0.001$) relative to vehicle indicating
673 depolarization of the mitochondria membrane (Fig. 9G, H).

674

675 **Figure 9. TCAP-1 mediated calcium regulation in C2C12 cells.** A. TCAP-1 mediated increase
676 in intracellular DAG concentrations (mean and SEM shown; $n=6$). B. TCAP-1 mediated increase
677 in intracellular IP3 concentrations (mean and SEM shown; $n=6$). C. Increase in intracellular
678 TCAP-1 mediated Ca^{2+} concentrations and inhibition by the IP3 receptor (2-APB) and

679 phospholipase C (U73122) antagonists; **D.** Quantification of data shown in C (n=6). **E.** Uptake in
680 Ca^{2+} -mediated Rhod-2 into mitochondrial membranes. **F.** Quantification of data shown in E based
681 on the change at 200 seconds. **G.** Decrease in Rhod-123 immunofluorescence in mitochondrial
682 membranes as a result of TCAP-1 administration. This decrease in Rhod-123 indicates a decrease
683 in mitochondrial membrane depolarization. **H.** Quantification of the data indicated in G based on
684 the changes at 225 seconds. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.)

685

686 Although these studies are similar with previous investigations of teneurins, TCAPs and LPHNs
687 in neurons, this is the first study to examine teneurin/TCAP and LPHN activity in skeletal muscle
688 function. To determine whether the TCAP-1 activation was dependent upon the LPHN receptors,
689 these genes were knocked-down (KD) using siRNA oligonucleotides, or knocked-out (KO) using
690 CRISPR in the C2C12 cells. Using the C2C12 cells, the LPHN-1 and -3 expression was reduced
691 using the siRNA oligonucleotides. The LPHN-1 receptor mRNA was reduced about an 80%
692 ($p < 0.01$) relative to the WT cells. Transfection with either the LPHN-1 siRNAs or the null vector
693 (NT) did not significantly change mRNA expression relative to the WT control (Fig. 10A).
694 Similarly, the LPHN-3 siRNA-associated oligonucleotides significantly ($p < 0.01$) decreased its
695 mRNA expression about 65% relative to the WT cells. There were no significant changes in
696 mRNA expression of the LPHN-1 transcript in either the LPHN-1 KD or the NT cells (Fig. 10B).
697 Despite the reduced expression of these receptors, cell morphology was normal (Fig. 10C). TCAP-
698 1 increased cytosolic Ca^{2+} in cells transfected with the NT control, however, relative to the NT
699 control, TCAP-1 did not increase Ca^{2+} in either the LPHN-1 and -3 siRNA-transfected cells, which
700 showed a significant decrease ($p < 0.01$ and $p < 0.001$, respectively) in intracellular Ca^{2+}
701 concentrations (Fig. 10D,E). However, because both LPHN-1 and -3 siRNA oligonucleotides
702 unexpectedly reduced intracellular Ca^{2+} concentrations by similar amounts, we repeated this study

703 by ablating the LPHN-1 and -3 receptors using CRISPR methods. The E5U7 target reduced LPHN-
704 1 expression by about 90% ($p < 0.01$) whereas, the E5D3 target reduced mRNA levels by about
705 95% ($p < 0.05$) relative to the WT cells (Fig. 11A). A significant decrease ($p < 0.05$) in LPHN-1
706 mRNA levels were present in both sets of transgenic cells (Fig. 11B). Importantly, cell morphology
707 was normal in the transgenic cells (Fig. 11C). CRISPR-based KO of the LPHN-3 gene were
708 unsuccessful after numerous attempts (data not shown; see discussion), hence studies were
709 performed with the LPHN-1 KO only. Similar to that achieved using the siRNA knock-down
710 cells, both CRISPR-associated KO transgenic cells (E5U and E5D) reduced intracellular Ca^{2+}
711 levels by about 60% ($p < 0.001$) (Fig. 11 D,E). Taken together, both the siRNA- and CRISPR-
712 associated methods to reduce mRNA expression indicate that the TCAP-1 associated intracellular
713 Ca^{2+} flux was mediated primarily by its interactions on LPHN-1 and -3.

714

715 **Figure 10: siRNA knockdown of LPHN-1 and -3 in C2C12 cells.** A. Cells transfected with the
716 LPHN-1-targetting siRNAs showed a significant reduction ($p < 0.01$) in LPHN-1 mRNA
717 expression. B. Cells transfected with the LPHN-3 targeting mRNA significantly ($p < 0.01$) reduced
718 LPHN-3 mRNA expression. C. Cells treated with either siRNAs showed normal morphology. D.
719 Changes in Ca^{2+} accumulation in cells transfected with either LPHN-1 or -3 siRNA
720 oligonucleotides. E. Quantification of the data shown in 'D' at 200 seconds.

721 **Figure 11: .CRISPR-based knockouts of the LPHN-1 and -3 genes in C2C12 cells.** The two
722 clones (E5U7) (A) and (E5D3) (B) significantly reduced LPHN-1 expression relative to NT and
723 WT cells. C. The morphology of the C2C12 cells were normal in both transfected cell lines. D.
724 Changes in cytosolic Ca^{2+} accumulation in the various cell types. E. Quantification of the data
725 shown in 'D' after 200 seconds. F, G, H, I. Changes in NADH production as determined by the
726 resasurin assay. J. Quantification of the data shown in F-I, K. Reduction in PGA-1 α mRNA

727 expression by PCR in E5U7 and E5D3 clones. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.
728 Mean \pm SEM indicated $n=7-8$.

729

730 To corroborate these findings with the previous observations that TCAP-1 could regulate energy
731 metabolism in skeletal muscle cells, the action of TCAP-1 on NADH production via a resazurin
732 assay was performed on the CRISPR LPHN KO cells (Fig 11F-I). From 30-150m, the TCAP-1-
733 treated WT C2C12 cells showed a significant increase ($p<0.01$; $p<0.05$, respectively) in
734 fluorescence compared to the vehicle (Fig. 11F). LPHN-1 E5U7 (Fig. 11G) and E5D3 (Fig.11H).
735 KOs did not show an increase after TCAP-1 treatment, whereas the NT control cells showed a
736 significant ($p<0.01$ increase after 120m (Fig. 11I). FCCP treatment, indicating cell viability
737 induced significant increases ($p<0.001$; $p<0.0001$) across all cell types (Fig. 11 F,G,H,I).

738 Given that the *in vivo* studies showed that TCAP-1 can modulate MHCI and that TCAP-1 may
739 also affect other hormones and signalling factors, it was unclear if the purported fibre changes
740 observed in TA muscle was a direct result of TCAP-1. Therefore, because PGC-1 α is a
741 transcription factor that up-regulates MHCI expression [46], the TCAP-1 actions on PGC-1 α was
742 measured by qRT-PCR. Following the TCAP-1 treatment (100nM), neither the E5U7 nor the
743 E5D3 cells showed any significant increases, whereas, both the WT cells and NT controls showed
744 about a 50% increase ($p<0.05$) in PGC-1 α expression (Fig. 11K). This indicated that TCAP-1 has
745 the potential to directly influence MCH fibre expression at the transcriptional level.

746

747

748

749 **Discussion**

750 This study describes a novel mechanism underlying skeletal muscle physiology. This investigation
751 is the first to show a functional relationship between teneurins and latrophilins (LPHN) with
752 respect to skeletal muscle function in mammals using rodent models. We have previously
753 established that there is a functional peptide on the distal tip of the teneurin extracellular region
754 which we have termed ‘teneurin C-terminal associated peptide’ (TCAP) and is highly active in the
755 CNS. Now, our data indicates that TCAP-1 affects skeletal muscle strength and fatigue, *in vivo*,
756 via a glucose-associated and, likely, an aerobic mitochondrial-based mechanism. This mechanism
757 is consistent with our previous findings in neurons and the CNS. Moreover, TCAP-1 interacts with
758 the putative teneurin receptors, LPHN-1 and -3 to activate the PLC-IP3-DAG pathway to regulate
759 intracellular Ca²⁺ flux that ultimately regulates glucose importation and mitochondrial activity.
760 The hypotheses developed from the *in vivo* studies were subsequently tested *in vitro* utilizing the
761 rat skeletal cell line, C2C12, to establish a cellular model upon which to base the *in vivo* actions
762 of TCAP-1 with respect to skeletal muscle dynamics.

763 A critical aspect of this study was the utilization of TCAP-1 as a peptide analogue of the distal C-
764 terminal region of teneurins. The genomic structure of the TCAP region of teneurins indicated that
765 it possessed a potentially cleavable peptide [22,24]. A synthetic version of TCAP-1 was developed
766 by replacing the N-terminal glutamine with pyroglutamyl acid and amidating the C-terminal
767 residue [24]. The resultant synthetic peptide was highly efficacious at regulating neural function,
768 behaviour and reproductive physiology in rodents [24,31,33,36-38] indicating that TCAP-1, itself,
769 possessed independent biological functions. Thus, rat/mouse TCAP-1 was utilized in this study
770 based on our previous work with this peptide. As indicated in Fig.1, the primary structure of rat
771 and mouse TCAP-1 is identical, and consequently, the same peptide was utilized for all *in vivo*

772 and *in vitro* experiments. However, it is important to point out that, although there are four
773 paralogous forms of teneurins and TCAP in vertebrates, albeit, with significant primary structure
774 conservation among them, our study has utilized only TCAP-1. TCAP-1 was utilized, therefore,
775 as a proxy for all TCAPs present in the organism or tissue, to determine the potential to regulate
776 skeletal muscle function. Based on our previous studies of this peptide and its level of primary
777 structure conservation, evidence indicates that our supposition is valid.

778 Elucidation of the teneurin/TCAP-LPHN network is complex. To date, there are 4 teneurins found
779 in vertebrates, each of which possesses a TCAP at its extracellular tip [4,17,18,23,24,26].
780 Moreover, the 3 latrophilin paralogues (LPHN1-3) have been identified in the vertebrate genome
781 [29,47,48]. A clear stoichiometry among teneurins and the LPHNs has been only partially
782 resolved. Both ligand and receptor proteins possess multiple domains that interact with a variety
783 of peripheral ligands in the extracellular matrix, the membrane and intracellularly as well
784 [10,13,14,21,22]. TCAP, as an amphiphilic peptide, that may be cleaved [14,22], or expressed
785 separately [33,34] is a ‘wild-card’ in this perplexity of molecular interactions. Although TCAP is
786 clearly bioactive with respect to cytoskeletal reorganization [32,33,37,38], glucose regulation
787 [3,39], signal transduction [24,38,39,49], metabolism [24,32,34,39] and stress-associated
788 behaviour [36, 50-54], these studies have focused at the neurological level. Despite this emphasis,
789 few studies [55] regarding the role of teneurins/TCAP and LPHN on skeletal muscle physiology
790 have been reported.

791 Our goal in examining the role of TCAP-1 and LPHN-1 and -3 was not intended to establish a
792 specific molecular interaction, per se, but rather to show that the teneurin/TCAP-LPHN system
793 plays an important role in skeletal muscle metabolism. Consequently, our investigation that
794 paralogues of teneurins, TCAPs and LPHNs were present both in hind-limb skeletal muscle

795 preparations and in C2C12 cells provided the basis of this molecular system with respect to skeletal
796 muscle physiology. Because TCAP-1 co-localization to cell membranes was associated with the
797 dystroglycan (DG) complex [38], we used DG antibodies to delineate the sarcolemma. In this
798 study, the interaction of the teneurin-3 and LPHN-1 in the TA muscle occurred at specific nodes
799 in the sarcolemma rather than being spread throughout all regions of DG labelling (see Fig. 3B).
800 Our data indicates that teneurin-3 is the dominant teneurin in skeletal muscle tissue. However, the
801 antisera available for the teneurins limited us to visualize histochemically only teneurin-3-like
802 epitopes, although PCR expression indicated that the teneurin-3 transcript was expressed. All 4
803 TCAPs were expressed by PCR although TCAP-3 showed low expression. In contrast, expression
804 of these transcripts in C2C12 cells indicated high expression of teneurin-3 along with all 4 TCAPs
805 (see Fig. 6A,B). This is the first time we have observed high TCAP expression without the
806 corresponding teneurin expression using PCR (Lovejoy, unpublished observations), although we
807 have only focused on neurons previously. We acknowledge that it can be difficult to reconcile the
808 expression patterns of teneurins using the antibodies available at the time, however, it is important
809 that both elements are present. These data may indicate a fundamental difference among teneurin
810 and TCAP expression in skeletal cells relative to neurons.

811 Our data indicated that TCAP-1, like in neurons [39], regulates glucose-mediated aerobic-based
812 energy metabolism in skeletal muscle. Several observations support this hypothesis. First, *in vivo*,
813 ir-LHPN primarily labeled small and intermediate muscle fibres in the rat TCA muscle (see Fig.
814 3C,D), that are typically associated with aerobic action. Second, with respect to MHC proteins,
815 TCAP-1 showed the greatest increase in MCHI expression in both short-term and long-term
816 TCAP-1 treatment of rats. Muscle kinetics are dependent, in part, on the relative proportion of
817 muscle fibers types. Although the TA muscle consists of 95% type-II muscle (fast-twitch

818 glycolytic muscle fiber type), TCAP-1 imparts traits of type-I fibers (slow-twitch and oxidative)
819 which possess greater MHC expression. Importantly, both short-term ($p<0.01$) and long-term
820 TCAP-1 ($p<0.01$) administration (Fig.3 E,F) increased in the MCHI fibre expression, although in
821 short-term TCAP-1- treated animals, MHCII expression was specifically decreased in MHCIIa,
822 ($p<0.05$); MHCIIx ($p<0.01$) and MHCIIb ($p<0.05$) mRNA expression, where only MHCIIb
823 showed a decrease ($p<0.01$) in the long-term treated rats. These expressional changes of MHC
824 transcription were corroborated by the expression of PGC-1 α , a critical transcriptional co-factor
825 that regulates the mitochondrial actions of myosin chain transcription. Using the C2C12 cells as a
826 model, TCAP-1 increased the transcription of PGC-1 α and was inhibited ($p<0.05$) by CRISPR-
827 mediated KO of the LPHN-1 gene. Thus the TCAP-1-mediated Ca²⁺ surge may activate CaMKIV
828 and CaN transcriptional regulators to promote the transcription of PGC-1 α . Overall, this indicates
829 that TCAP-1 is increasing slow-twitch gene expression, consistent with the *in vivo* contractile
830 kinetics observed in Fig 5. Third, rats treated with TCAP-1 enhanced baseline contractile kinetics
831 under basal and fatigue conditions using both short- and long-term TCAP-1 administration. Fourth,
832 a single dose of TCAP-1 increased uptake of ¹⁸F-2-deoxyglucose in rat hind-limb regions as
833 determined by fPET analysis. Fifth, because Ca²⁺ and ATP are required for proper muscle
834 contraction initiation and relaxation, TCAP-1 significantly increased GLUT4 expression (Fig.8A),
835 increased ³H-2-deoxyglucose uptake (Fig. 8D), ATP (Fig. 8E) and NADH (Fig. 8F) concentrations
836 and increased the protein expression of SDH in the TCA cycle (Fig. 8G,H) using the C2C12
837 myoblasts and myotubules. Taken together, these studies support the hypothesis that TCAP-1
838 regulates glucose uptake and metabolism in skeletal muscle cells in a similar manner previously
839 described in neurons [39].

840 Glucose is the main energy nutrient for skeletal muscle function, thus, up-regulation of glucose
841 metabolism could impact skeletal muscle activity. Therefore, the influence of TCAP-1 on these
842 contractile kinetic parameters indicates that TCAP-1 modulates Ca^{2+} levels to enhance SR-
843 sarcomere coupling. During fatigue, cytosolic Ca^{2+} levels accumulate due to inefficient SR-
844 sarcomere coupling, thereby reducing muscle function as was observed in contractile kinetic
845 parameters such as peak twitch force and 1/2RT. TCAP-1 treatment significantly increased both
846 parameters, indicating a clear role in Ca^{2+} modulation. Moreover, these results also suggest that
847 TCAP-1 increases ATP production rate to meet the energetic demands of the muscle during
848 fatigue. After the contraction, Ca^{2+} is cleared from the cytosol and re-uptaken into the SR via the
849 associated Ca^{2+} -ATPase (SERCA) pumps. SERCA pumps are high energy-consuming channels
850 that account for 20-50% of the energy turnover in a single contraction cycle [43,56]. Prolonged
851 stimulation results in the rapid depletion of ATP, thus reducing SERCA activity, ultimately leading
852 to the accumulation of cytosolic Ca^{2+} . However, because TCAP-1 increases glucose uptake into
853 the muscle, it provides additional substrates for energy metabolism which could maintain SERCA
854 activity during fatigue. This is corroborated by the finding that TCAP-1 had significantly faster
855 1/2RT during fatigue compared to vehicle treatment and by the increase in the type-1 muscle fiber
856 associated gene transcription that occurred in both short-term and long-term actions of TCAP-1.

857 From these studies, it is clear that TCAP-1 targets the mitochondria as it has potent actions upon
858 Ca^{2+} modulation and glucose signaling. As TCAP-1 increases Ca^{2+} uptake into the mitochondria,
859 likely from shuttling Ca^{2+} from the SR, this stimulates enzymes in the TCA cycle, specifically
860 glycerol phosphate dehydrogenase, pyruvate dehydrogenase phosphatase, isocitrate
861 dehydrogenase and oxoglutarate dehydrogenase [57,58]. This activates mitochondrial respiration
862 via the ETC and leads to increased energetic output, as seen by increases in ATP, NADH and

863 SDH. Thus, this may explain enhanced metabolism and function results under TCAP-1 treatment.
864 Previous studies in neurons have established that the IP3-DAG pathway is activated in response
865 to TCAP-1. In this study, we showed that a similar situation occurs in C2C12 cells and, likely, in
866 skeletal muscle. TCAP-1 treatment of C2C12 cells increase intracellular Ca^{2+} flux that can be
867 blocked using IP3 receptor (2-APB) and phospholipase C (U73122) inhibitors. Moreover, this
868 increase in intracellular Ca^{2+} is likely responsible for the depolarization of the mitochondrial
869 membranes. This work supports a previous report that SR-associated Ca^{2+} release was directed
870 toward the mitochondria in skeletal muscle [59].

871 These studies are consistent with previous observations in immortalized mouse neurons [39] and
872 in zebrafish [34]. Although these previous studies indicate a relationship with TCAP-1 and LPHN
873 action on Ca^{2+} flux involving the IP3-DAG pathway [39,60] other studies indicate that the LPHN-
874 mediated AMP-PKA pathway may also be activated by teneurins and TCAP [61,62]. For example,
875 TCAP-1 and -3 can increase cAMP levels in immortalized mouse neurons [24,37,49]. Further,
876 studies of vertebrate teneurins have also implicated activation of the PKA- cAMP cascade [31].
877 However, our goal in this study was to establish a mechanism by which TCAP-1 can regulate
878 energy metabolism in C2C12 cells, and for this reason we have focused on a Ca^{2+} -associated
879 mechanism, as it aligns with previous studies. However, we acknowledge that given the
880 complexity of teneurin-LPHN actions, other signal transduction systems such as ERK-MEK [38]
881 are also likely required for the full set of teneurin- and TCAP-mediated LPHN actions on cells.
882 Ancient-evolving peptide-protein systems will likely impinge on more than one intracellular signal
883 cascade events because they evolved before many of the later intracellular signaling transducing
884 pathways [31].

885 The mitochondria are ultimately responsible for supplying aerobic-based energy requirements to
886 eukaryotic cells. We have previously shown the relationship of TCAP-1 mediated energy
887 production and the mitochondria in the mouse neurons [39] and in zebrafish metabolism [34],
888 however this was the first study to establish the link between the teneurin/TCAP-LPHN system
889 and mitochondria in skeletal muscle cells. Although we have not studied mitochondria respiration
890 directly in this study, previously we showed that TCAP-3-treated zebrafish [34] increased both
891 basal and respiratory reserve capacity. Although total mitochondrial respiration is linked to proton
892 leak and ATP-linked respiration [63], no TCAP-3-associated actions on the latter could be
893 detected, but proton leak was increased in these studies.

894 The role of the teneurins, TCAP and LPHNs, together, has previously not been examined in
895 skeletal muscle. Therefore, we utilized both siRNA- and CRISPR-based methods to determine if
896 the reduced activity of the LPHN-1 and -3 receptors would attenuate TCAP-1-mediated
897 intracellular actions. Both CRISPR-based KOs of LPHN-1 significantly reduced the TCAP-1
898 associated increase in intracellular Ca^{2+} . We were unsuccessful to create a LPHN-3 KO however.
899 The length of the LPHN-3 gene in mice is about 10 times that of the LPHN-1 gene due to much
900 longer intronic sequences. Thus, this extended sequence may have played a role in the lack of
901 viability of the CRISPR- associated LPHN-3 oligonucleotides. Moreover, TCAP-1-mediated
902 intracellular Ca^{2+} was established using pharmacological antagonists of the PLC-IP3-IP3R
903 pathway of the SR. This rise in intracellular Ca^{2+} led to increased cellular glucose, concomitant
904 with increases in ATP and NADH production. Indeed, the TCAP-1-mediated increase in
905 intracellular Ca^{2+} corroborated with mitochondrial membrane hyperpolarization and increased
906 succinate dehydrogenase activity indicating that TCAP-1 also acted to increase mitochondrial
907 activity. However, the siRNA oligonucleotides did inhibit the actions of both receptors as

908 indicated by the significant reduction in the TCAP-1-mediated cytosolic Ca²⁺ response. siRNA
909 KDs of the LPHN-1 and -3 has been successfully used in the past using the mouse pancreatic β-
910 cell line, MIN6 [64] where the authors showed that LPHN-3 KDs reduced insulin secretion by
911 reducing cAMP levels via a Gi-mediated pathway. Although we have not examined the direct role
912 of TCAP-1 on pancreatic insulin release, this study is consistent with our supposition that TCAP,
913 itself, is associated with glucose regulation *in vivo* [3,39]. In our current study, however, our
914 experiments showed that ablation of LPHN-1 or -3 reduced TCAP-1-mediated Ca²⁺ concentrations
915 toward baseline levels. These results surprised us. We expected that the KO or KD of either
916 receptor would render only a partial suppression of the Ca²⁺ response. Because this was not the
917 case, one possibility is that there is an interaction among the LPHN isoforms specifically, or their
918 combined actions with the teneurins.

919 Teneurin and LPHN interaction is complex where the stoichiometry among the 4 teneurin and 3
920 LPHN paralogues has not been ascertained. Although studies using vertebrate models establish
921 clear evidence of teneurin-LPHN interaction [14,22,28,31], the specific correspondence of any
922 teneurin with any LPHN as cognitive pairs has yet to be established. The teneurins are
923 multifunctional transmembrane proteins that have TCAP at their distal extracellular tip. Even less
924 is understood regarding the promiscuity of the LPHNs with respect to TCAP interactions.
925 Previously, Silva and his associates [31] showed that the teneurin-2 region possessing the TCAP
926 unit was required for full binding to the LPHN-1 (Lasso), and Husic and her colleagues [32]
927 showed that the transgenic expression of teneurin-1 TCAP co-precipitated with the transgenic
928 over-expressed hormone binding domain (HBD) of LPHN-1 and, moreover, modulated the cell
929 adhesion characteristics of HEK293 cells overexpressed with the LPHN-1 mRNA. In this current
930 study, we established that both LPHN-1 and teneurin-3 transcripts were present and are co-

931 localized in the sarcolemma (Fig.3). These data corroborate with the PCR expression data
932 indicating that these mRNA transcripts show the highest expression, but are not meant to suggest
933 that this is indicative of cognitive ligand and receptor pairs, per se.

934 There is evidence that LPHN paralogues interact with each other. In our LPHN-1 and -3
935 attenuation studies, the reduction of one receptor inhibited the TCAP-1 mediated Ca^{2+} actions of
936 the other receptor. It is possible that there is a minor Ca^{2+} response mediated by the non-target
937 LPHN, but too low to detect with our assay conditions, although this seems unlikely. Homo-and
938 heterophilic oligomerization is a characteristic of the GPCRs, but this has not been well-studied in
939 Adhesion GPCR family members [65,66]. In LPHN-1, the C-terminal fragment and N-terminal
940 fragment is cleaved *in vivo* and re-associates with the α -latrotoxin (α LTX) mutant ligand LTX^{N4C}
941 [67], although this phenomenon has not been studied in detail in other LPHN paralogues despite
942 the high degree of conservation among these domains. Further, studies using the ‘*stachel*’ peptide
943 have provided additional insight into potential LPHN paralogue interactions with each other
944 [64,68,69]. The *stachel* peptide represents the N-terminus of the C-terminal fragment following
945 cleavage of the conserved GPCR-autoproteolysis (GAIN) region [70]. The recombinant expressed
946 *stachel* peptide induces diverse G-protein associations across LPHN paralogues, whereas α LTX
947 favours G11 signalling via LPHN-1 [60,71,72]. We and others have previously shown that the
948 TCAP amino acid sequence resembles that of Secretin GPCR family ligands and α LTX [26], thus
949 we posit that TCAP may represent the endogenous ligand that α LTX co-evolved with to become
950 a toxin.

951 One of the questions we did not address in this study was the trigger that stimulates TCAP to
952 regulate skeletal muscle physiology. Our hypothesis, at this time, is that TCAP is liberated locally
953 in the sarcolemma either by a direct cleavage of the teneurin [14,22,23] or by independent mRNA

954 transcription, translation and release from skeletal muscle or other local tissues [33,34]. We have
955 shown in the past that TCAP-1 can increase teneurin transcription in immortalized neurons [24],
956 but we have yet to establish this *in vivo*. Although TCAP is highly expressed in the brain, our
957 studies showing its presence in as a circulating hormone in serum has been equivocal (Lovejoy,
958 unpublished observations). As this is a critical aspect of TCAP action, this is a goal for upcoming
959 studies.

960 In summary, our data in this study indicate that TCAP-1 regulates energy metabolism in skeletal
961 muscle via an insulin-independent mechanism, and by doing so, modulates contractile kinetics, via
962 Ca^{2+} dynamics and ATP production. Together, these data describe a previously unknown
963 mechanism to regulate skeletal muscle dynamics. These data provide the foundation for a proposed
964 mechanism of TCAP-1 action in skeletal muscle. TCAP-1 interacts with LPHN-1 and -3 to
965 stimulate the activation of G-protein-coupled PLC leading to the increased conversion of PIP3 into
966 IP3 and DAG. Increased IP3 levels stimulates the IP3R on the SR, opening Ca^{2+} channels to
967 increase cytosolic Ca^{2+} levels. Cytosolic Ca^{2+} is imported into the mitochondria likely via the Ca^{2+}
968 uniporter (MCU) which stimulates the TCA cycle and electron transport chain (ETC). Enhanced
969 ETC activity results in increased proton extrusion from the mitochondrial matrix and
970 hyperpolarization of mitochondrial membrane potential. This ultimately results in increased ATP
971 and NADH production, as well as increased SDH-ATP levels. Ca^{2+} is subsequently pumped out
972 of the mitochondria likely via Na^+/Ca^{2+} exchangers (NCX), thus restoring homeostatic levels of
973 Ca^{2+} . Moreover, we showed that the TCAP-1 increased cellular energy availability by increased
974 glucose importation into cells likely due to increased GLUT4 expression. The TCAP-1 mediated
975 mechanism is likely due to its interactions with LPHNs -1 and -3.

976

977 **Acknowledgments**

978 We thank the Canadian Natural Sciences and Engineering Research Council (NSERC) for
979 Discovery Grants to Profs. D.A. Lovejoy, M. Locke and L. Buck and for NSERC Post-Graduate
980 Scholarships to Dr. Andrea Reid, Yani Chen, Mei Xu and Mia Husic and an Ontario Graduate
981 Scholarship to Thomas Dodsworth. We also thank Protagenic Therapeutics Inc. for operational
982 funding to Prof. D.A. Lovejoy

983

984 ***Author Contributions:***

985 Dr. Andrea Reid performed most of the experiments in this study, and wrote the initial draft of this
986 paper. Dr. David Hogg developed many of the calcium-associated experiments and supervised the
987 calcium studies and analyses, Dr. D. Barsyte and T. Dodsworth developed the strategy, preparation
988 and analyses of the siRNA and CRISPR cell lines. Prof. P. Biga and R. Reid provided the guidance
989 on the role of TCAP with respect to mitochondrial respirations and NADH assays, Dr. A. Slee
990 provided critical information on TCAP-1 kinetics. Prof. L. Buck provided the guidance for the
991 studies on energy metabolism in cells, and Prof. M. Locke developed and supervised the *in vivo*
992 studies on muscle metabolism. Mei Xu designed and performed the initial experiments to
993 determine the energy actions of TCAP in cells, Yani Chen developed the methods and analysed
994 the uptake of glucose in muscle cells in *in vivo* and *in vitro*. Mia Husic established some of the
995 methods for the cell culture of the transgenic cells and Prof. D. Lovejoy oversaw the entire research
996 program and analyses, and prepared the final drafts of the manuscript.

997

998 **References**

999

- 1000 1. Ni T, Yue J, Sun G, Zou Y, Wen J, Huang J. Ancient gene transfer from algae to animals:
1001 mechanisms and evolutionary significance. *BMC Mol Biol* 2012; 12: 83.
- 1002 2. Ramulu HG, Raoult D, Pontarotti P. The rhizome of life: what about metazoans? *Cell Infect*
1003 *Microbiol.* 2012; 2: 1–11.
- 1004 3. Lovejoy DA, Hogg DW. Information processing in affective disorders: Did an ancient peptide
1005 regulating intercellular metabolism become co-opted for noxious stress sensing? *BioEssays* 2020;
1006 2000039: 1-8.
- 1007 4. Chand D, De Lannoy L, Tucker RP, Lovejoy DA. Origin of chordate peptides by horizontal
1008 protozoan gene transfer in early metazoans and protists: Evolution of the teneurin C-terminal
1009 associated peptides. *Gen Comp Endocrinol* 2013; 188: 144-150.
- 1010 5. King N, Hittinger CT, Carroll SB. Evolution of key cell signaling and adhesion protein families
1011 predates animal origins. *Science* 2003; 301: 361–363.
- 1012 6. King N, Westbrook MJ, Young SL, Kuo A, Abedin M, Chapman J. et al. The genome of the
1013 choanoflagellate *Monosiga brevicollis* and the origin of metazoans. *Nature* 2008; 451: 783–788.
- 1014 7. Strotmann R, Schrock K, Boselt I, Staubert C, Russ A, Schoneberg T. Evolution of GPCR:
1015 Change and continuity. *Mol Cell Endocrinol.* 2011; 331: 170-178.
- 1016 8. Tucker RP. Horizontal gene transfer in choanoflagellates. *J Exp Zool B Mol Dev Evol* 2013;
1017 320: 1–9.
- 1018 9. Zhang D, de Souza RF, Anantharaman V, Iyer LM, Aravind L. Polymorphic toxin systems:
1019 comprehensive characterization of trafficking modes, mechanism of action, immunity and ecology
1020 using comparative genomics. *Biol Direct.* 2012; 7:18.

- 1021 10. Baumgartner S, Martin D, Hagios C, Chiquet-Ehrismann R. Ten-m, a *Drosophila* gene related
1022 to tenascin is a new pair-rule gene. *EMBO J.* 1994; 13: 3728-3740.
- 1023 11. Hong W, Mosca TJ, Luo L. Teneurins instruct synaptic partner matching in an olfactory map.
1024 *Nature* 2012; 484: 201–207.
- 1025 12. Kenzelmann D, Chiquet-Ehrismann R, Leachman NT, Tucker RP. Teneurin-1 is expressed in
1026 interconnected regions of the developing brain and is processed in vivo. *BMC Dev Biol.* 2008; 8:
1027 30.
- 1028 13. Levine A, Bashan-Ahrend A, Budai-Hadrian O, Gartenber D, Menasherow S, Wides R. Odd
1029 Oz: a novel *Drosophila* pair-rule gene. *Cell* 1994; 77: 587-598.
- 1030 14. Li J, Shalev-Benami M, Sando R, Jiang X, Kibrom A, Wang J. et al. Structural basis for
1031 teneurin function in circuit-wiring: a toxin motif at the synapse. *Cell* 2018; 173: 735-748.
- 1032 15. Mosca TJ, Hong W, Dani VS, Favaloro V, Luo L. Trans-synaptic Teneurin signalling in
1033 neuromuscular synapse organization and target choice. *Nature* 2012; 484: 237-241.
- 1034 16. Rubin BP, Tucker RP, Martin D, Chiquet-Ehrismann, R. Teneurins: a novel family of neuronal
1035 cell surface proteins invertebrates, homologous to the *Drosophila* pair-rule gene product Ten-m.
1036 *Dev Biol* 1999; 216: 195-209.
- 1037 17. Tucker RP, Chiquet-Ehrismann R. Teneurins: a conserved family of transmembrane proteins
1038 involved in intercellular signalling during development. *Dev Biol* 2006; 290: 237-245.
- 1039 18. Young TR, Leamey CA. Teneurins: important regulators of neural circuitry. *Int J Biochem*
1040 *Cell Biol* 2009; 41: 990–993.
- 1041 19. Minet AD, Chiquet-Ehrismann R. Phylogenetic analysis of teneurin genes and comparison to
1042 the rearrangement hotspot elements of *E. coli*. *Gene* 2000; 257: 87–97.

- 1043 20. Minet AD, Rubin BP, Tucker RP, Baumgartner S, Chiquet-Ehrismann R. Teneurin-1, a
1044 vertebrate homologue of the *Drosophila* pair-rule gene *ten-m*, is a neuronal protein with a novel
1045 type of heparin-binding domain. *J Cell Sci* 1999; 112: 2019-2032.
- 1046 21. Oohashi T, Zhou XH, Feng K, Richter B, Mörgelin M, Perez MT, et al. Mouse *ten-m/Odz* is
1047 a new family of dimeric type II transmembrane proteins expressed in many tissues. *Cell Biol*
1048 1999; 145: 563-577.
- 1049 22. Jackson VA, Meijer DH, Carrasquero M, van Bezouwen LS, Lowe ED, Kleanthous C, et al.
1050 Structures of Teneurin adhesion receptors reveal an ancient fold for cell-cell interaction. *Nat*
1051 *Commun* 2018; 9:1079.
- 1052 23. Lovejoy DA, Al Chawaf A, Cadinouche A. Teneurin C-terminal associated peptides: An
1053 enigmatic family of neuropeptides with structural similarity to the corticotrophin releasing factor
1054 and calcitonin family of peptides. *Gen Comp Endocrinol* 2006; 148: 299-305.
- 1055 24. Wang L, Rotzinger S, Barsyte-Lovejoy D, Qian X, Elias CF, Bittencourt JC et al. Teneurin
1056 proteins possess a carboxy terminal corticotropin-releasing factor-like sequence that modulates
1057 emotionality and neuronal growth. *Mol Brain Res* 2005; 133: 253-265.
- 1058 25. Aravind L, Anantharaman V, Zhang D, de Sousa RF, Iyer LM. Gene flow and biological
1059 conflict systems in the evolution of eukaryotes. *Front Cell Infect Microbiol* 2012; 2: 89.
- 1060 26. Michalec OM, Chang B, Lovejoy N, Lovejoy DA. Corticotropin-releasing factor (CRF) and
1061 its relationship to an ancient peptide family. *Front Endocrinol* 2020; 11: 529.
- 1062 27. Sekar R, Chow BKC. Role of the secretin peptide family and their receptors in the
1063 hypothalamic control of energy homeostasis. *Horm Metab Res* 2013; 45:945-954.
- 1064 28. Araç D, Li J. Teneurins and latrophilins: two giants meet at the synapse. *Curr Opin Struct*
1065 *Biol* 2019; 54: 141-151.

- 1066 29. Fredricksson R, Lagerstrom L, Lundin LG, Schioth H. The G protein coupled receptors in the
1067 human genome form five main families. Phylogenetic analysis, paralogon groups and fingerprints.
1068 Mol. Pharmacol. 2003; 63: 1256-1272.
- 1069 30. Fredriksson R, Schiöth H, The repertoire of G-protein coupled receptors in fully sequenced
1070 genomes. Mol Pharmacol 2005; 67: 1414-1425.
- 1071 31. Silva JP, Lelianova VG, Ermolyuk YS, Vysokov N, Hitchen PG, Berninghausen O et al.
1072 Latrophilin 1 and its endogenous ligand Lasso/teneurin-2 form a high-affinity trans-synaptic
1073 receptor pair with signaling capabilities. Proc Natl Acad Sci USA 2011; 108: 12113-12118.
- 1074 32. Husic M, Barsyte-Lovejoy D, Lovejoy DA. Teneurin C-terminal associated peptide (TCAP)-
1075 1 and latrophilin interaction in HEK293 cells: Evidence for modulation for intercellular adhesion.
1076 Front. Endocrinol. 2019; 10: 22.
- 1077 33. Chand D, Casatti CA, DeLannoy L, Song L, Kollara A, Barsyte-Lovejoy D, et al. C-terminal
1078 processing of the teneurin proteins: Independent actions of a teneurin C-terminal associated
1079 peptide in hippocampal cells. Mol Cell Neurosci. 2013; 52: 38-50.
- 1080 34. Reid RM, Reid AL, Lovejoy DA, Biga PR. Teneurin C-terminal associated peptide (TCAP)-3
1081 increases metabolic activity in zebrafish. Front Mar Sci. 2021; 7: 591160.
- 1082 35. Tan L, Xu K, Vaccarino F, Lovejoy DA, Rotzinger S. Repeated intracerebral teneurin C-
1083 terminal associated peptide (TCAP)-1 injections produce enduring changes in behavioral
1084 responses to corticotropin-releasing factor (CRF) in rat models of anxiety. Behav Brain Res 2008;
1085 188, 195-200.
- 1086 36. Tan LA, Al Chawaf A, Vaccarino FJ, Boutros JC, Lovejoy DA. Teneurin C-terminal
1087 associated peptide (TCAP)-1 increases dendritic spine density in hippocampal neurons and
1088 decreases anxiety-like behaviors in rats. Physiol Behav 2011; 104: 199-204.

- 1089 37. Al Chawaf A, St. Amant K, Belsham DD, Lovejoy DA. Regulation of neurite outgrowth in
1090 immortalized hypothalamic cells and hippocampal primary cultures by teneurin C-terminal
1091 associate peptide-1 (TCAP-1). *Neuroscience* 2007; 144: 1241-1254.
- 1092 38. Chand D, Song L, De Lannoy L, Barsyte-Lovejoy D, Ackloo S, Boutros PC et al. C-terminal
1093 region of teneurin-1 co-localizes with dystroglycan and modulates cytoskeletal organization
1094 through an ERK-dependent stathmin- and filamin A-mediated mechanism in hippocampal cells.
1095 *Neuroscience* 2012; 219: 255-270.
- 1096 39. Hogg DW, Chen Y, D'Aquila AL, Xu M, Husic M, Tan LA et al. A novel role of the
1097 corticotropin-releasing hormone (CRH) regulating peptide, teneurin C-terminal associated peptide
1098 (TCAP)-1 on glucose uptake into the brain. *J Neuroendocrinol* 2018; 30: e12579.
- 1099 40. Richter EA, Hargraves M. GLUT4 and skeletal muscle glucose uptake. *Physiol Rev* 2013; 93:
1100 993-1017.
- 1101 41. Santos JM, Ribeiro SB, Gaya AR, Appell HJ, Duarte JA. Skeletal muscle pathways of
1102 contraction-enhanced glucose uptake. *Int J Sports Med* 2008; 29: 785–794.
- 1103 42. Sayer AA, Dennison EM, Syddall HE, Gilbody HJ, Philips DIW, Cooper C. Type 2 diabetes,
1104 muscle strength, and impaired physical function: the tip of the iceberg? *Diabetes Care* 2005; 28:
1105 2541-2542.
- 1106 43. Holwerda AM, Locke M. Hsp25 and Hsp72 content in rat skeletal muscle following controlled
1107 shortening and lengthening contractions. *Appl Physiol Nutr Met* 2014; 39: 1380–1387.
- 1108 44. Maher F. Immunocolocalization of GLUT1 and GLUT3 glucose transporters in primary
1109 culture neurons and glial. *J Neurosci Res* 1995; 42: 459-469.
- 1110 45. Uemura E, Greenlee HW. Insulin regulates neuronal glucose uptake by promoting
1111 translocation of glucose transporter GLUT3. *Exp Neurol* 2006; 198: 48-53.

- 1112 46. Liang H, Ward WF. PGC-1 α : a key regulator of energy metabolism. *Adv Physiol Educ* 2006;
1113 30:154-151.
- 1114 47. Davletov BA, Shamotienko OG, Lelianova VG, Grishin V, Ushkaryov YA. Isolation and
1115 biochemical characterization of a calcium-independent alpha-latrotoxin-binding protein. *J Biol*
1116 *Chem* 1996; 271: 23239-23245.
- 1117 48. Silva J-P, Ushkaryov YA. The latrophilins, “split-personality” receptors. *Adv Exp Med Biol*
1118 2010; 706: 59-75.
- 1119 49. Qian X, Barsyte-Lovejoy D, Chewpoy RB, Wang L, Gautam N, Wang N et al. Characterization
1120 of teneurin C-terminal associated peptide (TCAP)-3 from rainbow trout hypothalamus. *Gen Comp*
1121 *Endocrinol* 2004; 137: 205-216.
- 1122 50. Al Chawaf A, Xu K, Tan L, Vaccarino F, Lovejoy DA, Rotzinger S. Corticotropin-releasing
1123 factor behaviours are modulated by intravenous administration of teneurin C-terminal associated
1124 peptides. *Peptides* 2007; 28: 1406-1415.
- 1125 51. Erb S, McPhee M, Brown ZJ, Kupferschmidt DA, Song L, Lovejoy DA. Repeated intravenous
1126 administrations of teneurin-C terminal associated peptide (TCAP)-1 attenuates reinstatement of
1127 cocaine seeking by corticotropin-releasing factor (CRF) in rats. *Beh Brain Res* 2014; 269: 1-5.
- 1128 52. Kupferschmidt D, Lovejoy DA, Rotzinger S, Erb S. Teneurin C-terminal associated peptide
1129 (TCAP)-1 blocks the effects of corticotropin-releasing factor (CRF) on the reinstatement of
1130 cocaine seeking and expression of cocaine-induced behavioral sensitization. *Br J Pharmacol* 2011;
1131 163: 574-583.
- 1132 53. Rotzinger, S, Lovejoy DA, Tan L. Behavioral effects of neuropeptide ligands in rodent models
1133 of depression and anxiety *Peptides* 2010; 31: 736-756.

- 1134 54. Tan LA, Chand D, De Almeida R, Xu M, Colacci M, De Lannoy et al. Modulation of
1135 neuroplastic changes and corticotropin-releasing factor associated behaviour by a phylogenetically
1136 ancient and conserved peptide family. *Gen Comp Endocrinol* 2012; 176: 309-313.
- 1137 55. Ishii K, Suzuki N, Mabuchi Y, Ito N, Kikura N, Fukada S-I et al. Muscle satellite cell protein
1138 teneurin-4 regulates differentiation during muscle regeneration. *Stem Cells* 2015; 33: 3017-3027.
- 1139 56. Calderón JC, Bolaños P, Caputo C. The excitation–contraction coupling mechanism in
1140 skeletal muscle *Biophys Rev* 2014; 6:133–160.
- 1141 57. Denton RM. Regulation of mitochondrial dehydrogenases by calcium ions. *Biochim Biophys*
1142 *Acta* 2009; 1787: 1309-1306.
- 1143 58. Wan B, La Noue KF, Cheung JY, Scaduto RC. Regulation of citric acid cycle by calcium. *J*
1144 *Biol Chem* 1989; 264:13430-13439.
- 1145 59. Dias-Vegas AR, Cardova A, Valladares D, Llanos P, Hildago C, Gharardi G et al.
1146 Mitochondrial calcium increase induced by RyR1 and IP₃R channel activation after membrane
1147 polarizations regulates skeletal muscle metabolism. *Front Physiol* 2018; 9: 791
- 1148 60. Rahman MA, Ashton AC, Meunier F, Davletov BA, Dolly JO, Ushkaryov YA. Norepinephrine
1149 exocytosis stimulated by alpha-latrotoxin requires both external and stored calcium, and is
1150 mediated by latrophilin, G proteins and phospholipase C. *Phil Trans R Soc London B* 1999; 354:
1151 379-386.
- 1152 61. J Lang, Y Ushkaryov, A Grasso, C B Wollheim. Ca²⁺-independent insulin exocytosis induced
1153 by alpha-latrotoxin requires latrophilin, a G protein-coupled receptor. *EMBO J* 1998; 17:648-57.
- 1154 62. Sando R, Südhof TC. Latrophilin GPCR signaling mediates synapse formation. *Elife*.
1155 2021;10:e65717.

- 1156 63. Rolfe DF, Brand MD. The physiological significance of mitochondrial proton leak in animal
1157 cells and tissues *Biosci Rep* 1997; 17: 9-16.
- 1158 64. R othe J, Thor R, Winkler J, Knierim AB, Binder C, Huth S, et al. Involvement of the adhesion
1159 GPCRs latrophilin in the regulation of insulin release. *Cell Rep* 2019; 26: 1537-1584.
- 1160 65. Meza-Aguilar DG, Boucard AA. Latrophilins updated. *Biomol Concepts* 2014; 5: 457-478.
- 1161 66. Milligan G, Ward RJ, Marsango S. 2019 GPCR homo-oligomerization. *Curr. Opin. Cell*
1162 *Biol.* 57:40-47
- 1163 67. Volynski KA, Silva J-P, Lelianova VG, Atiqur Rahman M, Hopkins C, Ushkaryov YA.
1164 Latrophilin fragments behave as independent proteins that associate and signal on binding to
1165 LTX(N4C) *EMBO J* 2004; 23; 4423-4433.
- 1166 68 M uller A, Winkler J, Fiedler F, Sastraihardja T, Binder C, Schnabel R et al. Oriented cell
1167 division I the *C. elegans* embryo is coordinated by G-protein signaling dependent on the Adhesion
1168 GPCR LAT-1. *PLOS Genetics* 2015; 11: e1005624.
- 1169 69. Nazarko O, Kibrom A, Winkler J, Leon K, Stoveken H, Salzman G, et al. A comprehensive
1170 mutagenic screen of the Adhesion GPCR latrophin/ADGRL1. *iScience* 2015; 3; 264-278.
- 1171 70. Liebscher I, Sch on J, Peterson SC, Fischer L, Auerback N, Demberg LM et al. A tethered
1172 agonist within the ectodomain activates the adhesion G protein-coupled receptors GPR126 and
1173 GPR133. *Cell Rep* 2014; 9: 2018-2026.
- 1174 71. Davletov BA, Meunier FA, Ashton AC, Matsushita H, Hirst WD, Lelianova VG, et al. Vesicle
1175 exocytosis stimulated by alpha-latrotoxin is mediated by latrophilin and requires both external and
1176 stored Ca²⁺. *EMBO J* 1998;17: 3909-3920.
- 1177 72. Lelianova VG, Davletov BA, Sterling A, Atiqur Rahman M, Grishin EV Totty NF et al. α -
1178 latrotoxin receptor, latrophilin, is a novel member of the secretin family of G protein-coupled

1179 receptors. J. Biol. Chem. 1997; 272:21504-21521.

Figure 1

A

RAT TCAP-1	QQLLGTGRVQGYDGYFVLSVEQYLELSDSANNIHFMQRQSEI-NH2
MOUSE TCAP-1	QQLLGTGRVQGYDGYFVLSVEQYLELSDSANNIHFMQRQSEI-NH2
RAT TCAP-2	QQLLSTGRVQGYEGYYVLPVEQYPELADSSSNIQFLRQNEI-NH2
MOUSE TCAP-2	QQLLSTGRVQGYEGYYVLPVEQYPELADSSSNIQFLRQNEI-NH2
RAT TCAP-3	.QLLSAGKVQGYDGYVLSVEQYPELADSANNIQFLRQSEI-NH2
MOUSE TCAP-3	.QLLSAGKVQGYDGYVLSVEQYPELADSANNIQFLRQSEI-NH2
RAT TCAP-4	QQVLNTGRVQGYDGGFFVTSVEQYPELSDSANNIHFMQRQSEM-NH2
MOUSE TCAP-4	QQVLNTGRVQGYDGGFFVTSVEQYPELSDSANNIHFMQRQSEM-NH2

bioRxiv preprint doi: <https://doi.org/10.1101/2021.10.25.465698>; this version posted October 25, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under a [CC-BY 4.0 International license](#).

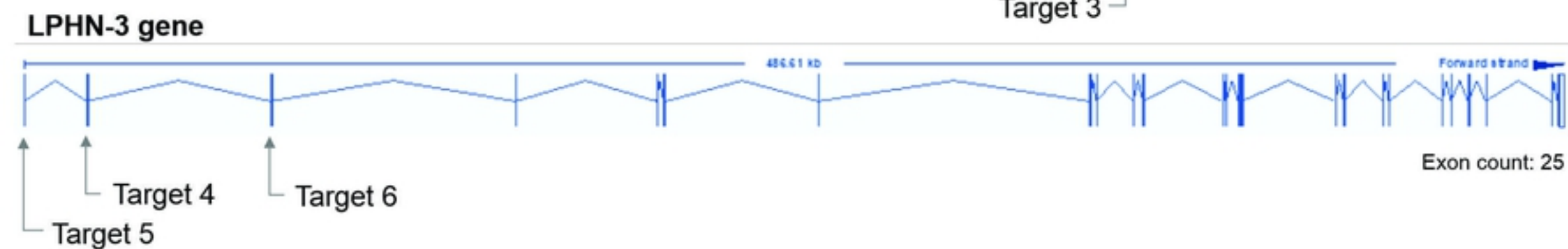
B

SYN. MOUSE TCAP-1	pEQLLGTGRVQGYDGYFVLSVEQYLELSDSANNIHFMQRQSEI-NH2
SC.MOUSE TCAP-1	pETHSSLELRVSLIGEQQFIGYENQSDQNYGLLAYFDRVGMS-NH2

A



B



C

LPHN-1		LPHN-3	
Target 1	5'-ACATTGTCAAATATGACCTG-3'	Target 4	5'-GAGCGCTCAACGGCTCATCG-3'
Target 2	5'-TGGAACCTACAAATACCTGG-3'	Target 5	5'-CACGATGCTTTTAGCACCTG-3'
Target 3	5'-CGTGGACTATGCCTTCAACA-3'	Target 6	5'-TCGAGAGCGCCAACTACGGG-3'

Figure 2

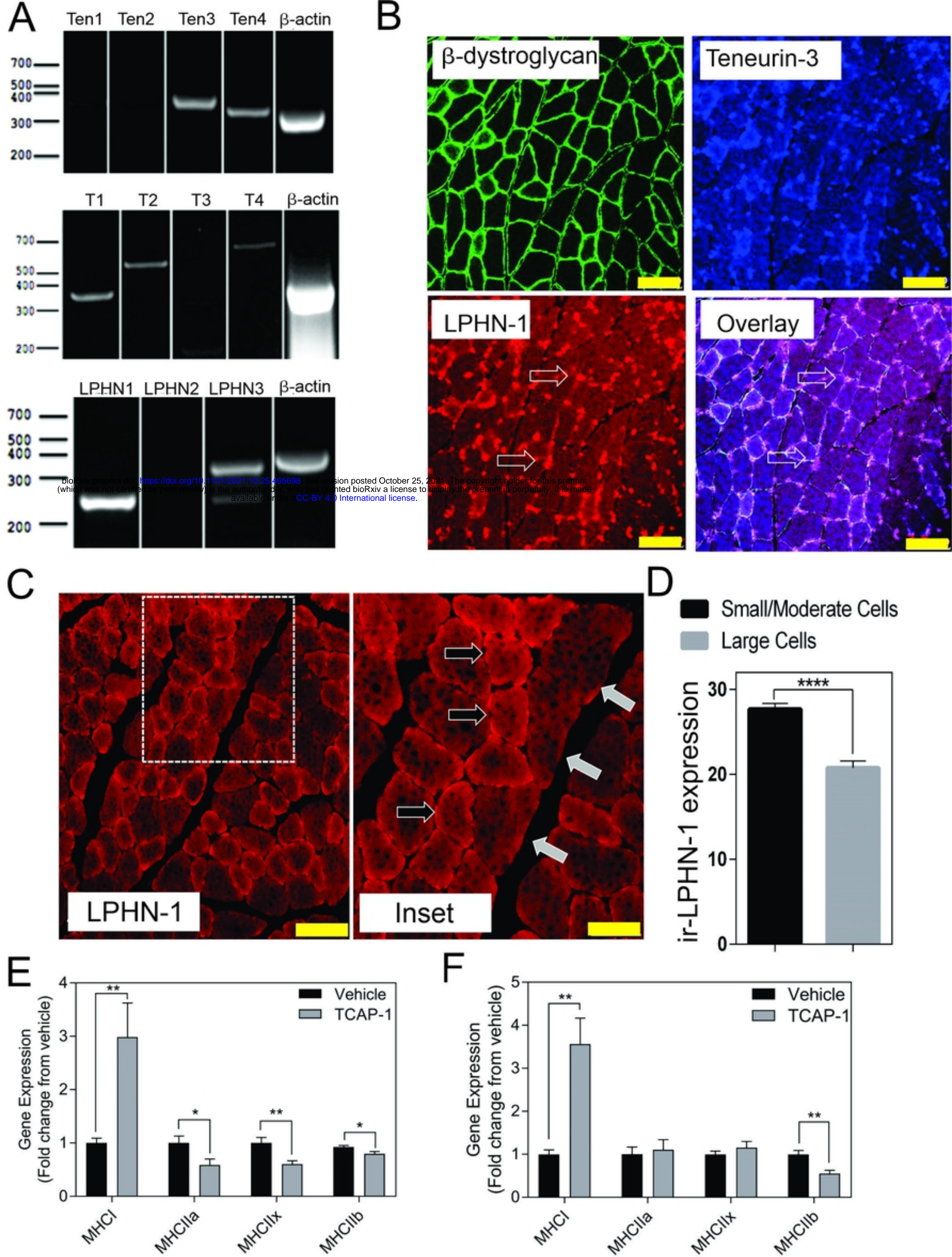


Figure 3

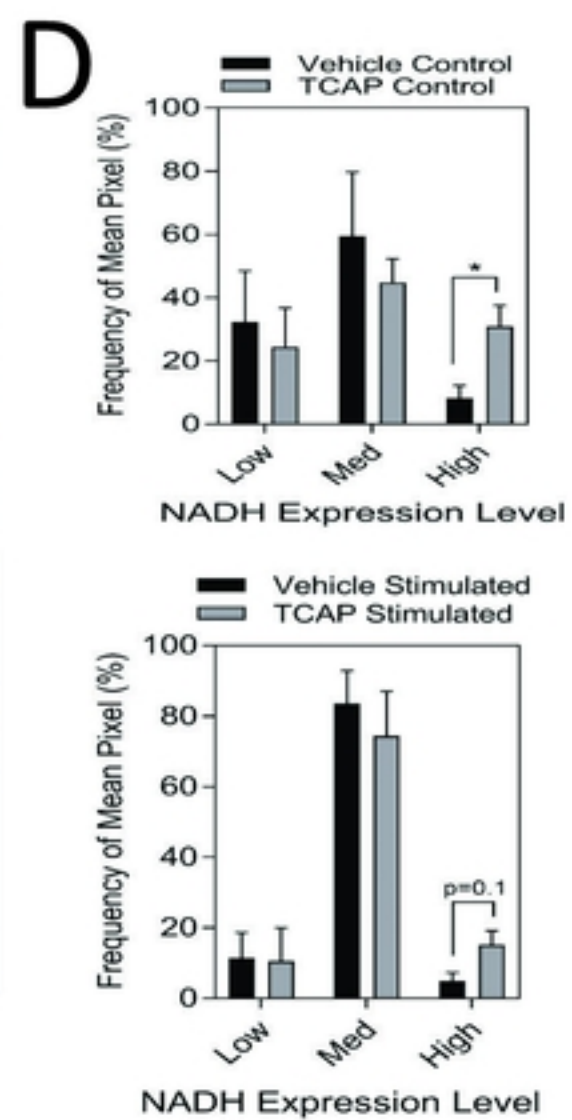
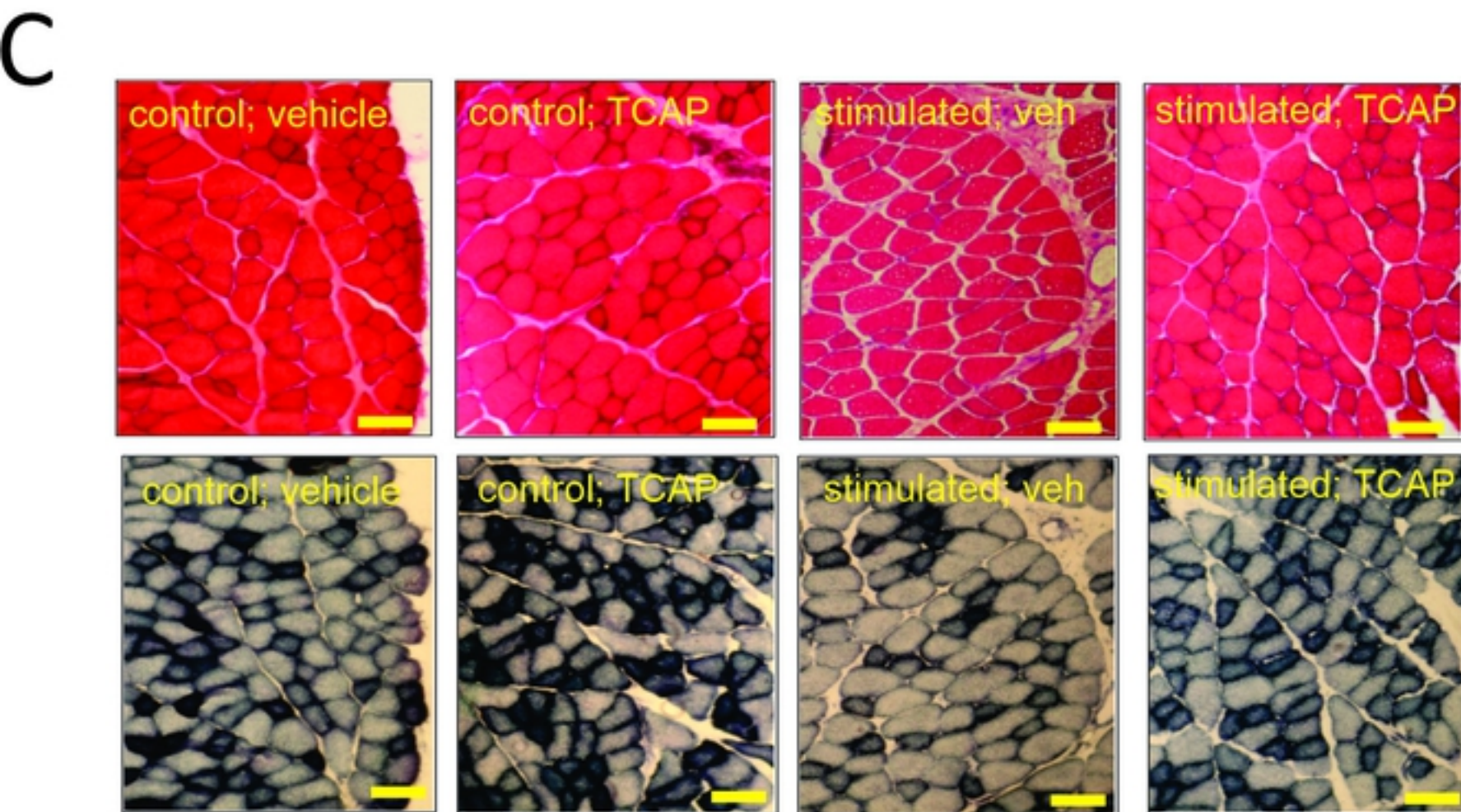
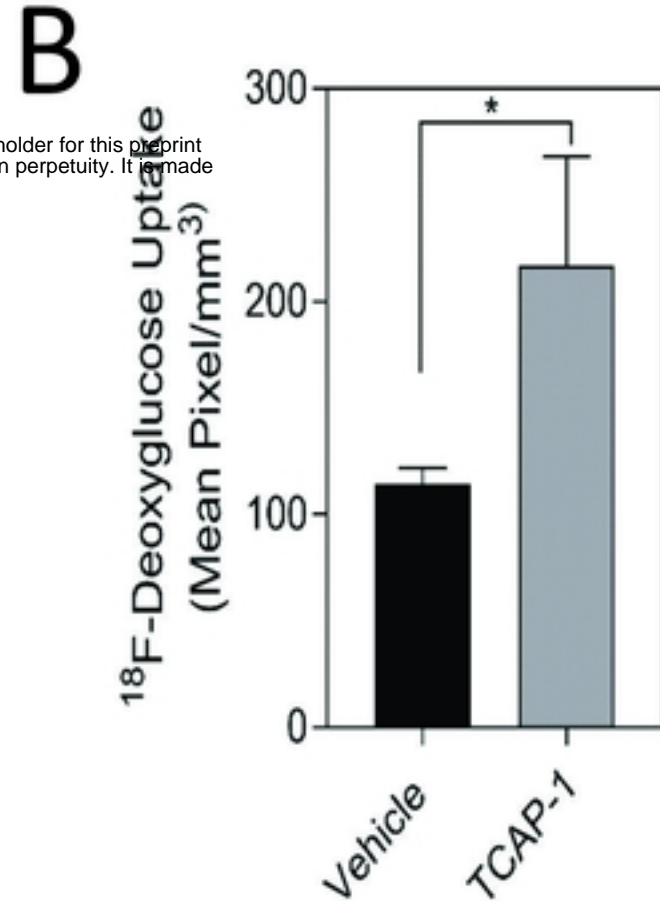
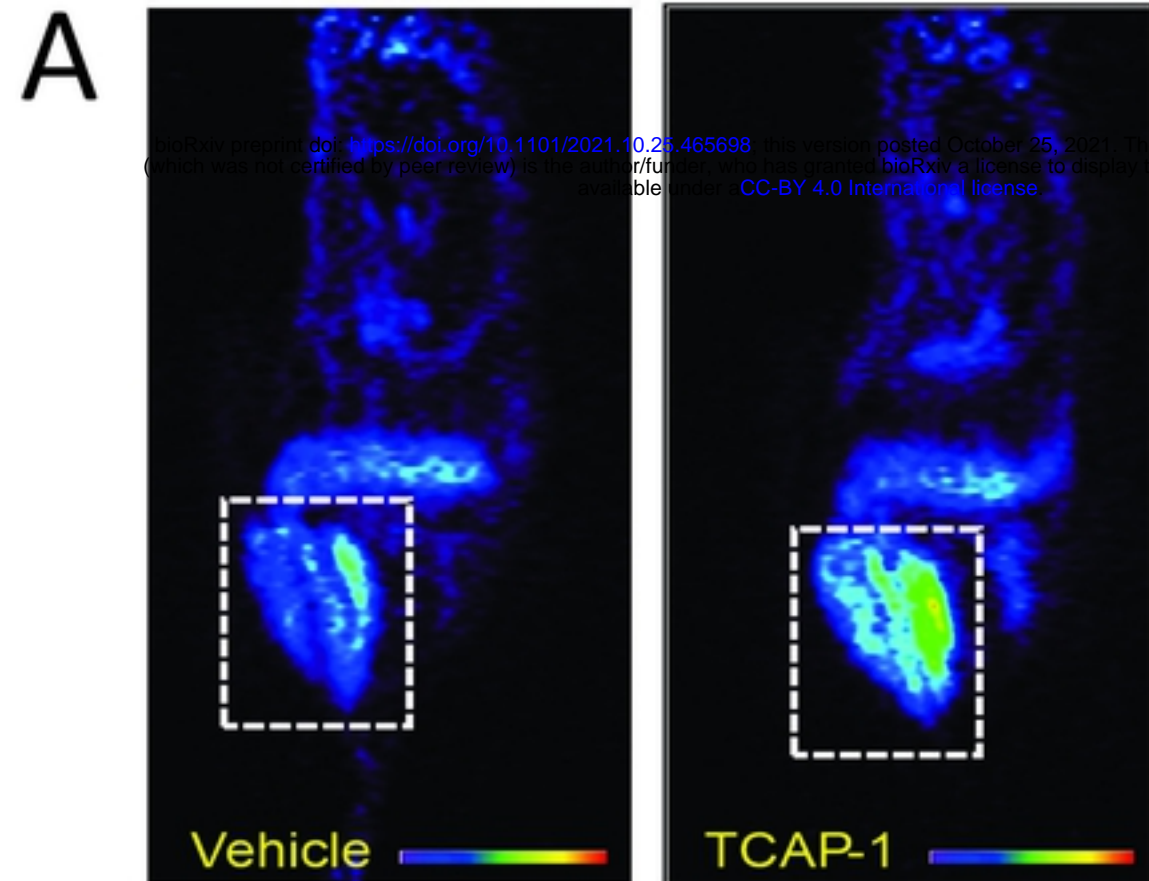


Figure 4

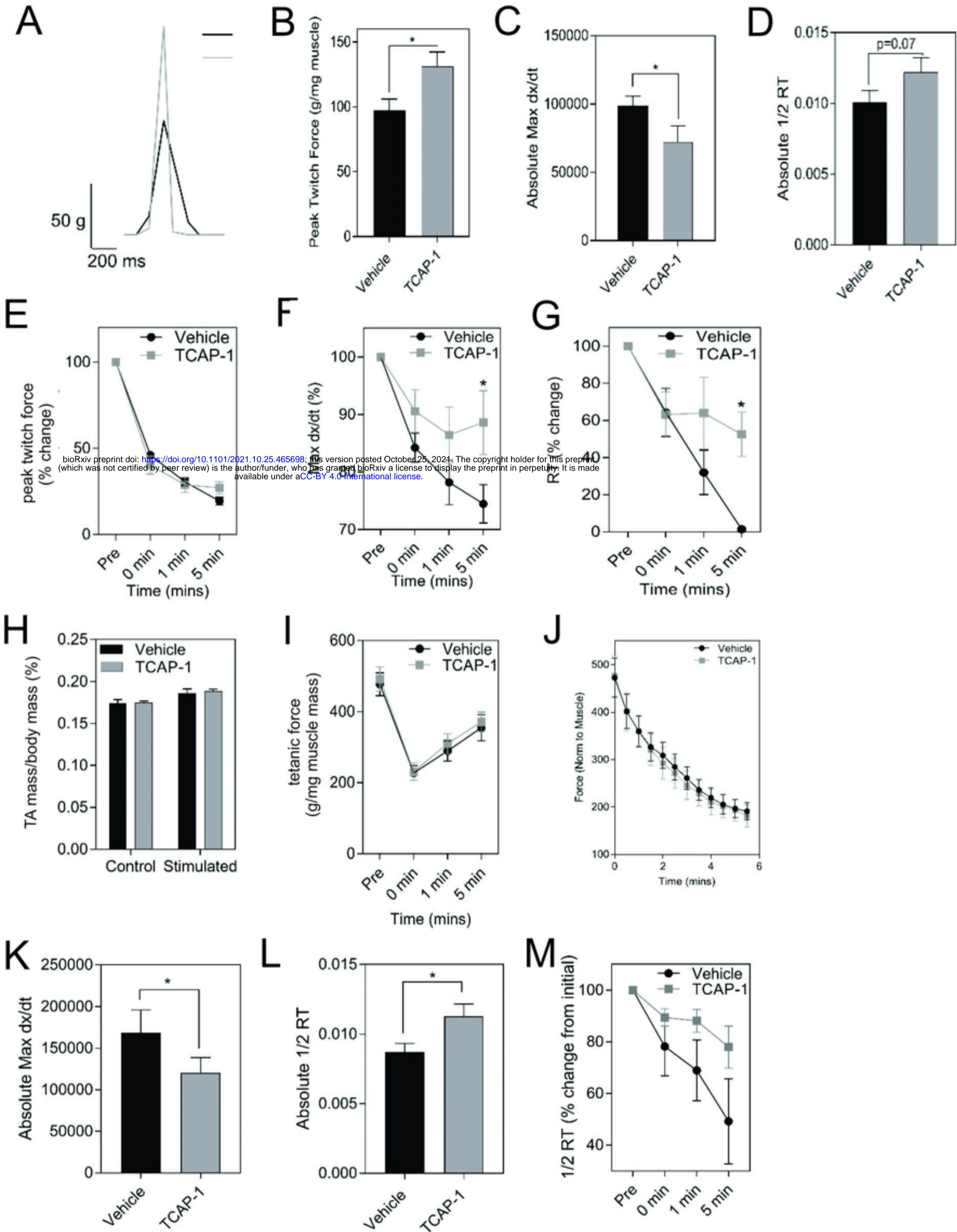


Figure 5

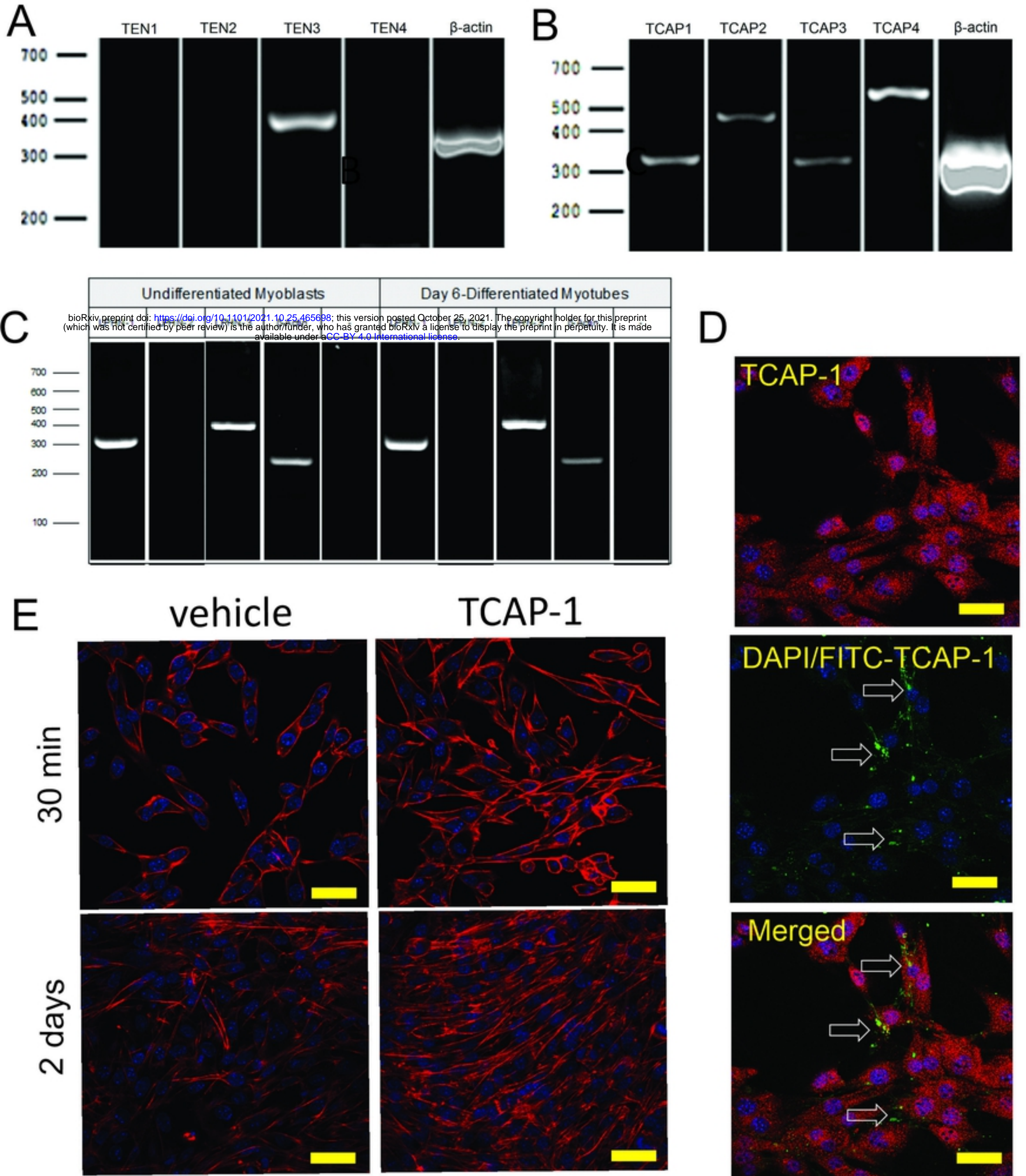


Figure 6

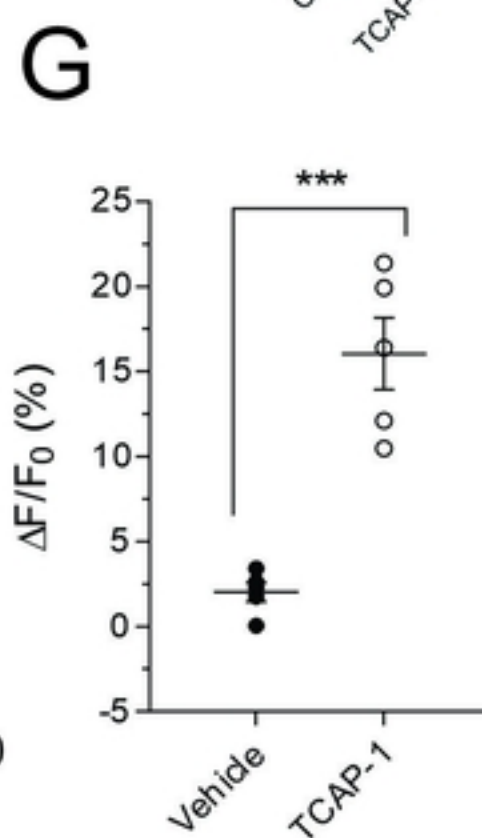
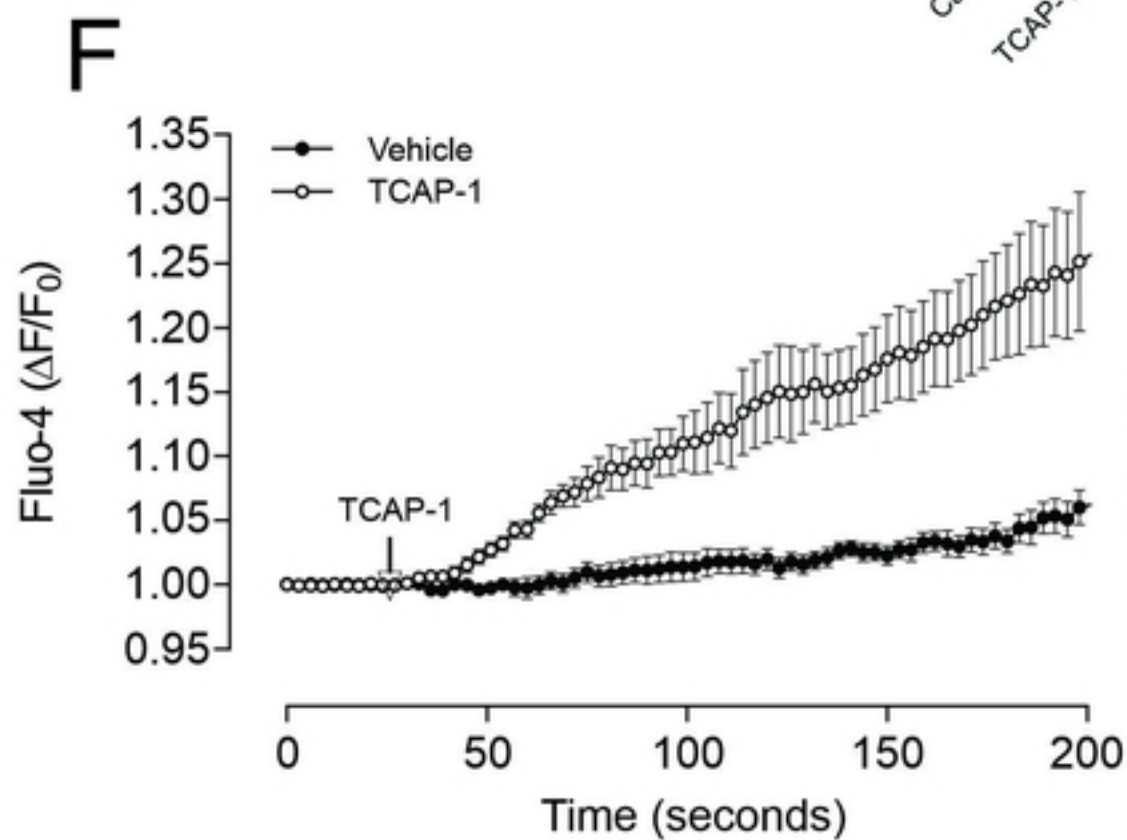
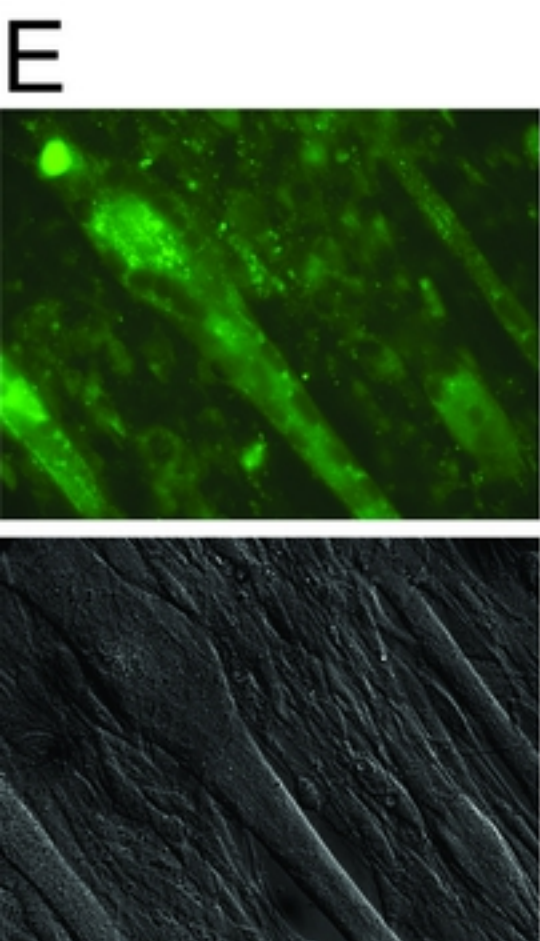
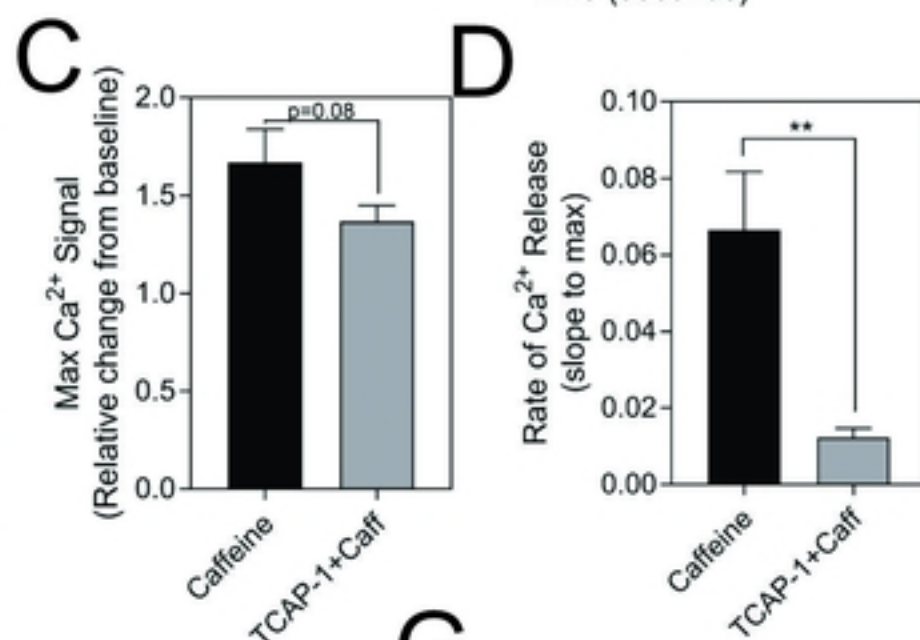
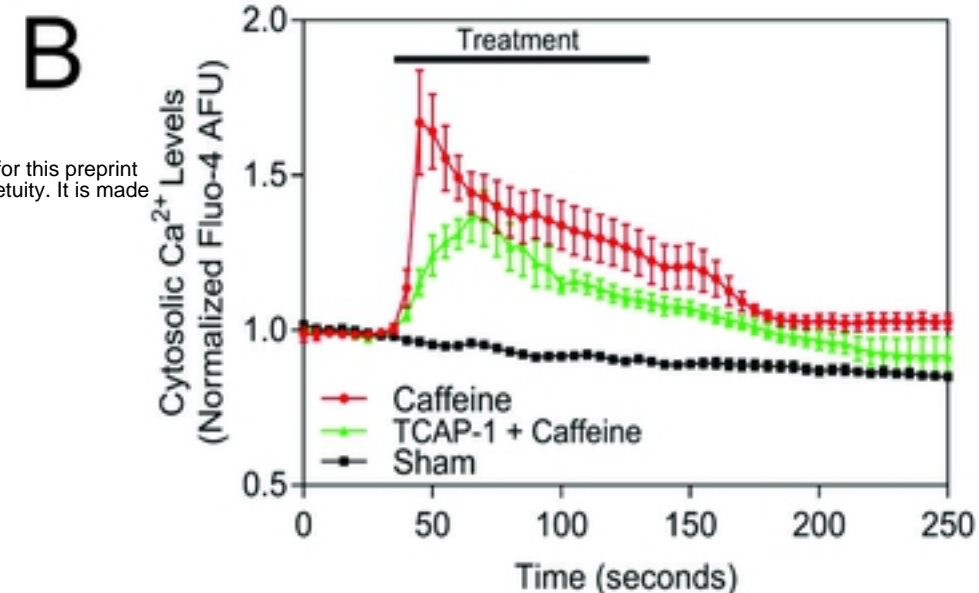
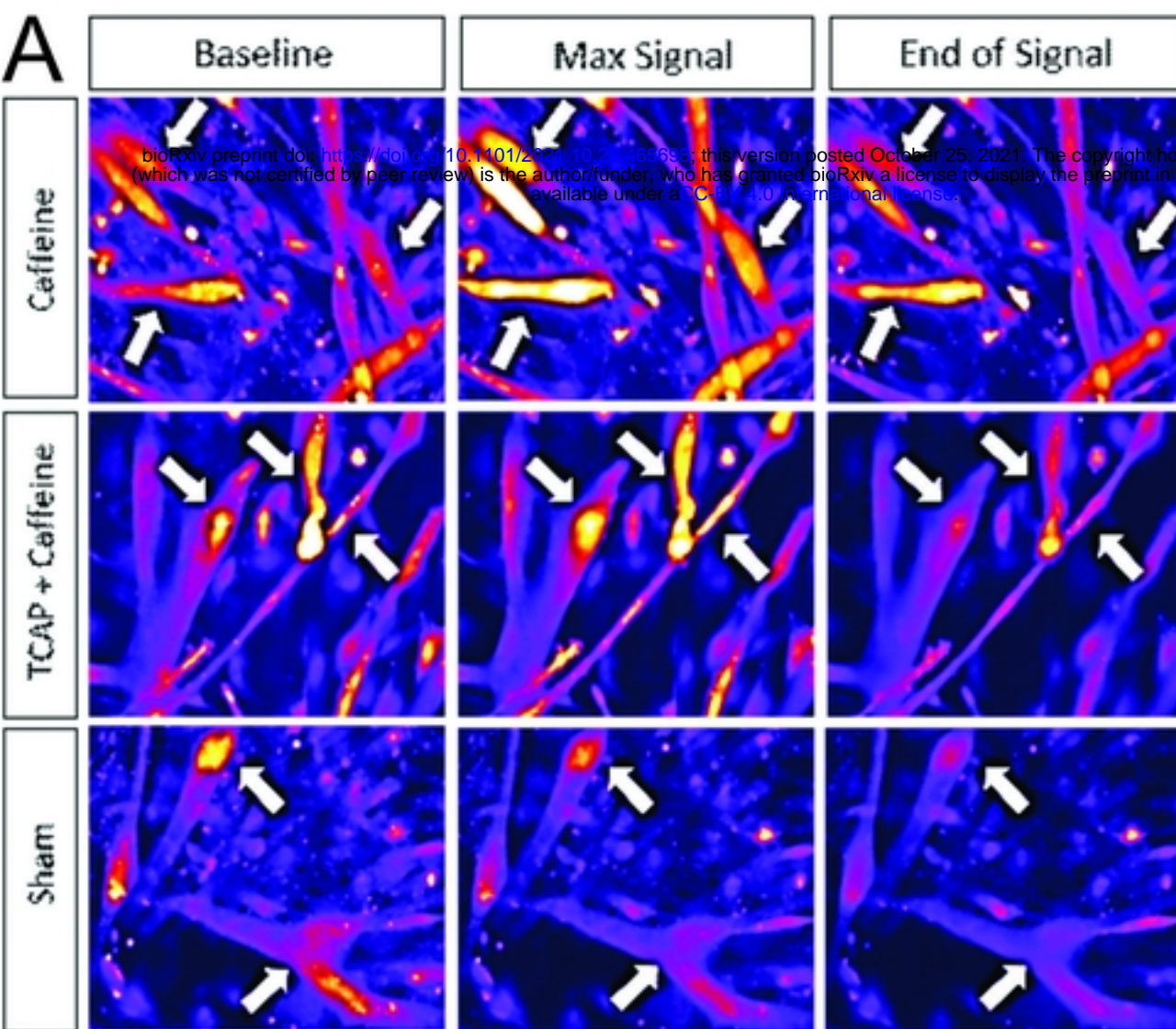


Figure 7

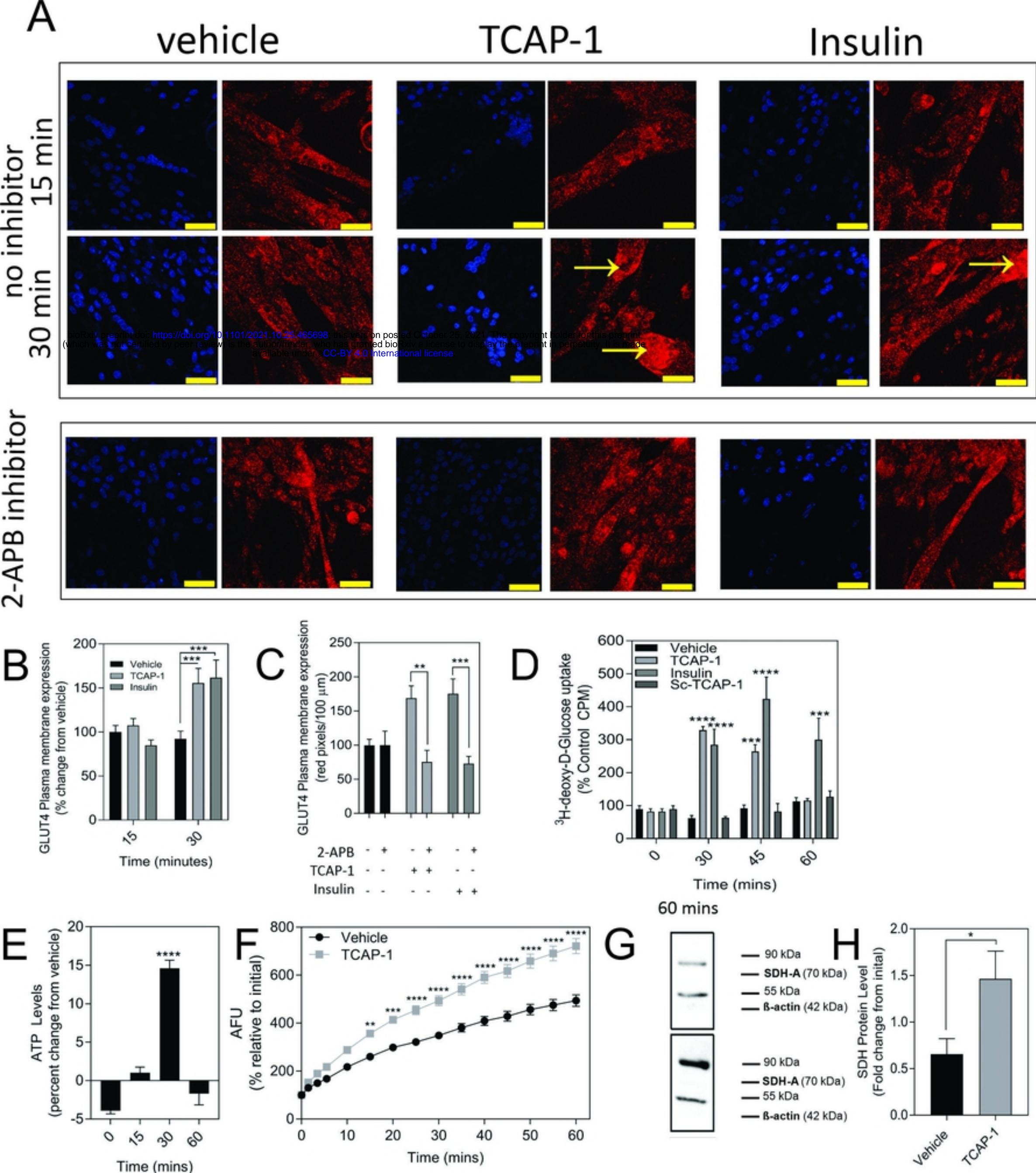


Figure 8

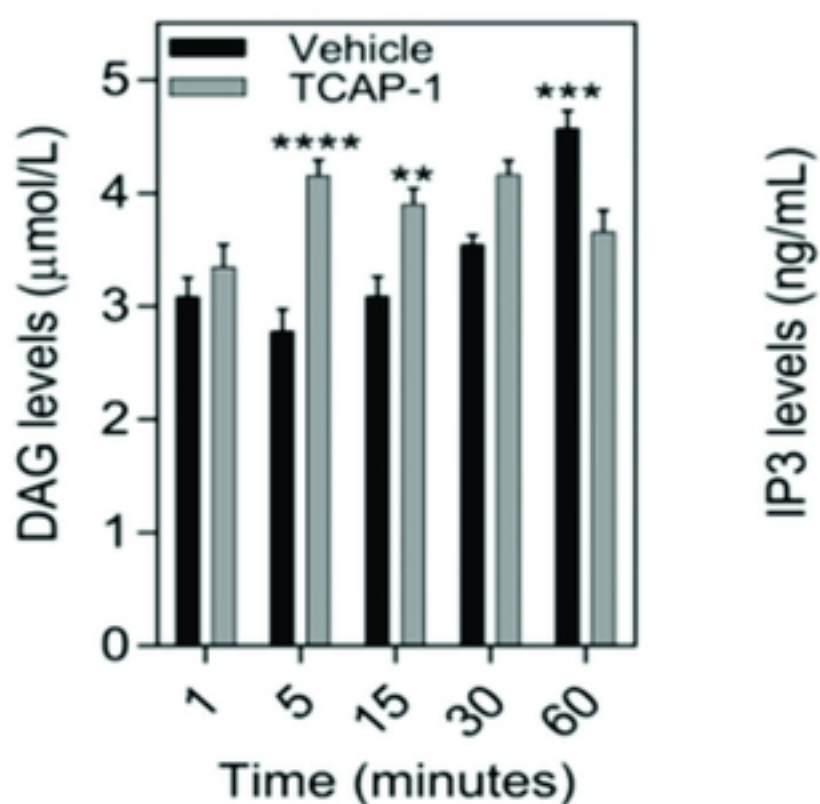
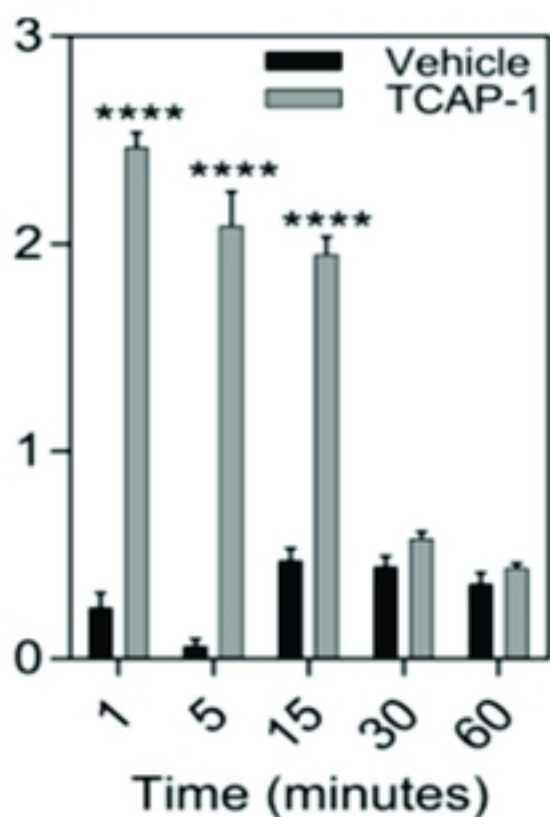
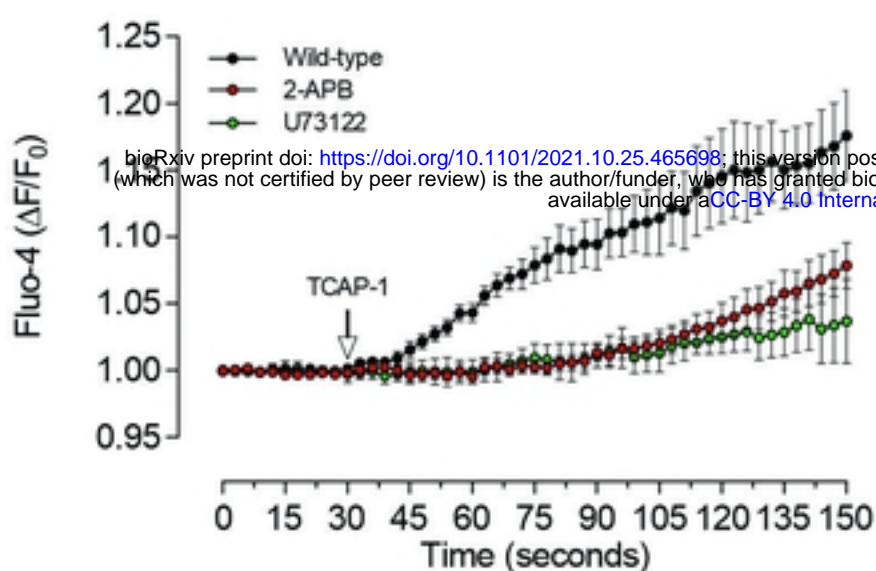
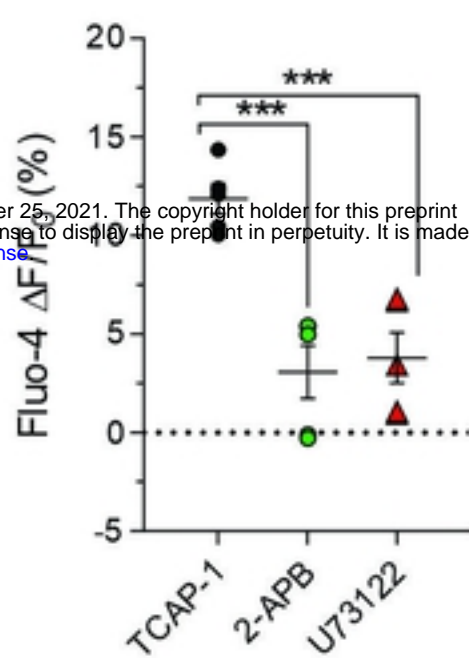
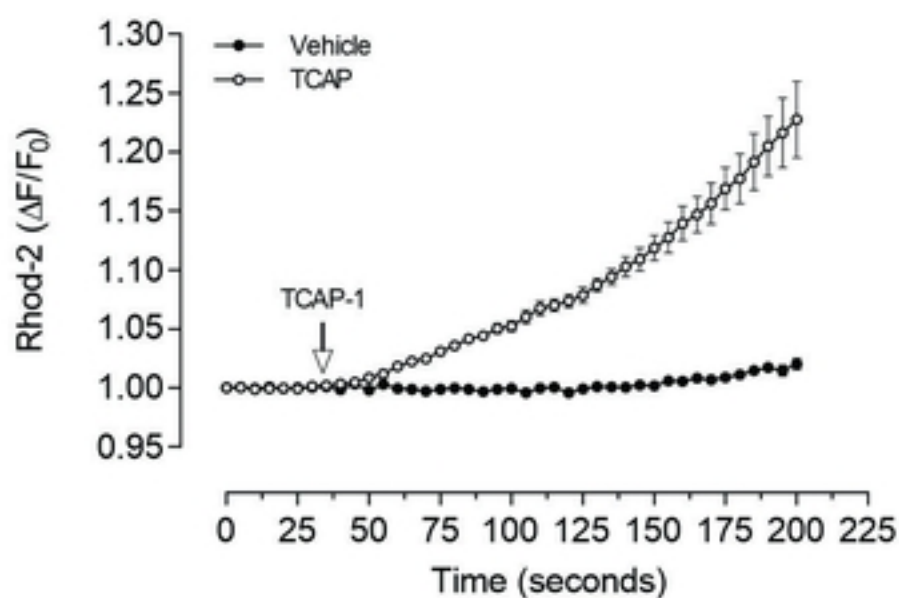
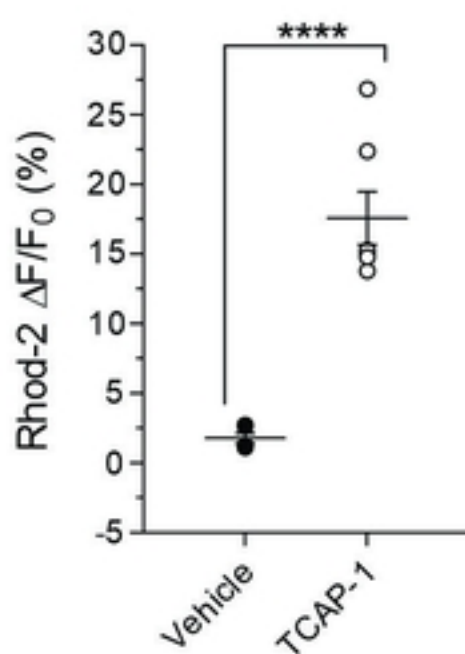
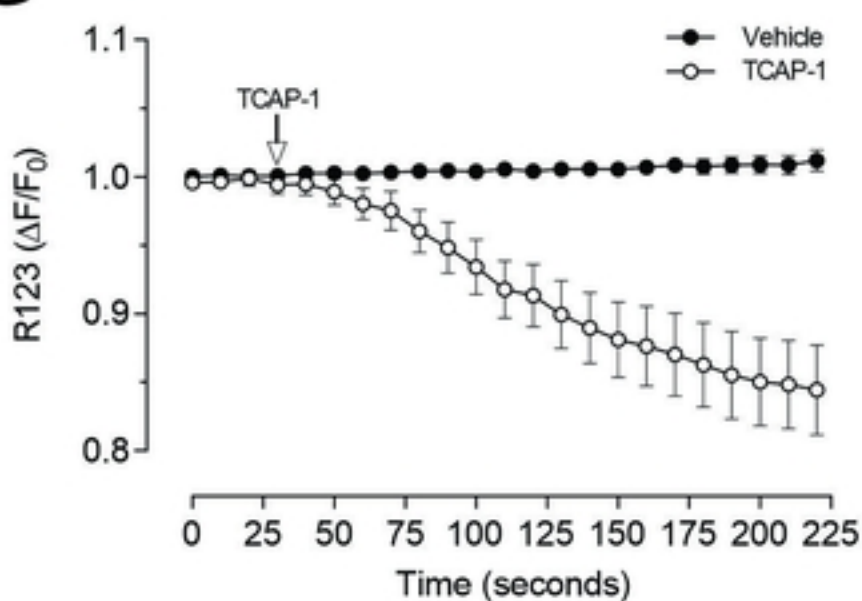
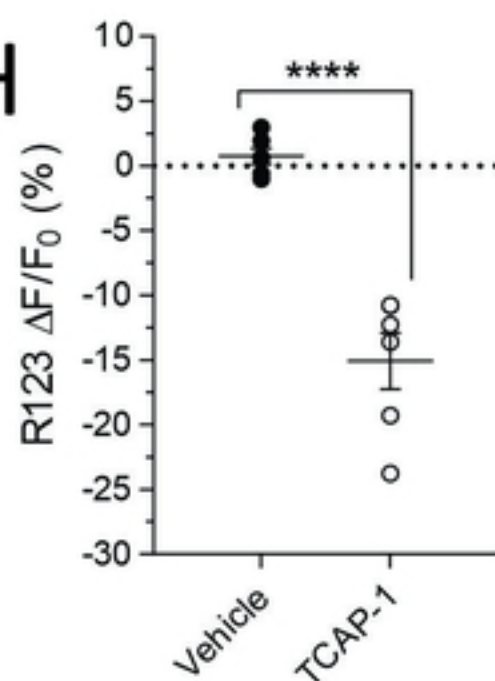
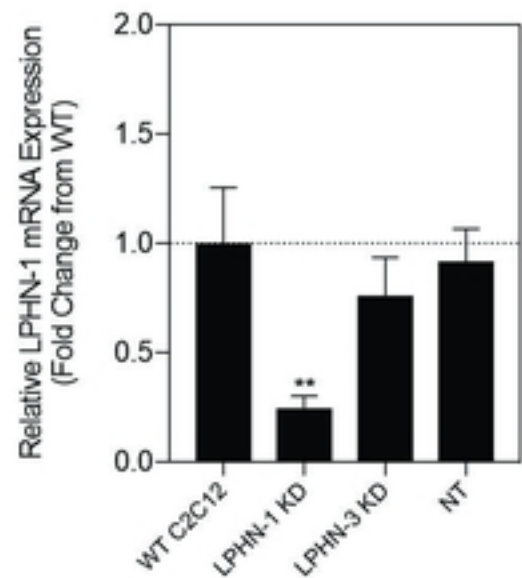
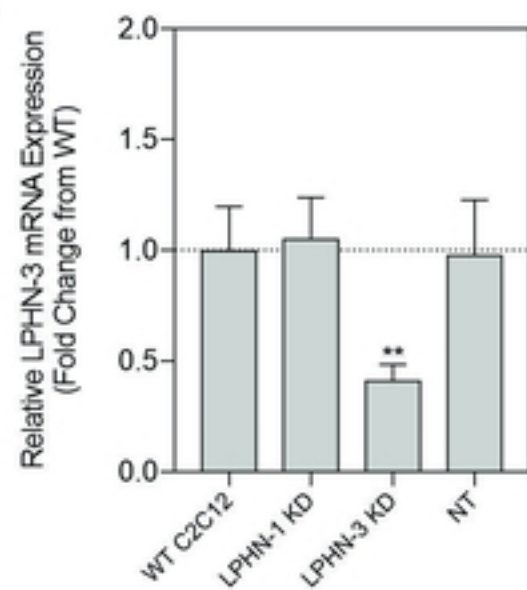
A**B****C****D****E****F****G****H**

Figure 9

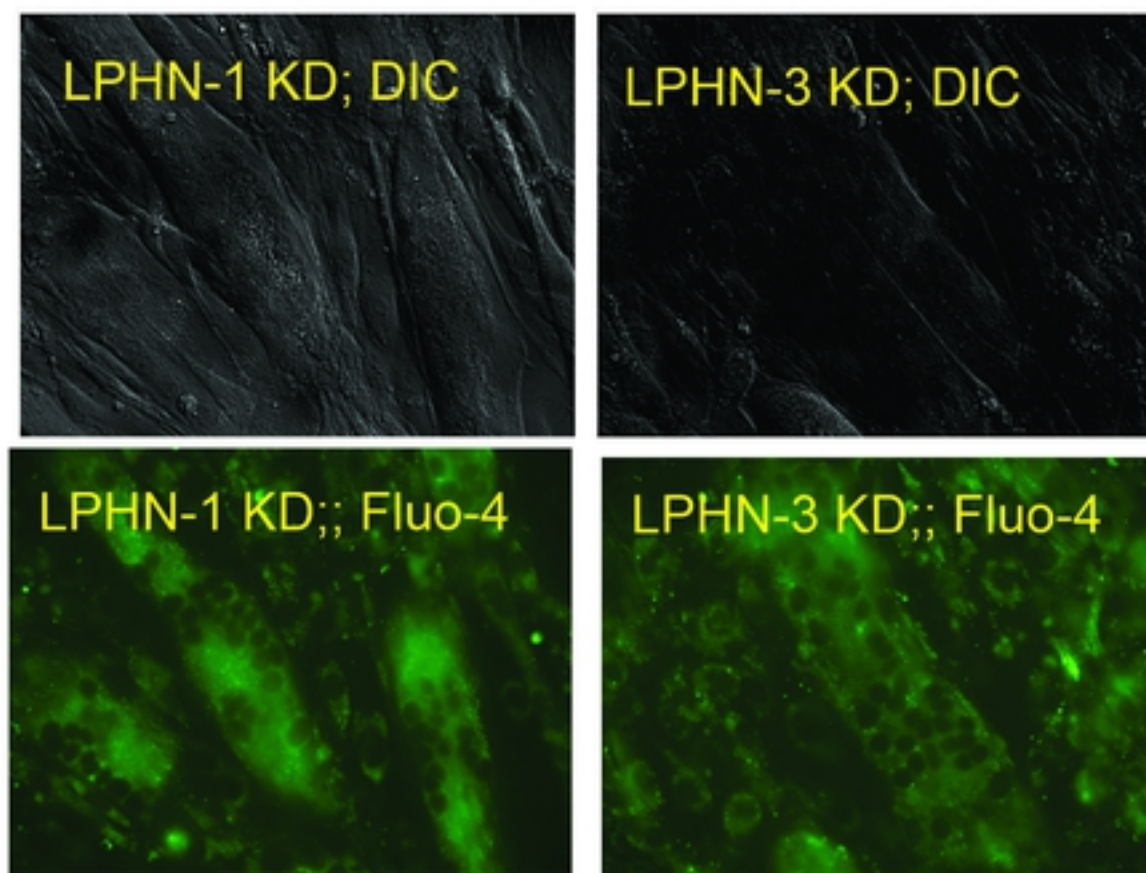
A



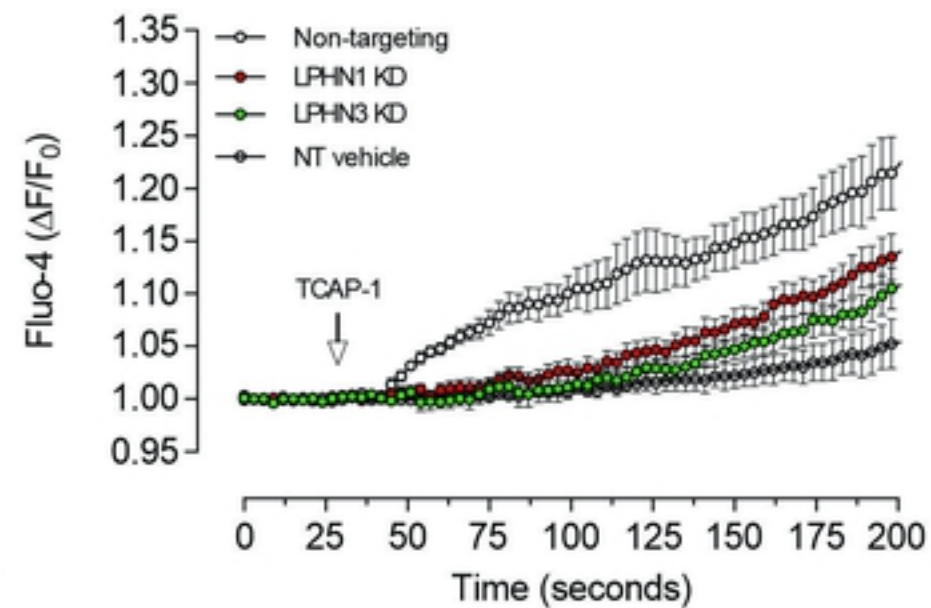
B



C



D



E

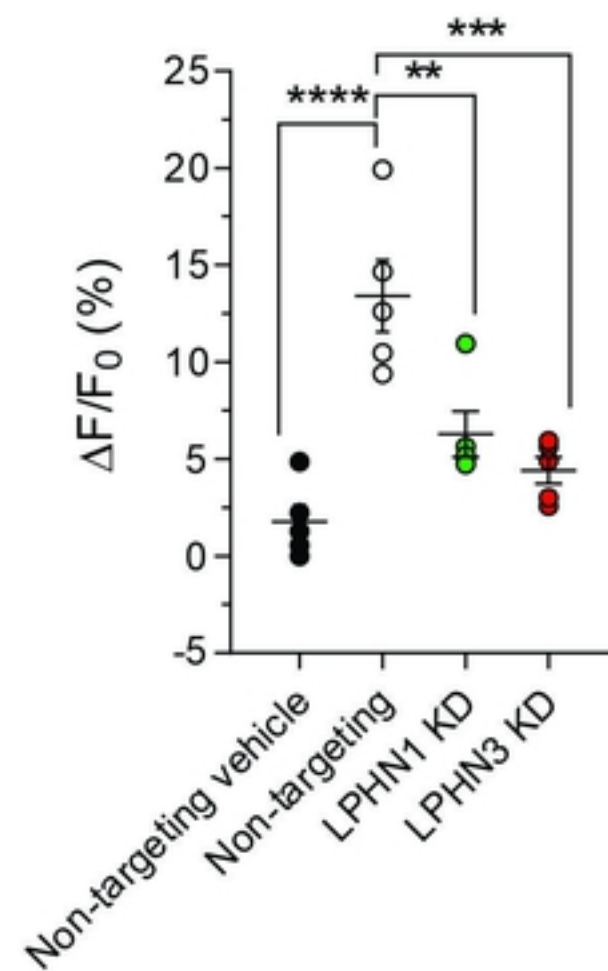


Figure 10

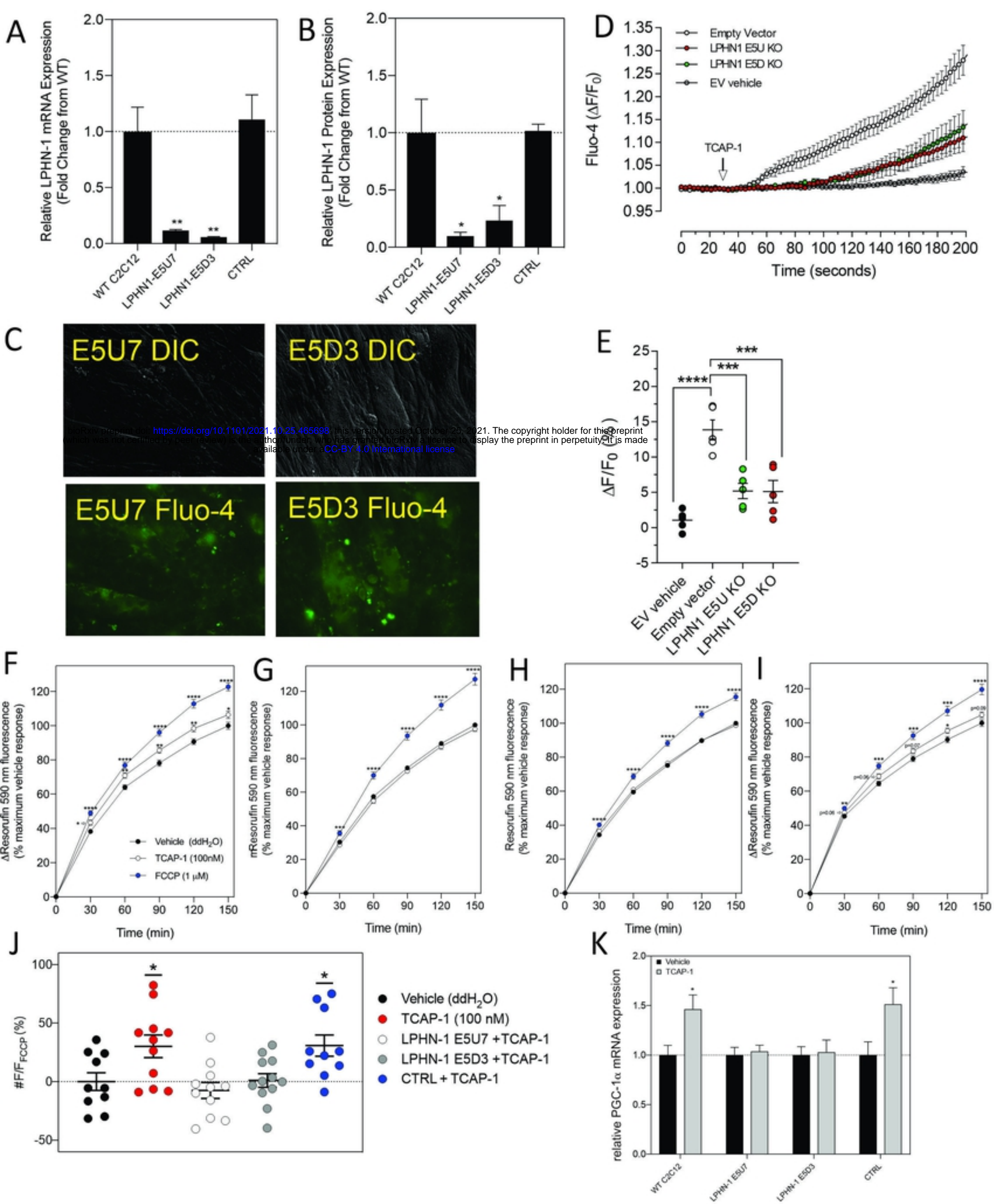


Figure 11