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1 **PCM1 labelling reveals myonuclear and nuclear dynamics in**
2 **skeletal muscle across species.**

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23 **Keywords: hypertrophy, regeneration, laminopathy, myonuclei, skeletal muscle, satellite cell, pericentriolar**
24 **material-1, macrophage.**

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27

28 ABSTRACT:

29 Myonuclei transcriptionally regulate muscle fibers during homeostasis and adaptation to exercise. Their
30 cellular location and quantity are important when characterising phenotypes of myopathies, the effect of
31 treatments and to understand the roles of satellite cells in muscle adaptation and muscle 'memory'. Difficulties
32 arise in identifying myonuclei due to their proximity to the sarcolemma and closely residing interstitial cell
33 neighbours. We aimed to determine to what extent PCM1 is a specific marker of myonuclei *in-vitro* and *in-vivo*.
34 Single isolated myofibers and cross-sections sections from mice and humans were studied from several models
35 including Wild-type and Lamin A/C mutant mice after functional overload, and damage and recovery in
36 humans following forced eccentric contractions. Fibers were immuno-labelled for PCM1, Pax7 and DNA. C2C12
37 myoblasts were also studied to investigate changes in PCM1 localisation during myogenesis. PCM1 labelled the
38 nuclear envelope of myonuclei in mature myofibers and in newly formed myotubes, but also labelled
39 centrosomes in proliferating myogenic precursors which may or may not fuse to join the myofiber syncytium.
40 It also labelled non-myogenic nuclei near the sarcolemma especially in regenerating areas of the
41 *Lmna*^{+/ Δ K32} mouse and damaged human muscle. While PCM1 is not completely specific to myonuclei, the
42 impact that PCM1+ macrophages and interstitial cells have on myonuclei counts would be small in healthy
43 muscle. PCM1 may prove useful as a marker of satellite cell dynamics due to the distinct change in localisation
44 during differentiation, revealing satellite cells in their quiescent (PCM1-), proliferating (PCM1+ centrosome),
45 and pre-fusion states (PCM1+ nuclear envelope).

46 INTRODUCTION:

47 Myonuclei influence transcriptional activity within myofibers in response to changes in activity or loading(1).
48 The large cytoplasmic volume of myofibers is maintained by multiple post-mitotic myonuclei regularly
49 distributed along the sarcolemma(2), except at specialised regions such as the myotendinous junction(3) and
50 neuromuscular junction(4). Changes in their distribution and number (through satellite cell (SC) fusion or
51 myonuclear loss), is of key importance in understanding muscle adaptation to age(2), supraphysiological
52 loading/growth(5-9), or exercise(10-12), and whether an increased number of nuclei per fiber can be retained
53 over long periods to support faster muscle growth when re-training or recovering from catabolic episodes such
54 as disuse(8, 11, 13-15). Several studies show that the skeletal muscle epigenome can be altered, potentially
55 allowing for accelerated muscle growth after previous training episodes(16-19), but it is still disputed whether

56 the maintenance of an increased number of myonuclei is a potential cellular adaptive mechanism enabling
57 rapid re-growth(8). Myonuclear maintenance is also important in myo/laminopathies, such as Lamin A/C
58 deficiency, in which faulty nuclear mechanics and impaired mechanically-activated gene transcription lead to
59 severe muscle dystrophy and skeletal muscle weakness(20, 21).

60 Myonuclei are generally spindle shaped, with a long axis of 20-30 μ m and short axis of 6-10 μ m. On transverse
61 cryosections, typically 5-12 μ m thick, it becomes a challenge to differentiate between myonuclei and other
62 nuclei that reside underneath the endomysium without automated approaches(22-25). Labelling with
63 dystrophin/laminin reveals whether a nucleus resides inside the sarcolemma (myonucleus), between the basal
64 lamina and sarcolemma (SC nucleus), or outside the basal lamina (stromal/vascular cell nucleus), and
65 determination of this position can be automated during image analysis(24, 25).

66 To identify myonuclei more reliably than through the positioning of DAPI relative to sarcolemmal labelling,
67 Winje *et al.*(26) reported that a pericentriolar protein, pericentriolar material-1 (PCM1), can be used as a
68 specific marker for myonuclei in skeletal muscle tissue. PCM1 prepares a cell to divide and supports the
69 stability of centrosomes during mitosis(27-29). PCM1 relocates to the nuclear envelope (NE) of post mitotic
70 nuclei, giving a ring appearance when nuclei are cut in thin transverse sections. However, PCM1 is ubiquitously
71 expressed and may thus be present in all cell types within skeletal muscle tissue(30).

72 Therefore, to make an independent assessment of PCM1 as a marker of myonuclei in a more varied set of
73 samples, we extracted single myofibers, myofiber bundles, as well as transverse sections to assess the
74 localisation and cell cycle stage of the nuclei within and near myofibers. We analyzed mouse fibers following
75 synergist ablation overload (functional overload) in WT and *Lmna*^{+/ Δ K32} mice and human fibers following
76 eccentric damage and recovery after electrical activation during imposed muscle stretch. We also investigated
77 PCM1 localisation during maturation in C2C12 myoblasts/myotubes. Our objective was to elucidate whether
78 PCM1 antibody labelling can be used on skeletal muscle cross-sections as a specific marker of myonuclei in any
79 case, or whether the protein may be expressed in proliferating myogenic, interstitial, or inflammatory cells.
80 We hypothesised that models of overload and regeneration that greatly expand the SC pool may be the most
81 affected and vulnerable to overestimation of myonuclear number.

82 **METHODS:**

83 **Mouse C2C12 Cell Culture**

84 C2C12 murine skeletal myoblasts(31, 32), from ATCC®, (Virginia, United States), were incubated on gelatin
85 (0.2%) coated plastic cover slips in 12 well-plates in humidified 5% CO₂ at 37°C in 1ml growth medium (GM)
86 containing DMEM, 10% FBS, 10% NCS, 1% l-glutamine (2mM final) and 1% penicillin–streptomycin solution.
87 Upon reaching confluence, cells were fixed with 0.5% PBS/BSA containing 2% paraformaldehyde or, to produce
88 multinucleated myotubes, myoblasts were differentiated by washing with PBS and transferring to low serum
89 media (LSM; DMEM with 2% horse serum, 1% l-glutamine and 1% penicillin–streptomycin). C2C12 cells
90 spontaneously differentiate under these conditions without additional growth factors(31) and were fixed after
91 7-days in LSM.

92 Fixed cells were incubated with a cocktail of antibodies/dyes including anti-PCM1 (HPA23370 Sigma Aldrich,
93 Merck) at 1:1000 and Phalloidin-FITC (Sigma P5282) at 1:500 overnight in immunobuffer (IB): PBS (10mM
94 phosphate pH-7.4, 150mM NaCl), 50mM glycine, 0.25% BSA, 0.03% saponin, 0.05% sodium-azide. An
95 appropriate secondary antibody for anti-PCM1, Goat Anti-Rabbit IgG-Alexa Fluor®594) (ab150077) was added
96 1:1000 the following day in IB. Following labelling, the plastic coverslips were removed, blotted dry and placed
97 on glass slides in mounting medium (Vectashield® with DAPI (1.5µg/ml), Burlingame, CA, USA) before
98 imaging. Experiments were performed at 3 different passages (5, 8, 11) and images taken from random regions
99 of interest comprising 3 technical replicates.

100 **Human Eccentric Damage and Recovery**

101 The Regional Scientific Ethical Committees of Copenhagen in Denmark approved this study (Ref: HD-2008-074)
102 and all procedures conformed to the Declaration of Helsinki. Young, healthy males ($n=2$), age; 20.5 ± 0.5 years,
103 height; 1.78 ± 0.02 cm, body mass; 76.5 ± 1.5 kg) gave informed consent and underwent a muscle injury
104 protocol of 200 forced lengthening contractions with electrical stimulation to activate the target muscle during
105 each contraction, as previously described (33). The protocol was performed on the vastus lateralis muscles of
106 one leg, leaving the other as internal control. The muscle biopsies analysed here are a subset of samples from
107 participants in whom extensive muscle damage was previously observed (33, 34).

108 Muscle biopsies were collected from both vastus lateralis muscles immediately before the damaging exercise
109 and at 2, 7 and 30-days from the damaged leg thereafter. Biopsies were taken under local anaesthetic (1%

110 lidocaine: Amgros I/S, Copenhagen, Denmark), using the percutaneous needle biopsy technique of Bergström
111 (35), with 5–6-mm-diameter biopsy needles and manual suction.

112 On extraction, biopsies were prepared for histology and single fiber analysis as previously described(34). Single
113 fibers bundles were pinned to maintain fiber length and covered in Krebs-Henseleit bicarbonate buffer
114 (containing 0.1% procaine) for 2 minutes, followed by Zamboni fixative (2% formaldehyde, 0.15% picric acid)
115 for 30 min, then transferred into fresh Zamboni fixative and placed in the fridge for approximately 4 hours.
116 Zamboni fixative was then replaced with 50% glycerol in PBS and moved to -20°C on the following day until
117 extraction.

118 **Functional overload of the mouse plantaris**

119 8 Wildtype (WT) and 10 *Lmna*^{+/ Δ K32} mice used in previous analyses(36) were included in this study and
120 underwent a sham operation or functional overload (FO) of the plantaris muscles, by aseptic tenotomy
121 of soleus and gastrocnemius muscles in both hind limbs(37). The cut distal tendons were folded proximally and
122 sutured to the proximal musculotendinous region leaving the plantaris intact. Animals recovered within 1-2
123 hours and were provided analgesia prior to and following surgery (Vetergesic© 0.3 mg/ml, SC:0.10mg/kg).

124 After 1-week of FO or sham surgery, animals were sacrificed by cervical dislocation, plantaris muscles were
125 dissected, and visible fat and connective tissue removed. Isolated plantaris muscles were frozen in isopentane
126 above liquid nitrogen for histological analysis or fixed in 4% paraformaldehyde at room temperature for one
127 hour for analysis of single muscle fibers. After fixation, PLN muscles were placed on ice and sucrose solution
128 was added in increasing molarity (0.5mM, 1mM, 1.5mM) and then frozen at -80°C in 2mM sucrose. Sucrose
129 frozen fibers were later placed on ice and transferred through decreasing molarity sucrose into IB for
130 mechanical isolation.

131 **Immunolabelling and analysis of single fibers and cross-sections.**

132 Single fibers were teased from fiber bundles under a stereomicroscope in IB. In some instances, mouse fibers
133 were extracted in bundles due to their small size and fragility during manual dissection. Primary antibody
134 cocktails of Pax7 (DSHB supernatant, 1:100) and anti-PCM1 (1:1000) were added to the petri dish in IB + plus
135 0.2% Triton-X100 overnight and then washed 3 x 10 minutes in IB. Secondary antibodies (Goat anti-mouse IgG-
136 Alexa Fluor®488 (ab150113) and Goat anti-rabbit IgG-Alexa Fluor®594 (ab150077) were added at 1:1000 in IB

137 for 2 hours before 3 x 10 minute washes in IB, before single fibers were mounted in DAPI (Vectashield® with
138 DAPI) onto glass slides before cover slipping and imaging.

139 For muscle cross-sections, snap-frozen muscle was sectioned at 10µm using an OTF5000 Cryostat (Bright
140 Instruments, UK) onto ThermoScientific™ SuperFrost Plus™ Adhesion slides (ThermoFisher Scientific Inc, USA).
141 Immunostaining and cover slipping were completed as above on glass slides, but adding antibodies anti-
142 collagen IV AB769 (Merck, Germany) at 1:500 and anti-CD68 MO718 (Dako, Denmark) at 1:500 for human
143 cross-sections. Secondary antibodies were donkey anti-goat Alexa Fluor®680 (a-21084) and donkey anti-mouse
144 Alexa Fluor®488 (ab150109) respectively at 1:1000 diluted in IB. A DSHB supernatant was used for dystrophin
145 on mouse sections MANDYS8 8H11 at 1:100 (DSHB deposited by Morris, G.E.), combined with a goat anti-
146 mouse IgG H&L Alexa Fluor®594 preadsorbed (ab150120) secondary antibody at 1:500 in IB.

147 **Imaging, PCM1 localisation and myonuclear analyses**

148 Cells/myofibers were imaged under 20x magnification and 30µm Z-stacks produced with 3µm steps using a
149 widefield fluorescent microscope (Leica DMB 6000, Germany) to determine PCM1 presence and localisation.
150 An A4 filter cube was used to image DAPI (EX: 340-380, EM: 450-490), L5 filter cube to image FITC and Alexa
151 Fluor® 488 (EX: 460-500, EM: 512-542) and an RHO filter cube to image Alexa Fluor®568/594 (EX:541-551,
152 EM:565-605). Images were taken with a monochrome DFC365 FX camera (Leica, Germany) and fluorescent
153 channels overlaid to determine PCM1 and Pax7 localisation. The number of Pax7+ cells per myofiber (not
154 normalised to fiber/sarcomeres per mm), and the percentage of Pax7+ cells that also displayed PCM1 positivity
155 were quantified on single myofibers from humans and mice across all timepoints and conditions. Manual
156 Pax7+ cell identification was performed on human cross-sections from control and 7-days after eccentric
157 damage, noting Pax7+ cells that were also PCM1+. Myonuclei identification was performed using DAPI and a
158 sarcolemmal marker, identifying myonuclei as nuclei whose centroid was clearly within the sarcolemmal
159 labelling. A second manual analysis was performed using PCM1 with sarcolemmal labelling to identify PCM1+
160 nuclei within the sarcolemmal boundary as above, as well as the total number of PCM1+ nuclei independent of
161 localisation to the sarcolemma. These measures and total DAPI+ nuclei were counted manually using the
162 multi-point tool on single fields of view using Image Jv1.53(38).

163 **Statistics**

164 Data were analyzed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Comparisons of PCM1
165 localisation *in-vitro* after 2 and 7 days in LSM was performed using a one-way ANOVA with Tukey's post hoc
166 testing. The number of Pax7+ cells per muscle fiber and the % of PCM1+ Pax7+ cells from human and mouse
167 isolated myofibers were also compared by one-way repeated measures ANOVA with Tukey's post-hoc testing.
168 Paired t-tests were performed to compare nuclear characteristics between control and 7-days post eccentric
169 damage on muscle cross-sections in humans. Bland-Altman analysis was performed using GraphPad Prism 8
170 software on the comparison of PCM1 labelling of myonuclei (subsarcolemmal PCM1) to manual quantification
171 of myonuclei through their subsarcolemmal positioning in control and 7-days post damage on human
172 transverse cross-sections. This analysis was also performed comparing myonuclei per fiber cross-section
173 measurements to manual quantification of all PCM1+ nuclei for WT & LMNA^{+/ Δ K32} control mice and after
174 functional overload.

175 **RESULTS:**

176 **PCM1 localisation changes consistently during C2C12 myoblast proliferation and differentiation into** 177 **myotubes *in-vitro*.**

178 As previously reported(39), PCM1 is present at the centrosome during proliferation in C2C12 mouse muscle
179 cells *in-vitro* (Figure 1A, C). When myoblasts are transferred to low serum media, a differentiation programme
180 is initiated in which PCM1 re-localises to form a perinuclear matrix around the myoblast nuclei seen as NE
181 staining, (Figure 1B). This change in localisation occurs during differentiation, preceding fusion of the
182 myoblasts into a multinucleated myotube(39). After 2-days in LSM, $75.7 \pm 9.3\%$ of nuclei had PCM1+
183 centrosomes, with a significantly lower amount ($17.5 \pm 9.2\%$, $P < 0.0001$) of nuclei with PCM1+ NEs (unfused).
184 There was a small population of already fused cells with PCM1+ NEs ($6.8 \pm 3.3\%$), significantly lower than the
185 population of cells with PCM1+ centrosomes ($P < 0.0001$), and populations of cells with PCM1+ NEs that had
186 differentiated but not yet fused, ($P < 0.019$), Figure 1E. Following 7-days in LSM, only $16.6 \pm 7.1\%$ of nuclei had
187 PCM1+ centrosomes and $11.6 \pm 4.1\%$ of cells had PCM1+ NEs but remained visibly unfused. There were
188 significantly more nuclei with PCM1+ NEs that had undergone fusion, $71.8 \pm 7.3\%$ which was significantly
189 higher than the remaining populations of nuclei, < 0.0001 , Figure 1F.

190 This change in PCM1 localisation may be required for appropriate cytoskeletal reorganisation, as motor
 191 proteins pull two myoblasts together before fusion(4). Once fused, PCM1 remains present at the NEs in the
 192 myotube, (Figure 1B/D). These results were also replicated in human primary cells, with similar observations
 193 during proliferation and early differentiation as seen in C2C12 cells (Data not shown).

194 *Insert Figure 1 Here*

195 **Human skeletal muscle degeneration and regeneration reveals multiple PCM1+ cell populations.**

196 We examined single muscle fibers/fiber bundles and transverse sections from human vastus lateralis biopsies
 197 taken before and 2, 7 and 30-days after eccentric damage, a model that has been shown to produce
 198 myogenesis in adult skeletal muscle(33, 34). On cross-sections, PCM1 labelling of myonuclei at the NE can
 199 appear either as a hollow ring, or as a single spot depending on where the nucleus is sectioned, (Figure 2A). On
 200 single fibers, PCM1 labelling of myonuclei appears mainly as a hollow ring, (Figure 2B-C). PCM1 protein was
 201 identified on some Pax7+ cells on both cross-sections (Figure 2A) and single isolated myofibers, (Figure 2C).
 202 Quantification of Pax7+ cells per fiber was performed revealing a significant increase in their number 7-days
 203 post eccentric damage (5.9 ± 5) compared with control (3.1 ± 2.2 , $P = 0.0002$) and 2-days post damage ($3.7 \pm$
 204 2.5 , $P = 0.0077$). The percentage of Pax7+ cells that also exhibited PCM1 positivity at their centrosome or NE
 205 was $9.5 \pm 1.5\%$ in the control state and increased at 2-days ($20 \pm 3\%$, $P = 0.14$), before peaking at 7-days ($29.5 \pm$
 206 3.5% , $P = 0.01$) post damage (Figure 2E). PCM1+/Pax7+ cells remain slightly elevated at 30-days post damage
 207 compared to baseline ($18.5\% \pm 3$, $P = 0.18$), with some Pax7+ cells exhibiting PCM1+ NEs which we believe are
 208 myocytes or pre-fusion cells, replicating our *in-vitro* observations of PCM1 stage-specific localisation.

209 *Insert Figure 2 Here*

210 We also manually quantified the number of myonuclei, PCM1+ myonuclei, total PCM1+ nuclei, Pax7+/PCM1-,
 211 Pax7+/PCM1+ and all DAPI+ nuclei (measurements are per fiber) on cross-sections from the same biopsies as
 212 the single fiber analysis. We selected to compare cross-sections from control and the 7-days post eccentric
 213 damage timepoint as this displayed the greatest amount of cellular remodelling(34). This analysis was
 214 performed on 2 participants, using 4 random images per biopsy, per timepoint, Table 1.

215 **Table 1: Assessment of nuclei populations on human muscle cross-sections in control and muscle 7-days**
 216 **post eccentric damage.** *Data is shown from 2 individuals. *Indicates a significant difference ($p = 0.05$).

<u>Per Fiber Cross-Section</u>	<u>Control</u>	<u>7-Days Post Damage</u>	<u>P-Value</u>
Pax7+ Nuclei	0.16 ± 0.03	0.17 ± 0.04	0.7
Pax7+/PCM1+	0.06 ± 0.02	0.09 ± 0.01	0.036*
Myonuclei (DAPI + Sarcolemmal Positioning)	2.04 ± 0.15	2.01 ± 0.08	0.827
PCM1+ Myonuclei	2.1 ± 0.17	2.16 ± 0.08	0.679
Total PCM1+ Nuclei	2.92 ± 0.5	3.89 ± 0.84	0.094
Total DAPI + Nuclei	4.43 ± 0.94	7.03 ± 2.27	0.048*

217

218 This analysis was extremely difficult in damaged/necrotic fibers due to the large density of nuclei within the
219 cytosol which is more easily seen on extracted myofibers (Figure 3A-B). While Pax7+ nuclei per fiber cross-
220 section did not differ between conditions (0.16 ± 0.03 vs. 0.17 ± 0.04 , $P = 0.7$), the number of Pax7+/PCM1+
221 SCs was higher (0.06 ± 0.02 vs. 0.09 ± 0.01 , $P = 0.036$) 7-days post eccentric damage. There was no difference
222 pre and post intervention in either manual counts of myonuclei (sub-sarcolemmal DAPI labelling) (2.04 ± 0.15
223 vs. 2.01 ± 0.08 , $P = 0.83$), or PCM1+ myonuclei counts (sub-sarcolemmal PCM1 labelling) per fiber cross-section
224 2.1 ± 0.17 vs. 2.16 ± 0.08 , $P = 0.7$). Bland-Altman analysis was performed and reported good agreement
225 between manual counting of myonuclei through DAPI labelling with sub-sarcolemmal positioning, versus PCM1
226 immunolabelling of myonuclei with subsarcolemmal positioning with 95% limits of agreement of -0.24 and
227 0.44. There was a slight bias (0.099 ± 0.17) toward PCM1 labelling reporting higher myonuclei numbers versus
228 manual counting as previously reported(15, 26), but both fell within agreement, Supplementary Figure 1.
229 However, 7-days post damage, there was a trend suggesting an increase in reported PCM1+ nuclei, despite no
230 change in PCM1+ myonuclei which is probably because of proliferating interstitial cells (2.92 ± 0.5 vs. $3.89 \pm$
231 0.84 , $P = 0.094$). The total number of DAPI+ nuclei did increase 7-days post damage, (4.43 ± 0.94 vs. $7.03 \pm$
232 2.27 , $P = 0.048$), likely reflecting the increase in infiltrating cells and interstitial nuclei(34, 40).

233 At 7-days, it can be observed on both cross-sections and isolated myofibers that there is extensive necrosis
234 and infiltration of other cell populations into damaged myofibers. Many of these cells within necrotic zones

235 have distinctive PCM1+ centrosomes and lay adjacent to intact fibers (indicated with an asterisk, Figure 3A-B,
236 Figure 4A-C). Using the same human biopsy material, it has previously been reported that these zones are full
237 of immune cells (CD68+) that infiltrate and remove severely damaged myofibers(34). Here we show that
238 CD68+ infiltrating cells also express PCM1 and appear across the entire damaged cytoplasm, or near to
239 myonuclei that remain at the basement membrane, Figure 4A-C. We also highlight that interstitial space in
240 skeletal muscle contains cells with PCM1+ NEs and cells with PCM1+ centrosomes near the myofiber
241 boundaries (Figure 2) which is exacerbated after damage (Figure 4).

242 *Insert Figure 3 Here*

243 *Insert Figure 4 Here*

244 **Defective muscle plasticity in *Lmna*^{+/ Δ K32} mutant mice following mechanical overload is accompanied by**
245 **higher numbers of PCM1+ Pax7+ cells and impaired nuclear spreading in-vivo.**

246 We recently reported(36) that under basal conditions WT and *Lmna*^{+/ Δ K32} mutant plantaris muscles are
247 equivalent in mass (WT = $0.69 \pm 0.02\text{mg}\cdot\text{g}^{-1}$ vs. *Lmna*^{+/ Δ K32} = $0.65 \pm 0.02\text{mg}\cdot\text{g}^{-1}$). Following 1-week of
248 mechanical overload, WT muscles make a robust hypertrophic response ($1.15 \pm 0.07\text{mg}\cdot\text{g}^{-1}$), but the mutant
249 presents defective hypertrophy ($0.84 \pm 0.05\text{mg}\cdot\text{g}^{-1}$). This reduced response to mechanical overload is
250 accompanied by defective myonuclear accretion, nuclear deformity, and an increase in both Pax7+ cells and
251 EdU+ fibers inside and outside of the myofiber (36). These adaptations and developed muscle pathologies can
252 be visualised in Figure 5A-E, with more data available in a recent publication by Owens *et al.*(36).

253 *Insert Figure 5 Here*

254 While we have previously reported an increase in satellite cells on cross-sections in response to functional
255 overload, our assessment on single fibers showed minimal difference in the number of Pax7+ cells per fiber
256 (not normalized to fiber length) between conditions, WT sham (2.58 ± 0.25), WT functional overload ($2.99 \pm$
257 0.42), *Lmna*^{+/ Δ K32} sham (1.96 ± 0.31), and *Lmna*^{+/ Δ K32} functional overload (2.58 ± 0.6), Figure 6F. This analysis
258 was performed by counting Pax7+ cells on 39 ± 3 , 52 ± 7 , 36 ± 4 , 41 ± 7 muscle fibers per group respectively.
259 This discrepancy may be explained by the fact that damaged/regenerating fibers that typically have more
260 Pax7+ cells in their proximity were more likely to break during mechanical isolation and were therefore not

261 successfully extracted and imaged. There was a small significant increase in the number of Pax7+ cells per fiber
262 between WT functional overload muscles and *Lmna*^{+/ Δ K32} sham muscles ($P=0.026$).

263 We then sought to assess PCM1 localisation on Pax7+ cells in this extensive muscle pathology where
264 regeneration and proliferative cells are common. From the single isolated myofibers assessed here, the
265 number of PCM1+ Pax7+ cells are comparable at baseline in WT ($8 \pm 3.55\%$) and mutant ($8.75 \pm 5.9\%$, $P =$
266 0.99), (Figure 6A, C). After overload, the percentage of PCM1+ Pax7+ cells were similar to sham muscles in the
267 WT but some individual samples showed that there was likely extensive proliferative activity through increased
268 PCM1+ Pax7+ cells ($16 \pm 6.65\%$, $P = 0.337$), Figure 6B). We identified a significant increase in the mutant ($22 \pm$
269 7.89% , $P = 0.02$) vs the WT sham control (Figure 6D). We also highlight that myotubes forming in the *Lmna*^{+/ Δ K32}
270 mutant after overload had more closely chained myonuclei (Figure 6E), characteristic of earlier development.
271 Conversely, myotubes present in WT exhibit appropriate nuclear spreading and peripheral migration (Figure
272 6B). The lack of or delay in this behaviour may be an additional factor contributing to defective hypertrophy
273 previously reported(36).

274 Lastly, we sought to identify the extent to which counting only PCM1+ nuclei would bias 'myonuclei' counts
275 across all for conditions in the mouse. Bland-Altman analysis comparing MyoVision myonuclei per fiber cross-
276 section measurements (previously reported(36)) with manual quantification of all PCM1+ nuclei, showed that
277 in all instances there was a bias in that counting all PCM1+ nuclei overestimated myonuclear number when
278 assessed by DAPI positioning relative to the sarcolemma using an automated, unbiased software programme.
279 This was most obvious in WT & *Lmna*^{+/ Δ K32} mutants (-0.18 vs -0.14) and increased 7-days post functional
280 overload (-1.18 vs -1.83) and 14-days post functional overload (-0.81 vs -0.53) due to the cellular proliferation
281 in the hypertrophying and regenerating muscles, (Supplementary Figure 2).

282 *Insert Figure 6 Here*

283 **DISCUSSION:**

284 Bergmann and colleagues first identified PCM1 in the NE of mature cardiomyocytes(41, 42). The Gundersen
285 laboratory later elegantly investigated PCM1 as a potential gold standard marker of myonuclei in skeletal
286 muscle from mice, rats, and humans(26). However, PCM1 is reportedly ubiquitously expressed at the pre-
287 mRNA level independent of cell lineage during mitosis, and is expressed in a range of cells as well as myofibers,

288 including schwann, immune, fibro-adipogenic, and endothelial cells, and in smooth muscle, tenocytes, SCs and
289 motor neurons/neuromuscular junction nuclei across multiple muscles, developmental stages and adulthood,
290 as assessed through single-nuclei RNA sequencing(30, 43). We observed the localisation of PCM1 within
291 several relevant models (WT mice, *Lmna*^{+/ Δ K32} mice and humans). Anti-PCM1 does label certain Pax7+ cells at
292 the centrosome, while previous reports have suggested it is specific to myonuclei(26). The pattern of labelling
293 in SCs is distinctively different to that in myonuclei, localizing to poles of the SC centrosome rather than the
294 NE, as seen in myonuclei. The percentage of PCM1+ SCs was dependent on the regenerative state and model
295 used leading to the proposal that such non-myonuclear immunolabelling reflects the extent of proliferative
296 expansion of interstitial cells most obviously in regenerating muscle. The number of Pax7+/PCM1+ cells
297 increase in human cross-sections and single fibers 7-days post eccentric damage and in *Lmna*^{+/ Δ K32} mice
298 following functional overload, but not to a significant extent following functional overload in WT mice.

299 There are distinct changes in PCM1 localisation both *in-vitro* and *in-vivo* that represent cell cycle stages of SCs;
300 first at the centrosome during prophase or anaphase, and then at the NE, when PCM1 forms an insoluble
301 perinuclear matrix as the nucleus exits the cell cycle into G0 to prepare for fusion into differentiated
302 myotubes(39, 44, 45). The change in PCM1 localisation from centrosome to NE occurs in single muscle cell
303 precursors and precedes fusion of two mononucleated cells into a myotube (Figure 1). This change in PCM1
304 localisation may be required for appropriate cytoskeletal reorganisation, as motor proteins pull two myoblasts
305 together before fusion(4). Single mononucleated Pax7+ cells with PCM1+ NEs we believe are indicative of 'pre-
306 fusion' cells. Srsen *et al.* previously reported that all Ki67+ proliferating myoblasts were also PCM1+ at the
307 centrosome and that upon PCM1 relocalisation to the NE, Ki67 positivity was lost(39). It has previously been
308 reported that such mononucleated cells with PCM1+ NEs express both embryonic myosin and myogenin which
309 are upregulated during differentiation(39). PCM1 may thus prove useful as a marker of the *in-vitro* cell cycle
310 and fusion index of myotubes due to a distinct change in localisation during differentiation.

311 PCM1 was also found in connective tissue/interstitial nuclei and within regenerating and necrotic myofibers in
312 nuclei that belong to proliferating macrophages. With this in mind, we advise caution on identifying all PCM1
313 labelling as indicative of myonuclei in skeletal muscle, without clear confirmation of sub-sarcolemma
314 positioning and the distinctive perinuclear patterning. Determining the number of non-myonuclei which are
315 PCM1+ is therefore important, especially if automated counting or subjective microanatomical analysis is

316 performed. From previous analyses, the number of myonuclei per fiber cross-section in mature skeletal muscle
317 ranges between 1.5 and 4.5, and for every 100 myonuclei there are about 1-5 SCs, or 0.04-0.25 Pax7+ cells per
318 fiber cross-section, depending on species, age, predominant fiber type composition, tissue section thickness
319 and the geometrical inclusion criteria to define a myonucleus(8, 26, 46-51). This means that the differences in
320 SC PCM1 positivity across our models would probably not affect overall myonuclei counting with PCM1 and
321 would be of minimal concern in healthy muscle and will be a valuable tool going forward in most instances.
322 However, we show a trend for an increase in total PCM1+ nuclei 7-days post eccentric damage in humans
323 (~33%, $P = 0.094$), which would probably reach significance in an appropriately powered experiment. In
324 addition, two studies have reported and noted higher myonuclei per fiber cross-section values (~11-16%)
325 when labelling with PCM1 in comparison to conventional positional methods^{26,52}, so we advise labs to
326 independently assess whether PCM1 is suitable for myonuclear counting depending on their muscle
327 phenotype, and especially if small differences between myonuclear number are to be expected between
328 conditions. Our investigation was performed across species and models but on small sample sizes. Therefore,
329 data presented may not be fully representative of whole muscles but highlights several cell populations other
330 than myonuclei that can be PCM1 positive within skeletal muscle.

331 While perinuclear PCM1 is not entirely specific to myonuclei, the impact of other PCM1+ nuclei would have
332 little effect on total myonuclei counts in most physiologically relevant models of hypertrophy yet may be
333 problematic in regenerative models or myo/laminopathies where there are more PCM1+ infiltrating
334 macrophages and interstitial cells. We suggest the use of PCM1, Pax7 and a basement membrane marker to
335 make completely sure the PCM1 labelling is associated with the perinuclear labelling of the myonucleus. PCM1
336 may also prove useful as a marker of both *in-vitro* and *in-vivo* SC dynamics due to the distinct change in
337 localisation during differentiation which reveals SCs in their quiescent (PCM1-), proliferating (PCM1+
338 centrosome) and pre-fusion state (PCM1+ NE).

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481

482 **Figure Legends:**

483 **Figure 1:** PCM1 localisation in C2C12 myoblast/myotube cultures. **(A)** Following 2 days in LSM, the culture
 484 contains both undifferentiated myoblasts which have characteristic PCM1+ centrosomes at the edge of their
 485 nucleus(*) and differentiated myotubes(#). **(B)** After 7-days in LSM, few undifferentiated myoblasts remain.
 486 Most nuclei are incorporated into multinucleated myotubes and show high PCM1 positivity. Scale bars indicate
 487 50µm. **(C)** Higher magnification imaging of PCM1 localised to the centrosome in the myoblast(*) after 2 days
 488 in LSM and **(D)** at the NE in the myotube(#) after 7 days in LSM. Scale bars indicate 25µm. **(E)** PCM1 localisation
 489 as a % of total DAPI+ nuclei after 2-days in LSM. **(F)** PCM1 localisation as a % of total DAPI+ nuclei after 7-days
 490 in LSM.

491 **Figure 2:** Cross-sections from control human muscle **(A)** illustrates the PCM1+ NE of transversely sectioned
 492 myonuclei associated with the myofiber border(m). Some nuclei within the endomysium have PCM1 staining
 493 at the centrosome(*) and NE(#). Pax7+ cells also exhibit PCM1 positivity at the centrosome even in control
 494 tissue(α). **(B)** Normal distribution of myonuclei in a control fiber with PCM1+ Nes. **(C)** A control human
 495 myofiber exhibiting a Pax7+ cell that is also PCM1+(α) alongside a myonucleus(m). Scale bars indicate 80µm.
 496 **(D)** The number of Pax7+ cells present per muscle fiber. **(E)** The number of proliferating Pax7+ cells (PCM1+
 497 centrosome) following damage caused by forced eccentric contractions. Data is presented from 100 SCs for
 498 each participant per timepoint, counted on 32 ± 8 muscle fibers (control), 27 ± 3 muscle fibers (2 days), 17 ± 6
 499 muscle fibers (7 days) and 24 ± 3 muscle fibers (30 days). The number of SCs was not normalized to fiber
 500 length which we note likely differed across extracted fibers. Significant differences are displayed as follows, $P <$
 501 0.05*, $P < 0.01^{**}$, $P < 0.001^{***}$.

502 **Figure 3:** Single human myofibers extracted 7 days post eccentric damage. **(A, B)** show 3 myofibers at different
 503 positions within a z-stack. The right most fiber is necrotic, indicated by infiltration of a dense population of
 504 nuclei belonging to proliferating macrophages with PCM1+ centrosomes(*). Several myonuclei have been
 505 highlighted with their distinctive PCM1+ NE (#). Scale bars indicate 100µm.

506 **Figure 4:** Human cross-sections from biopsies 7 days post eccentric damage. **(A, B)**. Large bodies of
 507 proliferating immune cells can be observed in necrotic zones and regenerating fibers identified through PCM1+

508 centrosomes(*), alongside myonuclei(m) and PCM1+ Pax7+ cells(α). **(C)** confirms that macrophages are PCM1+
 509 inside and around myofibers through CD68 labelling. Scale bars indicate 80 μ m.

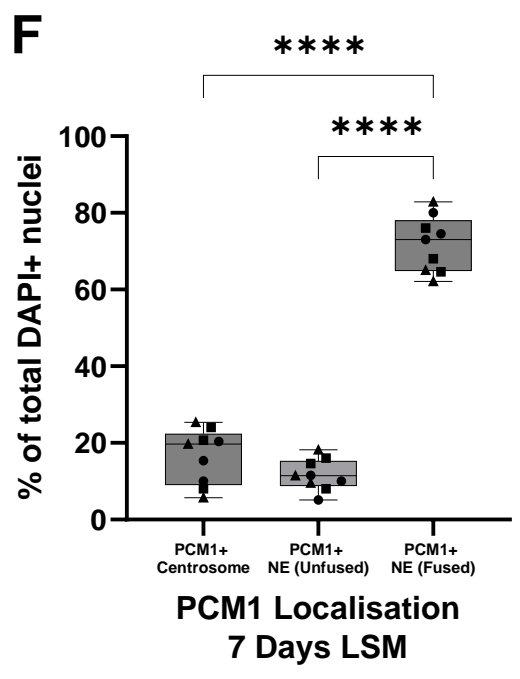
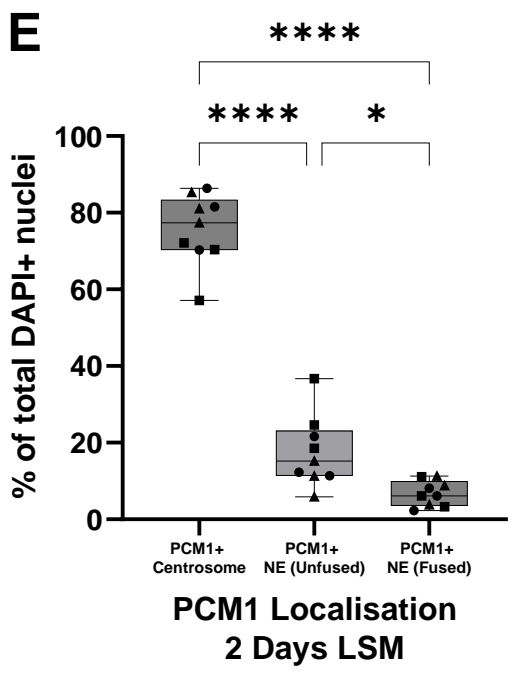
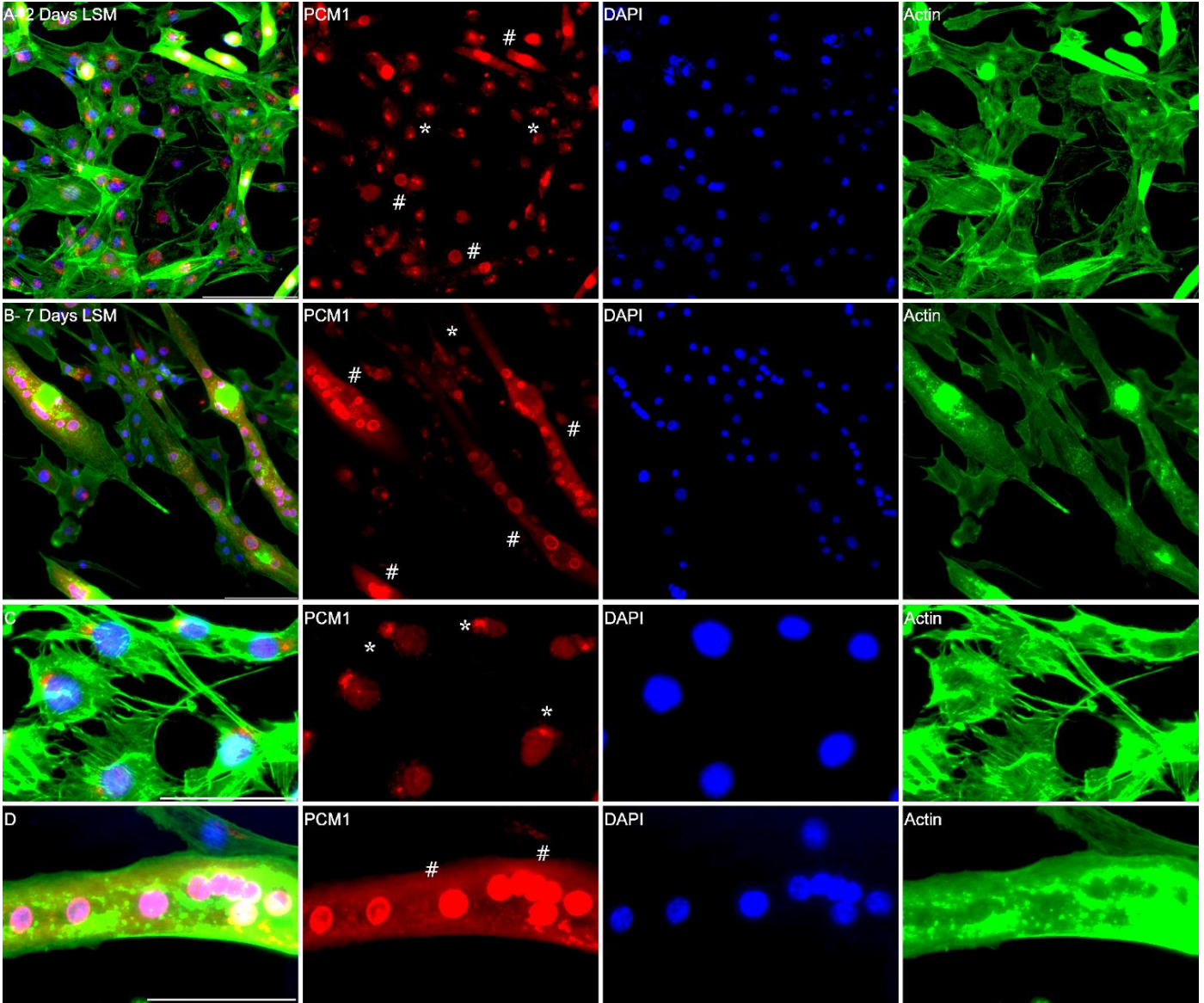
510 **Figure 5:** Cross sections showing morphology of WT and *Lmna*^{+/ Δ K32} mouse plantaris muscle. **(A)** WT muscle, 7
 511 days post a sham operation. **(B)** WT muscle 7 days post functional overload displays an increase in fiber cross-
 512 sectional area in comparison to the sham operated muscles. **(C)** *Lmna*^{+/ Δ K32} muscle 7 days post a sham
 513 operation. **(D, E)** *Lmna*^{+/ Δ K32} muscle 7 days post functional overload shows excessive dystrophin protein in
 514 regenerating areas with myofibres/myotubes containing centralised nuclei with a PCM1+ nuclear envelope.
 515 Scale bars indicate 50 μ m.

516 **Figure 6:** **(A)** Single extracted myofiber from WT sham operated mouse showing PCM1+ centrosome labelling
 517 around a single Pax7+ cell(α). Scale bars indicate 20 μ m. **(B)** PCM1 labels the NE of newly differentiated
 518 myonuclei during nuclear spreading following 1-week of functional overload in the WT mouse. PCM1 also
 519 labels a Pax7+ cell between an adjacent mature fiber(α). Scale bars indicate 50 μ m. **(C)** Single extracted
 520 myofiber from a sham operated *Lmna*^{+/ Δ K32} mouse, (α) is indicative of satellite cells. Scale bars indicate 50 μ m.
 521 **(D)** *Lmna*^{+/ Δ K32} fibers following 1 week of functional overload with a number of Pax7+ cells and centrally located
 522 myonuclei Scale bars indicate 20 μ m. **(E)** *Lmna*^{+/ Δ K32} intact myofibers adjacent to an early myocyte/myofiber
 523 following 1-week of functional overload. We note this was more frequent in the *Lmna*^{+/ Δ K32} mutants after
 524 overload compared to the WT. The nuclei appear to be in the alignment stage of myogenesis. Scale bars
 525 indicate 20 μ m. **(F)** Number of Pax7+ cells per fiber across all conditions (not normalised to fiber length),
 526 including number of fibers assessed per individual. **(G)** WT and *Lmna*^{+/ Δ K32} have similar levels of Pax7+ cells with
 527 PCM1+ centrosomes which is elevated following 1-week of functional overload. Data is presented from 100
 528 SCs each, counted on 39 \pm 3 muscle fibers (WT sham), 52 \pm 7 muscle fibers (*Lmna*^{+/ Δ K32} Sham), 36 \pm 4 muscle
 529 fibers (WT 1-week overload) and 41 \pm 7 muscle fibers (*Lmna*^{+/ Δ K32} 1-week overload). *Indicates statistical
 530 significance between conditions ($P < 0.05$).

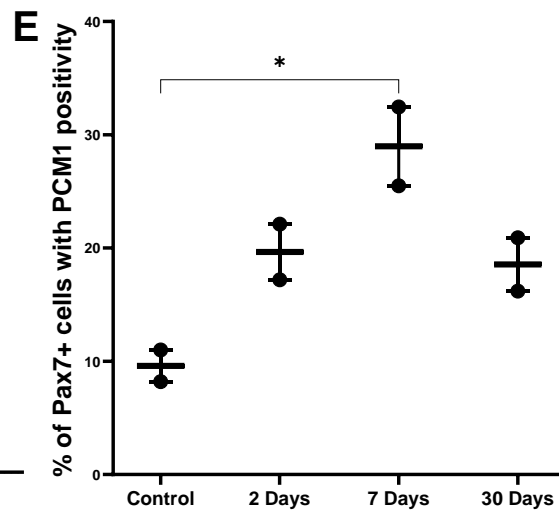
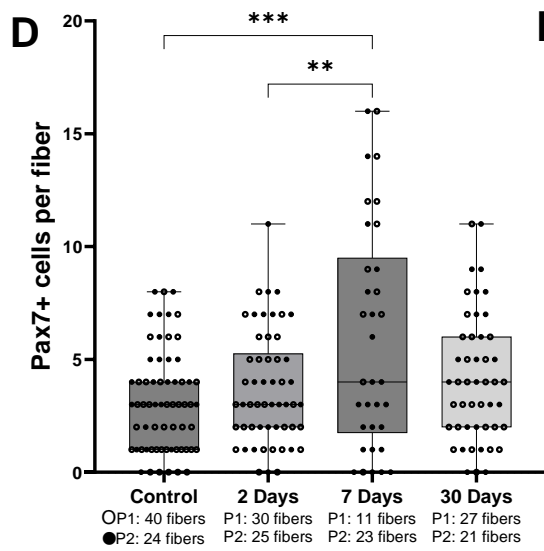
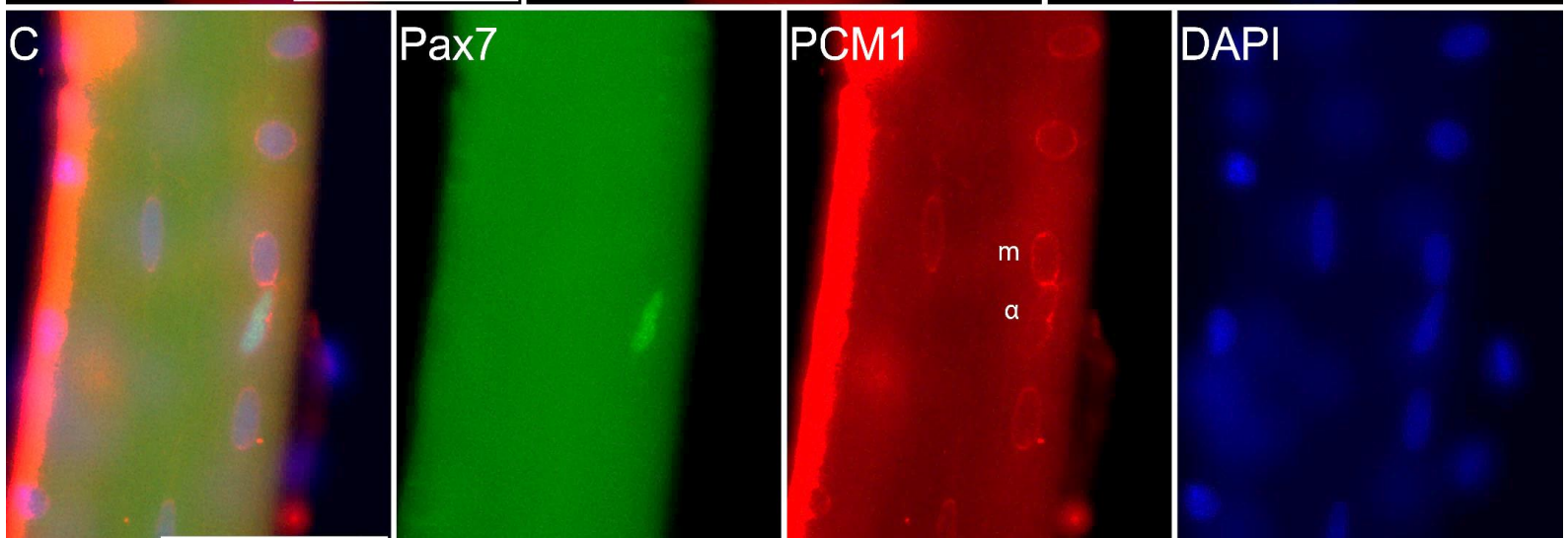
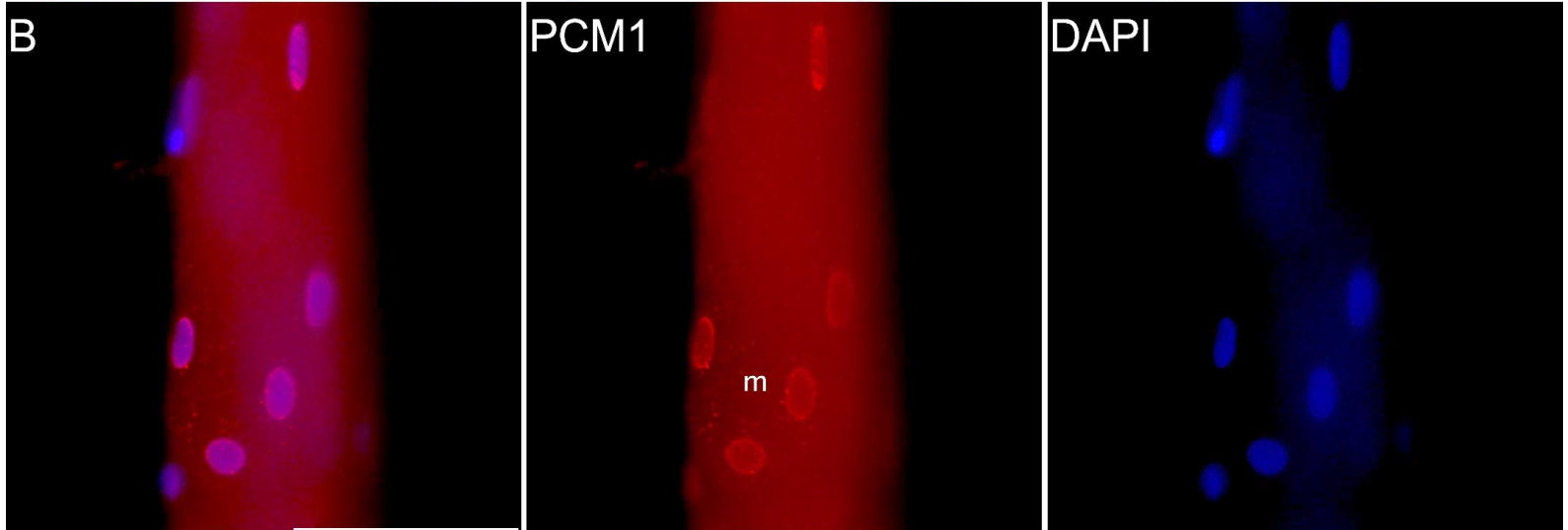
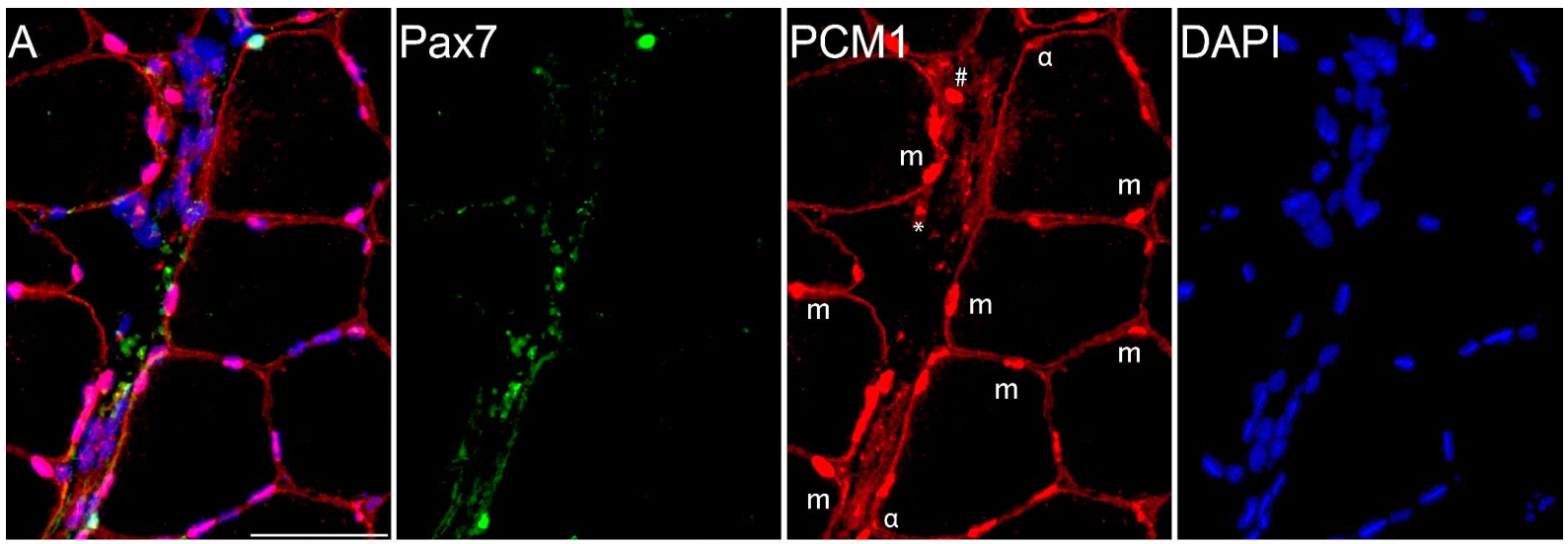
531 **Supplementary Figure 1:** Bland-Altman plot analysis comparing PCM1 labelling of myonuclei (subsarcolemmal
 532 PCM1) to manual quantification of myonuclei through their subsarcolemmal positioning in control and 7-days
 533 post damage on human transverse cross-sections.

534 **Supplementary Figure 2:** Bland-Altman plot analysis comparing myonuclei per fiber cross-section
535 measurements to manual quantification of all PCM1+ nuclei for WT & LMNA^{+/ Δ K32} control mice and after
536 functional overload.

F1



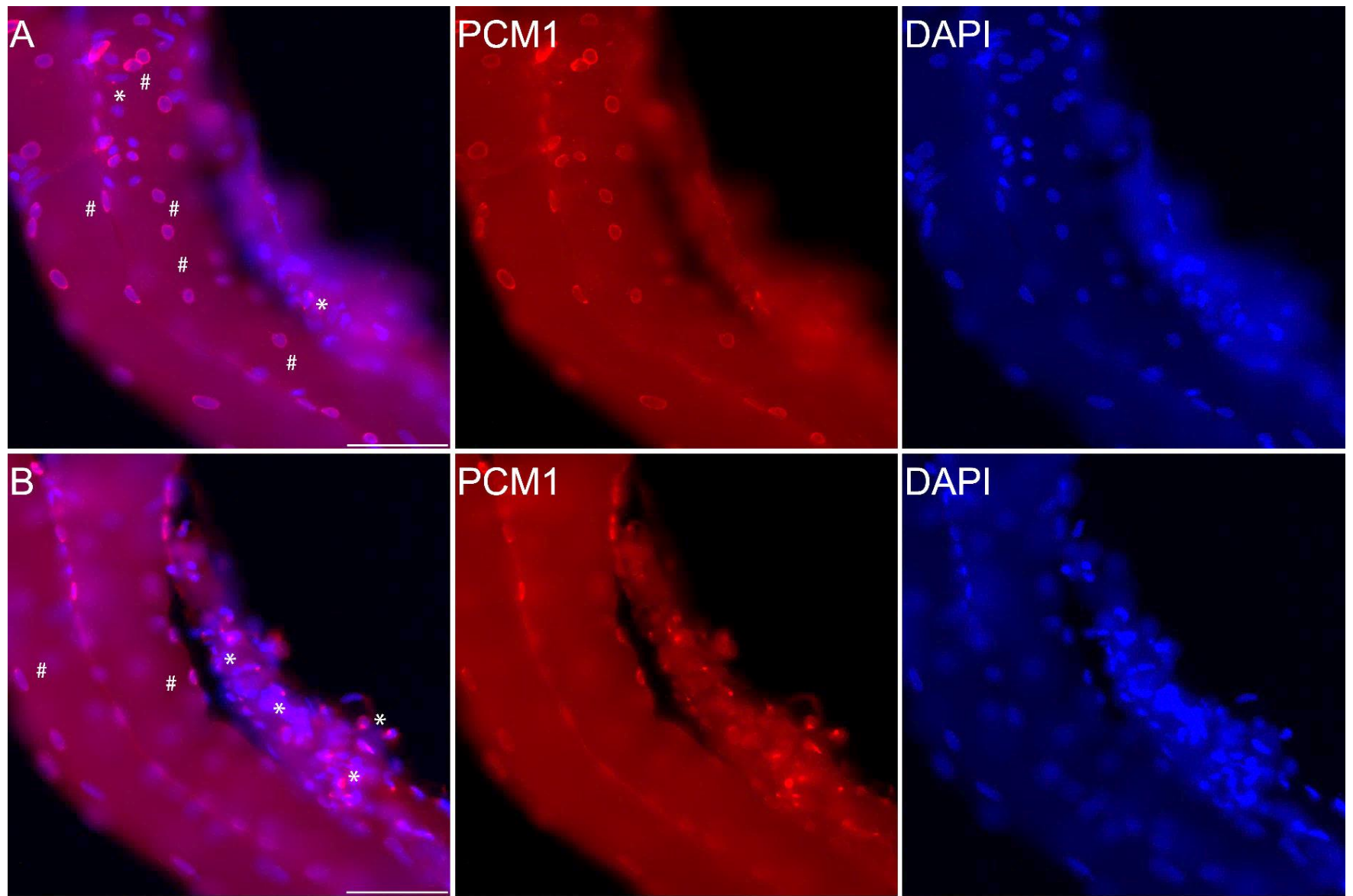
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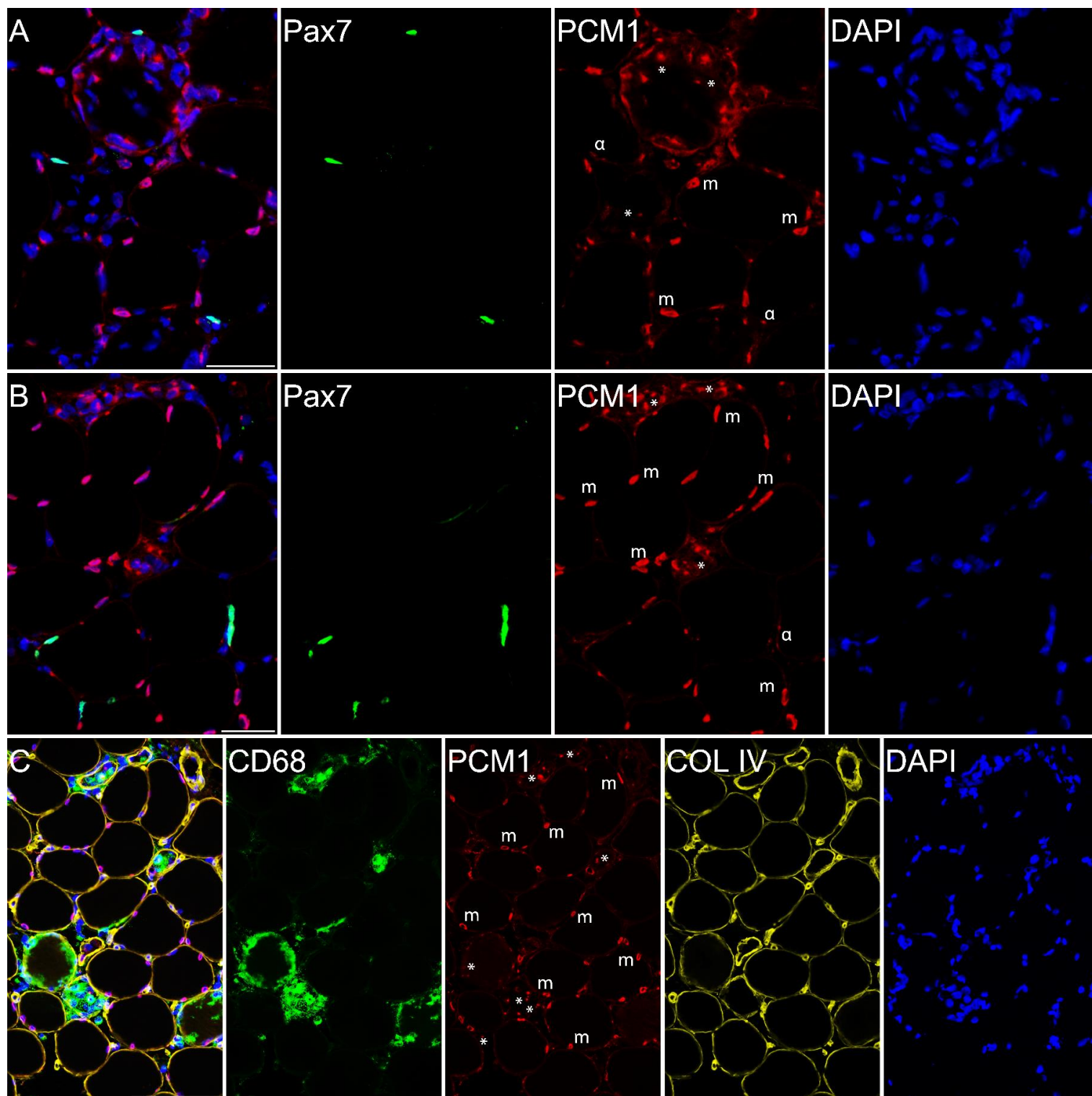
T1

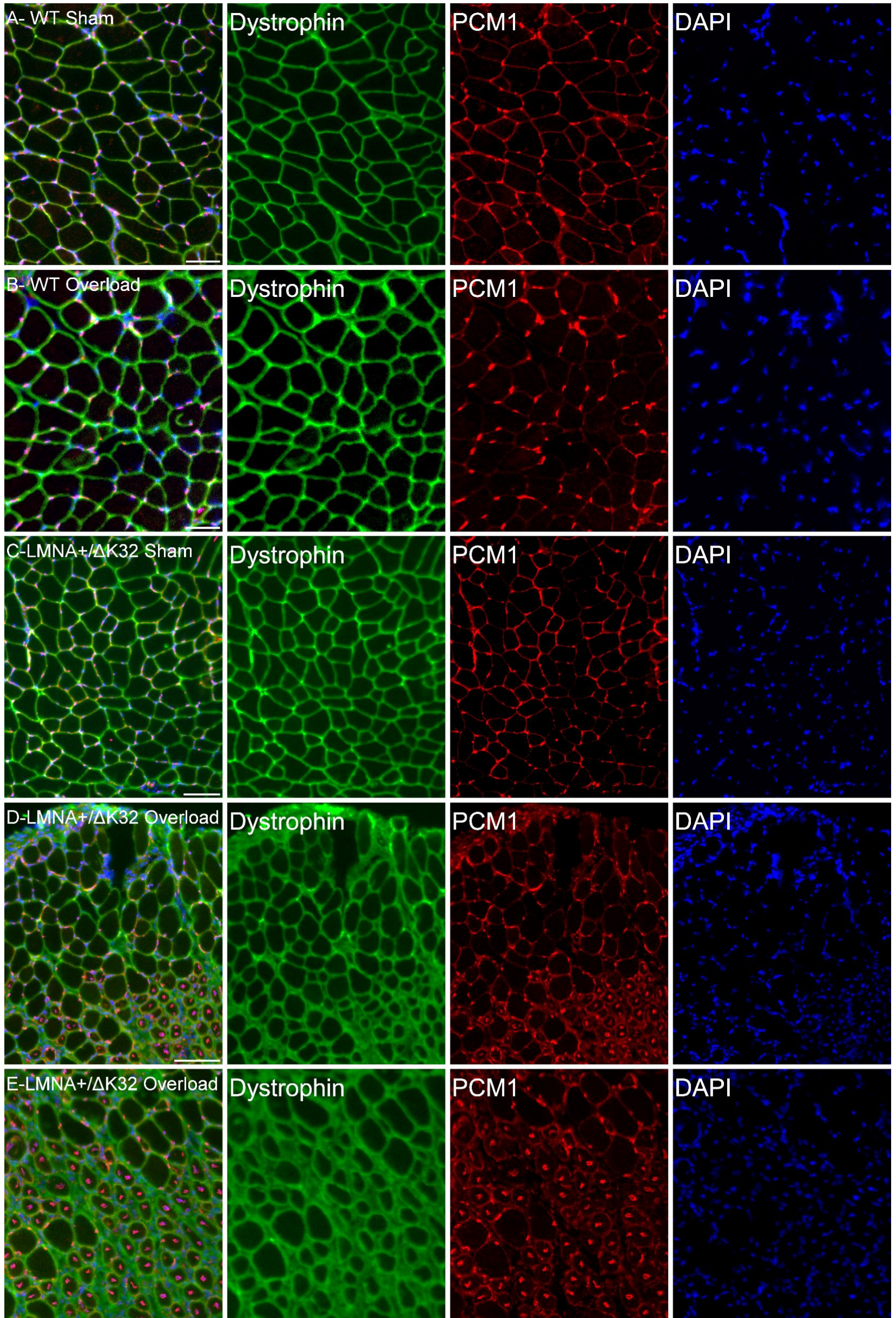
Per Fiber Cross Section	Control	7 Days Post Damage	P-Value
Pax7+ Nuclei	0.16 ± 0.03	0.17 ± 0.04	0.7
Pax7+/PCM1+	0.06 ± 0.02	0.09 ± 0.01	0.036*
Manually Counted Myonuclei (DAPI + Sarcolemmal Positioning)	2.04 ± 0.15	2.01 ± 0.08	0.827
PCM1+ Myonuclei	2.1 ± 0.17	2.16 ± 0.08	0.679
Total PCM1+ Nuclei	2.92 ± 0.5	3.89 ± 0.84	0.094
Total DAPI + Nuclei	4.43 ± 0.94	7.03 ± 2.27	0.048*

F3

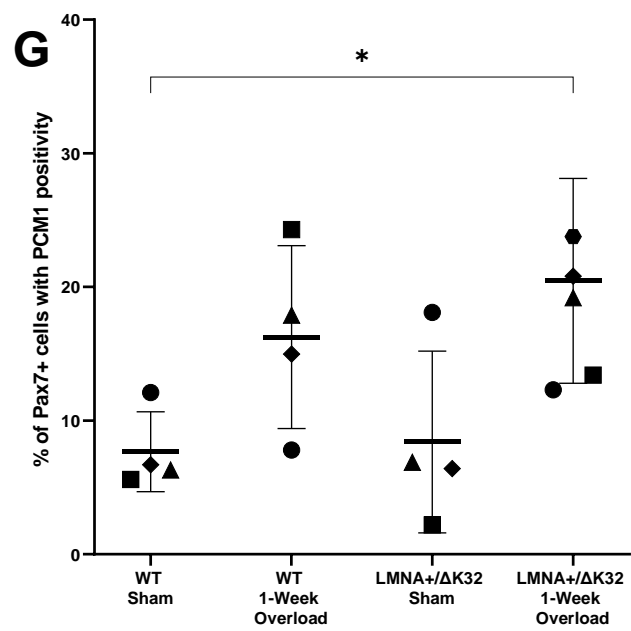
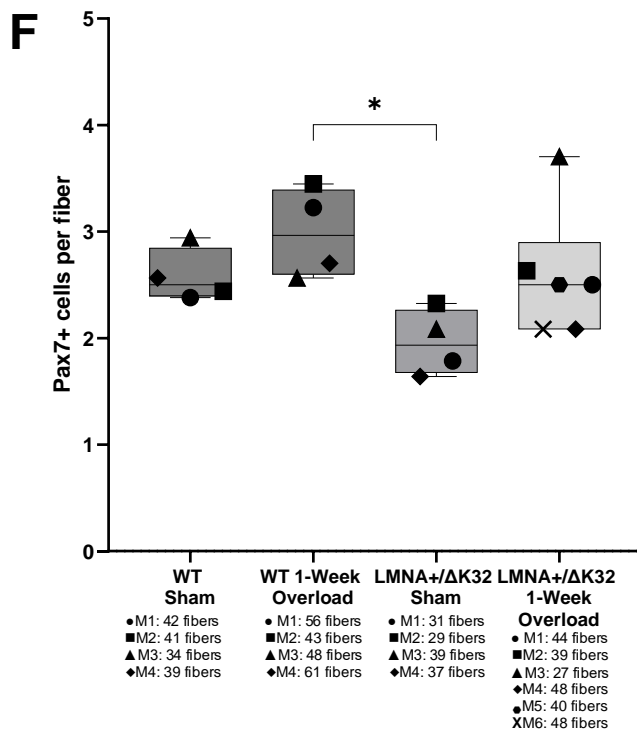
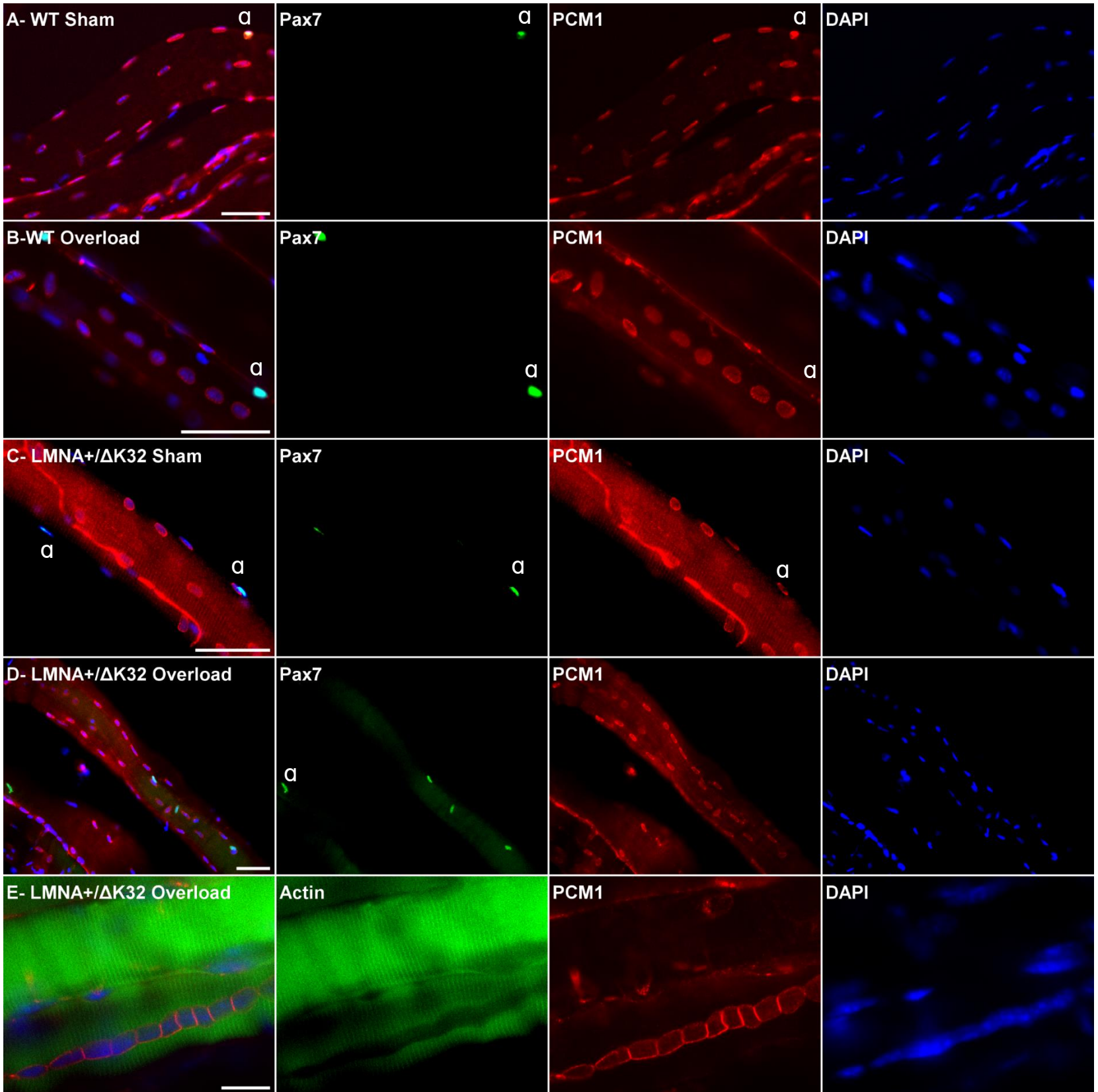


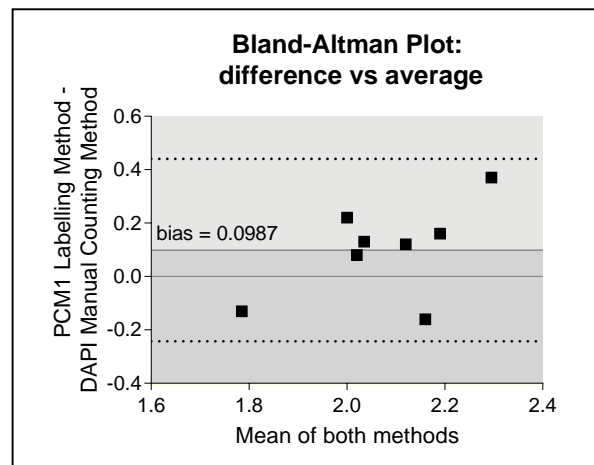
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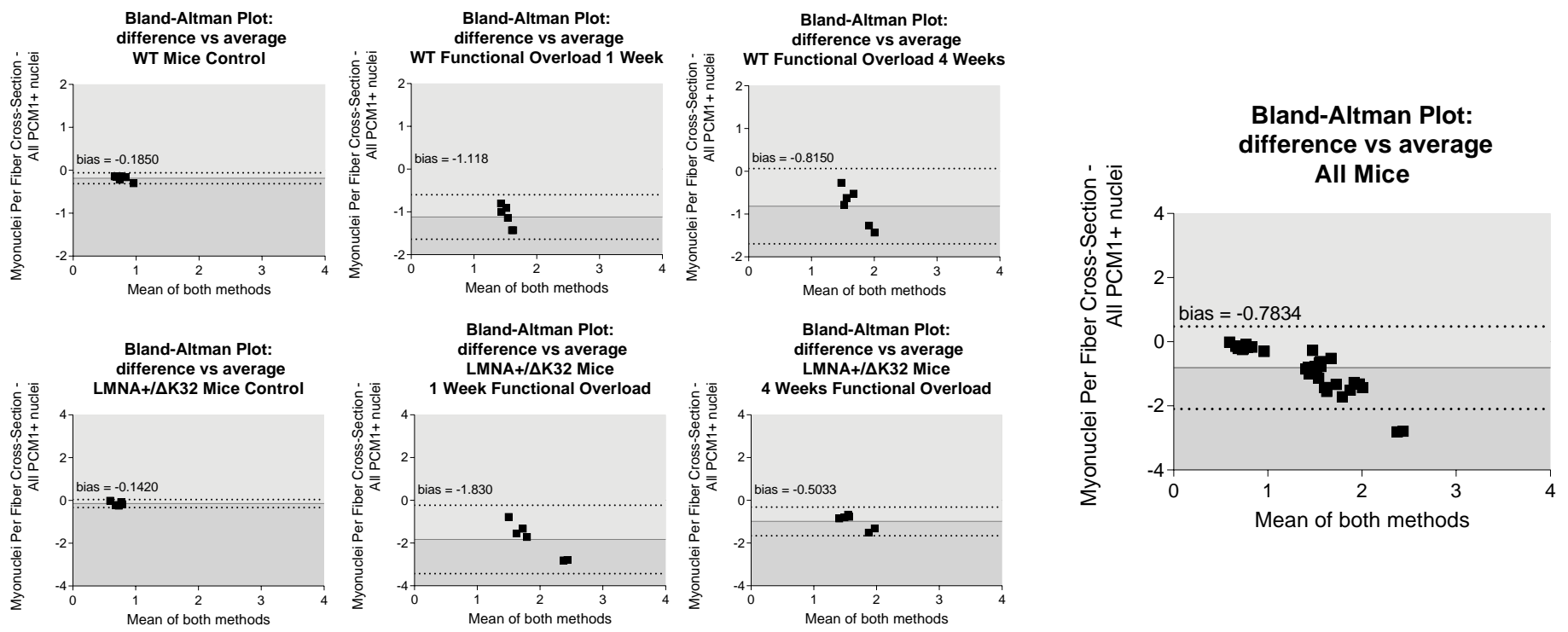


F6





Supplementary Figure 1: Bland-Altman plot analysis comparing PCM1 labelling of myonuclei (subsarcolemmal PCM1) to manual quantification of myonuclei through their subsarcolemmal positioning in control and 7-days post damage on human transverse cross-sections.



Supplementary Figure 2: Bland-Altman plot analysis comparing myonuclei per fiber cross-section measurements to manual quantification of all PCM1+ nuclei for WT & LMNA^{+/ΔK32} control mice and after functional overload.