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### Article

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

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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 subcellular 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 from mice and humans were studied from several models including  
35 wild-type and Lamin A/C mutant mice after functional overload, and damage and recovery in humans following  
36 forced eccentric contractions. Fibers were immuno-labelled for PCM1, Pax7 and DNA. C2C12 myoblasts were  
37 also studied to investigate changes in PCM1 localisation during myogenesis. PCM1 was detected at the nuclear  
38 envelope of myonuclei in mature myofibers and in newly formed myotubes, but also at centrosomes in  
39 proliferating myogenic precursors which may or may not fuse to join the myofiber syncytium. PCM1 was also  
40 detected in non-myogenic nuclei near the sarcolemma especially in regenerating areas of the *Lmna*<sup>+/ $\Delta$ K32</sup> mouse  
41 and damaged human muscle. While PCM1 is not completely specific to myonuclei, the impact that PCM1+  
42 macrophages and interstitial cells have on myonuclei counts would be small in healthy muscle. PCM1 may prove  
43 useful as a marker of satellite cell dynamics due to the distinct change in localisation during differentiation,  
44 revealing satellite cells in their quiescent (PCM1-), proliferating (PCM1+ centrosome), and pre-fusion states  
45 (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 rapid  
57 re-growth(8). Myonuclear maintenance is also important in myopathies and 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 $\mu$ m and short axis of 6-10 $\mu$ m. On transverse  
61 cryosections, typically 5-12 $\mu$ m thick, it becomes a challenge to differentiate between myonuclei and other nuclei  
62 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, Winje  
67 *et al.*(26) reported that a pericentriolar protein, pericentriolar material-1 (PCM1), can be used as a specific  
68 marker for myonuclei in skeletal muscle tissue. PCM1 prepares a cell to divide and supports the stability of  
69 centrosomes during mitosis(27-29). PCM1 relocates to the nuclear envelope (NE) of post mitotic nuclei, giving  
70 a ring appearance when nuclei are cut in thin transverse sections. However, PCM1 is ubiquitously expressed and  
71 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*<sup>+/ $\Delta$ K32</sup> 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 immuno-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. We  
80 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 (0.2%)  
85 coated plastic cover slips in 12 well-plates in humidified 5% CO<sub>2</sub> at 37°C in 1ml growth medium (GM) containing  
86 DMEM, 10% FBS, 10% NCS, 1% l-glutamine (2mM final) and 1% penicillin–streptomycin solution. Upon reaching  
87 confluence, cells were fixed with 0.5% PBS/BSA containing 2% paraformaldehyde or, to produce multinucleated  
88 myotubes, myoblasts were differentiated by washing with PBS and transferring to low serum media (LSM;  
89 DMEM with 2% horse serum, 1% l-glutamine and 1% penicillin–streptomycin). C2C12 cells spontaneously  
90 differentiate under these conditions without additional growth factors(31) and were fixed after 7-days in LSM.

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

### 99 **Human Eccentric Damage and Recovery**

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

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

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

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

### 116 **Functional overload of the mouse plantaris**

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

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

### 129 **Immunolabelling and analysis of single fibers and cross-sections.**

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

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

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

#### 145 **Imaging, PCM1 localisation and myonuclear analyses**

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

#### 161 **Statistics**

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

## 172 **RESULTS:**

### 173 **PCM1 localisation changes consistently during C2C12 myoblast proliferation and differentiation into** 174 **myotubes *in-vitro*.**

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

187 This change in PCM1 localisation may be required for appropriate cytoskeletal reorganisation, as motor proteins  
188 pull two myoblasts together before fusion(4). Once fused, PCM1 remains present at the NEs in the myotube,



189 (Figure 1B/D). These results were also replicated in human primary cells, with similar observations during  
190 proliferation and early differentiation as seen in C2C12 cells (Data not shown).

191 *Insert Figure 1 Here*

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

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

206 *Insert Figure 2 Here*

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

212 **Table 1: Assessment of nuclei populations on human muscle cross-sections in control and muscle 7-days post**  
213 **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	<b>0.036*</b>
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	<b>0.048*</b>

214

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

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

232 distinctive PCM1+ centrosomes and lay adjacent to intact fibers (indicated with an asterisk, Figure 3A-B, Figure  
233 4A-C). Using the same human biopsy material, it has previously been reported that these zones are full of  
234 immune cells (CD68+) that infiltrate and remove severely damaged myofibers(34). Here we show that CD68+  
235 infiltrating cells also express PCM1 and appear across the entire damaged cytoplasm, or near to myonuclei that  
236 remain at the basement membrane, Figure 4A-C. We also highlight that interstitial space in skeletal muscle  
237 contains cells with PCM1+ NEs and cells with PCM1+ centrosomes near the myofiber boundaries (Figure 2) which  
238 is exacerbated after damage (Figure 4).

239 *Insert Figure 3 Here*

240 *Insert Figure 4 Here*

241 **Defective muscle plasticity in *Lmna*<sup>+/ $\Delta$ K32</sup> mutant mice following mechanical overload is accompanied by higher**  
242 **numbers of PCM1+ Pax7+ cells and impaired nuclear spreading in-vivo.**

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

250 *Insert Figure 5 Here*

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

258 extracted and imaged. There was a small significant increase in the number of Pax7+ cells per fiber between WT  
259 functional overload muscles and *Lmna*<sup>+/ $\Delta$ K32</sup> sham muscles ( $P = 0.026$ ).

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

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

279 *Insert Figure 6 Here*

## 280 **DISCUSSION:**

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

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

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

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

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

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

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478

479 **Figure Legends:**

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

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

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

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

505 centrosomes(\*), alongside myonuclei(m) and PCM1+ Pax7+ cells( $\alpha$ ). **(C)** confirms that macrophages are PCM1+  
 506 inside and around myofibers through CD68 labelling. Scale bars indicate 80 $\mu$ m.

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

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

527 **Supplementary Figure 1:** Bland-Altman plot analysis comparing PCM1 labelling of myonuclei (subsarcolemmal  
 528 PCM1) to manual quantification of myonuclei through their subsarcolemmal positioning in control and 7-days  
 529 post damage on human transverse cross-sections. Available at <https://doi.org/10.6084/m9.figshare.21397053>.

530 **Supplementary Figure 2:** Bland-Altman plot analysis comparing myonuclei per fiber cross-section  
531 measurements to manual quantification of all PCM1+ nuclei for WT & LMNA<sup>+/ $\Delta$ K32</sup> control mice and after  
532 functional overload. Available at <https://doi.org/10.6084/m9.figshare.21397074>.