

Myomerger induces fusion of non-fusogenic cells and is required for myoblast fusion

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Abstract

Despite the importance of cell fusion for mammalian development and physiology, the factors critical for this process remain to be fully defined¹. This lack of knowledge has severely limited our ability to reconstitute cell fusion, which is necessary to decipher the biochemical mechanisms driving plasma membrane merger. Myomaker (*Tmem8c*) is a muscle-specific protein required for myoblast fusion^{2,3}. Expression of myomaker in fibroblasts drives their fusion with myoblasts, but not with other myomaker-fibroblasts, highlighting the requirement of additional myoblast-derived factors for fusion. Here, we demonstrate that *Gm7325*, named myomerger, induces the fusion of myomaker-expressing fibroblasts. Cell mixing experiments reveal that while myomaker renders cells fusion-competent, myomerger induces fusogenicity. Thus, myomaker and myomerger confer fusogenic activity to normally non-fusogenic cells. Myomerger is skeletal muscle-specific and only expressed during developmental and regenerative myogenesis. Disruption of myomerger in myoblast cell lines through Cas9-mutagenesis generated non-fusogenic myocytes. Genetic deletion of myomerger in mice results in a paucity of muscle fibers demonstrating a requirement for myomerger in normal muscle formation. Myomerger deficient myocytes exhibit an ability to differentiate and harbor organized sarcomeres, however remain mono-nucleated. These data identify myomerger as a fundamental myoblast fusion protein and establishes a system that begins to reconstitute mammalian cell fusion.

1 Main

2

3 The fusion of plasma membranes is necessary for numerous biological processes from
4 conception to the development of skeletal muscle, osteoclasts, trophoblasts, and giant cells.
5 The molecular regulation of fusion is poorly understood and the reconstitution of fusogenicity
6 has not been achieved in mammalian cells. Specifically, the factors that directly participate in
7 membrane coalescence have not been identified. The development of systems that
8 reconstitute fusion in the absence of other processes allow identification of nodal fusion
9 machinery and associated molecular mechanisms. For example, the *C. elegans* fusogen
10 epithelial fusion failure (Eff-1) is sufficient to fuse typically non-fusing cells^{4,5} and mechanisms of
11 intracellular membrane fusion were partially revealed through reconstitution of SNAREs on
12 synthetic membranes⁶⁻⁸. Thus, discoveries of specific fusion proteins and development of
13 reconstitution systems have been historically critical to decipher multiple types of membrane
14 fusion, however these systems are lacking for mammalian cellular fusion.

15 Myoblast fusion is a highly specific process essential for muscle formation, and while
16 numerous proteins have been shown to regulate mammalian myoblast fusion⁹⁻¹⁹, myomaker is
17 the only known muscle-specific protein absolutely required for this process². Expression of
18 myomaker in fibroblasts or mesenchymal stromal cells (MSCs) induces their fusion with muscle
19 cells^{20,21}. However, myomaker-fibroblasts do not fuse to each other indicating that additional
20 muscle-derived factors are required for fusion. To uncover potential factors, we compared genes
21 induced by expression of MyoD to their level of expression in 10T ½ fibroblasts. Of the top 100
22 MyoD-regulated genes not expressed in fibroblasts (Supplemental Information), we eliminated
23 genes not likely to be directly involved in fusion (transcription factors, sarcomeric and metabolic
24 genes) and focused on genes with transmembrane domains. This analysis yielded the following

25 five genes: *Tmem182*, *Gm7325*, *Cdh15*, *Tspan33*, and *Tm6sf1*, however *Cdh15* was omitted
26 from further analysis because it is not necessary for myoblast fusion or muscle formation²². We
27 retrovirally expressed each gene in myomaker⁺ GFP⁺ fibroblasts and assayed for fusion.
28 Appropriate expression in fibroblasts was verified through quantitative reverse transcription
29 polymerase chain reaction (qRT-PCR) analysis (Extended Data Fig. 1a). We observed mainly
30 mono-nucleated GFP⁺ cells in all cultures except when *Gm7325* was expressed where
31 widespread multi-nucleated cells were present (Fig. 1a). Based on the ability of *Gm7325* to
32 induce fusion of myomaker⁺ fibroblasts and the observations described below we named the
33 protein myomergers.

34 Multiple *Gm7325* transcripts are annotated in the University of California, Santa Cruz,
35 mouse genome. The shorter transcript contains a single exon and yields a protein with 84 amino
36 acids. In contrast, the longer transcript utilizes an upstream exon with an alternative start site
37 and results in a protein of 108 amino acids (Extended Data Fig. 2a). The single coding exon of
38 the short transcript is conserved in other mammalian genomes, including humans, while the
39 upstream alternative exon leading to the longer transcript is not highly conserved (Extended
40 Data Fig. 2b). For the initial screen we cloned the *Gm7325* locus into a retroviral vector, allowing
41 normal splicing and expression of both short and long transcripts. Transduction of myomaker⁺
42 fibroblasts with either myomergers-short (S) or myomergers-long (L) induced formation of multi-
43 nucleated cells, indicating both proteins are sufficient for fusion (Extended Data Fig. 2c).
44 Additionally, myomergers and myomaker together induced fusion of 3T3 fibroblasts and MSCs
45 (Extended Data Fig. 2d), suggesting these two genes could activate fusion in a multitude of cell
46 types.

47 Given that multi-nucleated cells could arise from fusion or endoreplication, we designed
48 a system to validate that multi-nucleated cells observed in fibroblasts expressing both

49 myomaker and myomaker were generated through fusion. We engineered two fibroblast cell
50 lines that both express myomaker, with one expressing GFP and the other expressing nuclear-
51 localized TdTomato (NLS-Tom). Myomaker⁺ GFP⁺ and myomaker⁺ NLS-Tom⁺ fibroblasts were
52 infected with a myomaker retrovirus or a control empty retrovirus, mixed, and fusion was
53 assessed (Fig. 1b). We observed cells with multiple nuclei containing both GFP and NLS-Tom
54 in fibroblasts expressing myomaker and myomaker indicating fusion (Fig. 1b). Quantification
55 of fusion revealed approximately 20% of nuclei were contained in syncytia in cultures where
56 fibroblasts were expressing both myomaker and myomaker (Fig. 1b). These results confirm
57 that the observed syncytial cells are formed through fusion and that expression of myomaker
58 and myomaker is sufficient to confer fusogenicity in non-fusogenic fibroblasts.

59 We also sought to determine the cell biology of fusion induced by myomaker and
60 myomaker. We mixed myomaker⁺ myomaker⁺ GFP⁺ fibroblasts with NLS-Tom⁺ fibroblasts
61 expressing myomaker or myomaker (Extended Data Fig. 3). Here we observed fusion of
62 myomaker⁺ myomaker⁺ GFP⁺ fibroblasts with myomaker⁺ NLS-Tom⁺ but not myomaker⁺ NLS-
63 Tom⁺ fibroblasts (Extended Data Fig. 3). This heterotypic nature of fibroblast fusion are
64 consistent with our previously reported heterologous fusion system between myoblasts and
65 fibroblasts². In that system, myomaker⁺ fibroblasts that did not express myomaker fused with
66 muscle cells, which express both myomaker and myomaker. We next utilized our heterologous
67 fusion system where fibroblasts were infected with GFP and either empty, myomaker, or
68 myomaker retrovirus, and then mixed with C2C12 myoblasts (Fig. 1c). In this assay, fusion is
69 detected through co-localization of GFP (fibroblasts) with myosin⁺ myotubes. Compared to
70 empty-infected GFP⁺ fibroblasts, we detected a significant increase in fusion between myosin⁺
71 cells with either myomaker⁺ GFP⁺ fibroblasts or myomaker⁺ GFP⁺ fibroblasts (Fig. 1c). However,
72 quantification of myosin⁺ GFP⁺ cells revealed that myomaker did not drive the fusion of

73 fibroblasts with muscle cells to the levels observed with myomaker (Fig. 1c). These data confirm
74 myomaker is required in both fusing cells for *in vitro* fusion, while myomerger is essential in one
75 fusing cell.

76 The only current information regarding *Gm7325* is its potential expression in embryonic
77 stem (ES) cells²³, therefore we interrogated its expression pattern more thoroughly. We
78 performed qRT-PCR on multiple tissues from postnatal (P) day 5 mice with primers to distinguish
79 the two potential mouse transcripts (Extended Data Fig. 4a). In neonatal tissues, we detected
80 expression of both myomerger transcripts only in skeletal muscle (Fig. 2a). Despite the evidence
81 of two myomerger transcripts, immunoblot analysis of skeletal muscle lysates from P5 mice
82 using a commercially available antibody identified a single band at approximately 12 kDa. This
83 band was absent in P28 lysates indicating that myomerger is downregulated after neonatal
84 development (Fig. 2b). Skeletal muscle exhibits a robust ability to regenerate due to the
85 presence of muscle stem cells, also known as satellite cells^{24,25}. We analyzed expression of
86 myomerger in *mdx*^{4cv} mice, which is a mouse model of muscular dystrophy that leads to chronic
87 cycles of degeneration and regeneration²⁶. Myomerger expression was detected in diaphragm
88 lysates from *mdx*^{4cv} mice, but not control diaphragms (Fig. 2c). Finally, we analyzed expression
89 of myomerger in a model of skeletal muscle overload-induced (MOV) hypertrophy and observed
90 up-regulation (Fig. 2d). Collectively, these data demonstrate that myomerger is expressed only
91 during development and is induced upon satellite cell activation in the adult.

92 We next sought to determine if myomerger is regulated as myoblasts differentiate. In
93 C2C12 cells, both *Gm735* transcripts were significantly elevated during differentiation (Extended
94 Data Fig. 4b). Similarly, myomerger protein levels were low in proliferating myoblasts (day 0),
95 but increased upon differentiation with expression maintained during myoblast differentiation and
96 fusion into myotubes (Fig. 2e). The short mouse myomerger protein, but not the long form, is

97 highly conserved among vertebrate species (Extended Data Fig. 4c). After transduction of
98 C2C12 cells with empty, myomerger-S, or myomerger-L we detected an increased upper band
99 in cells expressing either myomerger-S or myomerger-L that co-migrated with the 12 kDa
100 endogenous protein in the empty-infected C2C12 cells (Extended Data Fig. 4d). A lower band
101 was identified exclusively in myomerger-S lysates suggesting that complex mRNA or post-
102 translational processing results in the endogenous single 12 kDa band observed in WT C2C12
103 cells and muscle homogenates. Both myomerger proteins harbor a hydrophobic region close to
104 the N-terminus, where computational analysis of this region indicates a signal peptide or
105 transmembrane domain (Extended Data Fig. 4e). Given that both variants were found to confer
106 fusogenicity, the significance of the predicted domains is presently unclear. To understand
107 subcellular localization, we fractionated C2C12 cells on day 2 of differentiation and identified
108 myomerger in membrane fractions containing caveolin-3, a protein known to associate with both
109 heavy and light vesicles (Fig. 2f). Immunostaining of fibroblasts expressing myomerger-S or
110 myomerger-L shows that both proteins exhibit similar diffuse and vesicular localization (Fig. 2g).
111 Thus, myomerger associates with membrane compartments consistent with its ability to induce
112 fusion.

113 The ability of myomerger to induce fusion of myomaker-fibroblasts, and its muscle-
114 specific expression in the mouse, suggests that myomerger may play a critical role during
115 myogenesis. To begin to decipher the role of myomerger in myogenesis, we evaluated its
116 function during myoblast differentiation. We utilized CRISPR/Cas9 genome editing to disrupt
117 myomerger in C2C12 myoblasts. Two guide RNAs (gRNA) were designed to target the largest
118 exon of *Gm7325*, which resulted in a 166 base pair deletion thereby disrupting both mouse
119 transcripts (Extended Data Fig. 5a). C2C12 cells were transfected with a plasmid containing
120 Cas9 with an IRES-GFP and myomerger gRNAs, or transfected with only Cas9-IRES-GFP as a

121 control. Flow cytometry of GFP⁺ cells followed by genotyping through PCR analysis revealed
122 disruption of the myomerger locus (Extended Data Fig. 5b). Myomerger was not detectable in
123 myomerger KO C2C12 cells confirming efficient disruption of the locus (Fig. 3a). Control and
124 myomerger KO C2C12 cells were then analyzed for their ability to differentiate and form
125 myotubes. WT myoblasts differentiated, as indicated by myosin⁺ cells, and fused to form multi-
126 nucleated myotubes (Fig. 3b). In contrast, myomerger KO C2C12 cells exhibited the ability to
127 differentiate but lacked fusogenic activity to form myotubes (Fig. 3b). Indeed, quantification of
128 the differentiation index revealed no difference in the percentage of myosin⁺ cells between WT
129 and myomerger KO cultures (Fig. 3c). Additionally, quantification of fusion demonstrated that
130 myomerger KO myosin⁺ cells remain mono-nucleated while WT cells fuse (Fig. 3d). qRT-PCR
131 analysis for the myogenic genes *Myogenin*, *Myh4*, *Ckm*, and *Tmem8c* (myomaker) further
132 indicated that myomerger KO myoblasts activate the differentiation program (Fig. 3e).
133 Interestingly, myogenic transcripts were elevated in myomerger KO cells potentially suggesting
134 a feedback mechanism by which non-fusogenic cells attempt to further differentiate (Fig. 3e).
135 Infection of myomerger KO C2C12 cells with either myomerger-S or myomerger-L rescued the
136 fusion defect demonstrating that the phenotype in these cells is specifically due to the loss of
137 myomerger (Extended Data Fig. 5c). Western blot analysis from these lysates shows re-
138 expression of myomerger in KO cells (Extended Data Fig. 5d). As a potential mechanism for the
139 lack of fusion in myomerger KO myocytes, we examined expression and localization of
140 myomaker. On day 2 of differentiation, myomerger KO cells exhibited normal expression and
141 localization of myomaker (Extended Data Fig. 6a). Moreover, we did not detect widespread co-
142 localization between myomaker and myomerger suggesting that myomerger does not directly
143 regulate myomaker distribution (Extended Data Fig. 6b). These data reveal that myomerger is
144 necessary for myoblast fusion *in vitro*.

145 To examine the function of myomerger *in vivo*, we disrupted exon 3 using the same
146 CRISPR/Cas9 strategy described for C2C12 myoblasts. Injection of Cas9 and myomerger
147 gRNAs into blastocysts resulted in lethality of 9 of the 10 F₀ pups, suggesting that the high
148 efficiency of Cas9 lead to homozygous deletion of myomerger. The one remaining pup was
149 heterozygous for myomerger and was mated to generate *Gm7325*^{-/-} mice (Extended Data Fig.
150 7a). Sequencing the mutant PCR product from the heterozygous founder revealed the presence
151 of the same mutation as was achieved in C2C12 cells. We failed to observe any *Gm7325*^{-/-} mice
152 upon genotyping two litters at P7 suggesting that, in conjunction with the lethality detected
153 through initial generation of F₀ pups, myomerger is essential for life. Indeed, E17.5 *Gm7325*^{-/-}
154 embryos exhibited minimal skeletal muscle upon gross examination (Fig. 4a). Specifically, bones
155 of the limbs and rib cage were noticeable due to a scarcity of surrounding muscle as observed
156 in WT embryos. Myomerger KO mice also display a hunched appearance with elongated snouts,
157 hallmark characteristics in embryos with improper muscle formation (Fig. 4a). Detection of
158 myomerger by western blot of WT and *Gm7325*^{-/-} tongues showed elimination of myomerger
159 protein in KO samples (Extended Data Fig. 7b). E15.5 forelimb sections show that myomerger
160 KO myoblasts express myogenin indicating that specification and differentiation are activated
161 despite loss of myomerger (Fig. 4b). Moreover, histological analysis of multiple muscle groups
162 at E15.5 revealed the presence of myosin⁺ muscle cells and sarcomeric structures in myomerger
163 KO mice, (Fig. 4c and Extended Data Fig. 7c). While multi-nucleated myofibers were present in
164 WT mice, these structures were not readily detected in myomerger KO mice indicating that
165 genetic loss of myomerger renders myocytes non-fusogenic (Fig. 4c and Extended Data Fig. 7c).
166 Analysis of forelimbs from E17.5 WT and myomerger KO embryos confirm that myomerger KO
167 myoblasts are unable to properly fuse, although we did detect myocytes with two nuclei
168 (Extended Data Fig. 7d-f). These results, together with our *in vitro* analysis, reveals that

169 myomerger is required for muscle formation during mammalian development through regulation
170 of myoblast fusion.

171 In summary, we report the discovery of an additional muscle-specific factor required for
172 myoblast fusion and developmental myogenesis. While myomaker and myomerger are both
173 necessary for muscle formation, E17.5 myomerger KO embryos exhibit more myocytes
174 compared to embryos lacking myomaker suggesting that these two key myoblast fusion proteins
175 may have distinct functions. Given that myomaker and myomerger are both essential for
176 myoblast fusion, an understanding of their precise function will aid in the delineation of
177 mammalian plasma membrane fusion mechanisms. The fibroblast cell fusion system developed
178 here, through expression of myomaker and myomerger, provides a unique platform to decipher
179 fusion mechanisms. Moreover, the induction of cell fusion by two factors represents an avenue
180 for gene delivery to potentially any tissue.

181

182 **Methods**

183

184 **Cell Culture**

185 C2C12 cells, 10T 1/2 fibroblasts, and NIH/3T3 fibroblasts were purchased from American Type
186 Culture Collection and propagated in DMEM (Gibco) containing 10% heat-inactivated bovine
187 growth serum (BGS) and supplemented with antibiotics. C2C12 cells were differentiated by
188 switching to media containing 2% heat-inactivated horse serum (HS) and antibiotics. MSCs
189 were a gift from Jose Cancelas and described previously²⁷.

190

191 **Bioinformatic Analysis**

192 Microarray data from the GEO DataSet GSE34907²⁸ was interrogated using GEO2R analysis to
193 identify 1826 genes displaying an increase greater than 1 log fold-change in MyoD-expressing
194 fibroblasts. In parallel, a transcriptional profile of 10T 1/2 fibroblasts transduced with empty virus
195 was generated using RNA-seq analysis (paired-end library layout using Illumina sequencing
196 platform) and a list of all genes with RPKM values below 1.5 compiled using Strand NGS
197 software (Ver. 2.6; Build: Mouse mm10 (UCSC) using Ensembl transcript annotations). These
198 two gene lists were then compared to generate a final tally comprised of 531 genes that were
199 both upregulated in MyoD-expressing fibroblasts and had low or no detectable expression in
200 10T 1/2 fibroblasts. Finally, the top 100 genes were interrogated for genes that contain
201 transmembrane domains and not previously studied for their role during myoblast fusion.

202

203 **Animals**

204 We used a dual sgRNA targeting strategy to create *Gm7325*^{-/-} mice. We selected the sgRNAs
205 according to the on- and off-target scores from the web tool CRISPOR²⁹. The selected gRNAs

206 were 5'-GCAGCGATCGAAGCACCATC-3' and 5'-GAGGCCTCTCCAGAATCCGG-3' that target
207 exon 3 of *Gm7325*. The sgRNAs were *in vitro* synthesized using the MEGAshortscript T7 kit
208 (ThermoFisher) and purified by the MEGAclear Kit (ThermoFisher), as previously described³⁰.
209 sgRNAs (50 ng/ul of each) were mixed with 100 ng/ul Cas9 protein (ThermoFisher) and
210 incubated at 37°C for 15 min to form a ribonucleoprotein complex. We then injected the mix into
211 the cytoplasm of one-cell-stage embryos of the C57BL/6 genetic background using a piezo-
212 driven microinjection technique as described previously³⁰. Injected embryos were immediately
213 transferred into the oviducal ampulla of pseudopregnant CD-1 females. Live born pups were
214 genotyped by PCR with the following primers: F: 5'- GAAGGGAGGACTCCACACCC-3' and R:
215 5'-CGCCTGGACTAACCGGCTCC-3'. The edited allele was further confirmed by Sanger
216 sequencing. One heterozygous founder was obtained and mated with WT C57Bl6 mice to
217 eventually generate KO mice. *Mdx*^{4cv} mice were purchased from Jackson Laboratory (#002378).
218 Muscle overload of the plantaris muscle was achieved through bilateral synergistic ablation of
219 soleus and gastrocnemius muscles as described by others³¹. Briefly, the soleus and
220 gastrocnemius muscles were exposed by making an incision on the posterior-lateral aspect of
221 the lower limb. The distal and proximal tendons of the soleus, lateral and medial gastrocnemius
222 were subsequently cut and carefully excised. All animal procedures were approved by Cincinnati
223 Children's Hospital Medical Center's Institutional Animal Care and Use Committee.

224

225 **CRISPR-Mediated Genome Editing in C2C12 Cells**

226 Freshly plated low passage C2C12 cells were transfected with 4µg of a modified pX458 plasmid
227 (Addgene #48138, gift from Yueh-Chiang Hu), which contained a high fidelity Cas9, an optimized
228 sgRNA scaffold, and an IRES-GFP cassette. The same gRNAs used to generate KO animals
229 were used for C2C12 cells. 16 µL of Lipofectamine 2000 was used for this transfection. 5 x 10⁵

230 C2C12 cells were transfected in a 60 mm culture dish. Forty-eight hours after transfection GFP⁺
231 cells were sorted into 96 well plates using FACS. These cells were maintained in DMEM
232 containing 20% FBS with antibiotics at subconfluent densities. The cell lines were genotyped by
233 amplifying a 420 bp region surrounding the site of Cas9 activity using the primers used to
234 genotype *Gm7325*^{-/-} animals.

235

236 **Cloning and viral infection**

237 We initially cloned a region of the *Gm7325* locus, containing all genomic information for
238 expression of myomergershort and myomergerslong, from C57Bl6 mouse genomic DNA using
239 the following primers: F: 5'-AGTGATGCTGAATCCACCGCA-3' and R: 5'-
240 CCAATAACAACACACTGTCCT-3'. We cloned myomergershort and long coding sequences
241 from cDNA of differentiating C2C12 cells using the following primers: myomergerslong F: 5'-
242 ATGCCAGAAGAAAGCTGCACTG-3', myomergersshort F: 5'-
243 ATGCCCGTTCCATTGCTCCCGA-3', and a common myomergers R: 5'-TCA CTT CTG GGG
244 GCC CAA TCT C-3'. Myomergers cDNA and genomic DNA was cloned into the retroviral vector
245 pBabe-X² using EcoRI. Myomakers and GFP retroviral plasmids have been described previously².
246 NLS-TdTomato was subcloned from pQC-NLS-TdTomato (Addgene #37347) into the retroviral
247 vector pMX (Cell Biolabs). Plasmids containing cDNA for Tmem182, Tspan33, and Tm6sf1 from
248 the Mammalian Gene Collection were purchased from Open Biosystems and subcloned into
249 pBabe-X. Ten micrograms of retroviral plasmid DNA were transfected with FuGENE 6 (Roche)
250 into Platinum E cells (Cell Biolabs), which were plated 24 hours before transfection on a 10 cm
251 culture dish at a density of 3-4x10⁶ cells per dish. Forty-eight hours after transfection, viral media
252 were collected, filtered through a 0.45 µm cellulose syringe filter and mixed with polybrene
253 (Sigma) at a final concentration of 6 µg/ml. Target cells were plated on 10 cm culture dishes at

254 a density of 4×10^5 cells per dish 16-18 hours before infection. Eighteen hours after infection,
255 virus was removed, cells were washed with PBS and split into new 10 cm dishes.

256

257 **Cell fusion assays**

258 Cells were split 18 hours after retroviral infection and split again 24-48 hours later. At the second
259 split, cells were seeded for the fusion assay on 35-mm dishes ($3-4 \times 10^5$ cells per dish) or on 8-
260 well Ibidi slides (2×10^4 cells/well). Fusion was assessed 24-48 hours after seeding. For
261 heterologous fusion, cultures of fibroblasts and myoblasts mixed at a ratio of 1:1 (1.5×10^5 cells
262 for each) were induced to differentiate 24 hours after seeding and fusion was assessed on day
263 4 of differentiation.

264

265 **RNA extraction and quantitative RT-PCR (qRT-PCR)**

266 Total RNA was extracted from either mouse tissue or cultured cells with TRIZOL (Life
267 Technologies) according to manufacturer's protocol. cDNA was synthesized using High-
268 Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random primers. Gene
269 expression was assessed using standard quantitative PCR approach with Power Sybr® Green
270 PCR mastermix (Applied Biosystems). Analysis was performed on StepOnePlus Real-Time PCR
271 system (Applied Biosystems) with the following primers: myomergershort-F: 5'-
272 CAGGAGGGCAAGAAGTTCAG-3' and myomergershort-R: 5'-ATGTCTTGGGAGCTCAGTCG-3';
273 myomergerslong-F: 5'-ACCAGCTTTCATGCCAGAAG-3' and myomergerslong-R: 5'-
274 ATGTCTTGGGAGCTCAGTCG-3'; myomaker-F: 5'-ATCGCTACCAAGAGGCGTT-3' and
275 myomaker-R: 5'-CACAGCACAGACAAACCAGG-3'; Tm6sf1 F: 5'-
276 TTAGTGGTCCCTGGATGCTC-3" and Tm6sf1 R: 5'-GACGCACCAATGTGAGAAAA-3';
277 Tspan33-F: 5'-GGGGACGAGTTCTCCTTCG-3' and Tspan33 R: 5'-

278 TGCTTCTGCGTGCTTCATTAG-3'; Tmem182-F: 5'-GGCTCTCTTCGGAGCTTTGG-3' and
279 Tmem182-R: 5'-GGTGGCTGATTGGTGTACCAG-3'; myogenin-F: 5'-
280 CTACAGGCCTTGCTCAGCTC-3' and myogenin-R: 5'-GTGGGAGTTGCATTCACTGG-3';
281 Ckm-F: 5'-ACCTCCACAGCACAGACAGA-3' and Ckm-R: 5'-CAGCTTGAAC TTGTTGTGGG-3';
282 Myh4-F: 5'-GCAGGACTTGGTGGACAAAC-3' and Myh4-R: 5'-ACTTGGCCAGGTTGACATTG-
283 3'; GAPDH-F: 5'-TGC GACTTCAACAGCAACTC-3' and GAPDH-R: 5'-
284 GCCTCTCTTGCTCAGTGTCC-3'.

285

286 **Western blot analysis**

287 Cultured cells were washed two times with ice cold PBS, scraped into a conical tube, pelleted,
288 resuspended in lysis buffer (50 mM Tris-HCl, pH 6.8, 1mM EDTA, 2% SDS) and sonicated for a
289 total of 15 seconds (three 5 second pulses). Skeletal muscle tissues from mice were
290 homogenized with a bead homogenizer (TissueLyser II; Qiagen) in lysis buffer (10 mM Tris (pH
291 7.4), 1 mM EDTA, 1mM dithiothreitol, 0.5% Triton X-100, 2.1 mg/ml NaF) containing protease
292 and phosphatase inhibitor cocktails (5 µl/ml; Sigma-Aldrich). Both cells and tissue lysates were
293 centrifuged to pellet insoluble material and protein concentration was determined using Bradford
294 protein assay. Equal amounts of protein (5 µg for cells and 20 µg for tissues) were prepared with
295 loading buffer (1x Laemmli (Bio-Rad) with reducing agent (5% β-mercaptoethanol for cells and
296 100 mM DTT for tissues). Samples were heated at 37°C for 30 minutes and separated on a 20%
297 SDS-PAGE. The gels were subsequently transferred to a PVDF membrane (Millipore), blocked
298 in 5% milk in Tris-buffered saline/0.1% Tween-20 (TBS-T) and incubated with anti-sheep ESGP
299 antibody (1 mg/µl; R&D Systems) overnight at 4°C. Membranes were then washed with TBS-T
300 and incubated with Alexa-Fluor 647 donkey anti-sheep secondary antibody (1:5,000; Invitrogen).

301 Bands were visualized using the Odyssey® infrared detection system (LI-COR Biosciences).
302 GAPDH (1:5,000; Millipore) was used as a loading control.

303

304 **Subcellular fractionation**

305 C2C12 cells were harvested on day 2 of differentiation in ice cold hypotonic buffer (10 mM Tris-
306 HCl pH 8, 2 mM EDTA) and lysed using a dounce homogenizer. Lysates were then centrifuged
307 at 800 x g for 5 minutes at 4°C to separate nuclei and cell debris. That supernatant was then
308 centrifuged at 5000 x g for 10 minutes to pellet mitochondria and ER. ER and heavy vesicles
309 were further pelleted through centrifugation at 17,000 x g for 10 minutes. Finally, plasma
310 membrane, light vesicles, and organelles were pelleted at 100,000 x g for 20 minutes and the
311 supernatant from this spin was collected as the cytosolic fraction. All pellets were resuspended
312 in lysis buffer (50 mM Tris-HCl, pH 6.8, 1mM EDTA, 2% SDS) at volumes equal to the
313 supernatant. 8 µl of each fraction was separated by SDS-PAGE and analyzed for presence of
314 myomerger, caveolin-3, and tubulin. Caveolin-3 antibody (BD Transduction Laboratories
315 #610421) was used at 1:6700 and tubulin (Santa Cruz #SC-8035) at 1:50.

316

317 **Immunocytochemistry**

318 Cultured cells were rinsed with PBS and fixed in 4% paraformaldehyde (PFA)/PBS for 15
319 minutes at room temperature. Cells were subsequently permeabilized and blocked in 0.01%
320 Triton X-100/5% donkey serum/PBS for one hour at room temperature. Primary antibody diluted
321 in permeabilization/blocking buffer was incubated overnight. Cells were then washed with PBS
322 and incubated with secondary Alexa-Fluor antibodies (1:250) for 1 hour. A myomaker antibody
323 custom antibody was generated through YenZym Antibodies LLC. Rabbits were immunized with
324 amino acids #137-152 of mouse myomaker (MKEKKGLYPDKSIYTQ) after conjugation to KLH.

325 We used antigen-specific affinity purified products at a concentration of 4.3 $\mu\text{g}/\text{mL}$ for
326 immunostaining. Esgp (myomerger) antibody was used at a concentration of 1 $\mu\text{g}/\text{mL}$. Anti-
327 mouse myosin (my32, MA5-11748, ThermoFisher Scientific) antibody was used at 1:100.
328 Hoechst 33342 solution (ThermoFisher Scientific) was used to stain nuclei. Cells were imaged
329 using Nikon A1R+ confocal on a FN1 microscope (35 mm dishes) or Nikon A1R confocal on
330 Eclipse T1 inverted microscope (Ibidi slides).

331

332 **Histology and immunohistochemistry**

333 For cryosections, embryos were dissected, fixed in 4% PFA/PBS overnight at 4°C, washed in
334 PBS, incubated in 30% sucrose/PBS overnight and then in 1:1 mix of optimal cutting temperature
335 (O.C.T.) formulation and 30% sucrose prior to embedding in O.C.T.. Sections were cut at 10 μm
336 and then permeabilized with 0.2% Triton X-100/PBS, blocked with 1% BSA/1% heat inactivated
337 goat serum/0.025% Tween20/PBS and incubated with primary antibody overnight. Anti-mouse
338 myosin (my32, MA5-11748, ThermoFisher Scientific) antibody was used at 1:100, whereas
339 myogenin (F5D, Developmental Hybridomas) was used at a concentration of 2.56 $\mu\text{g}/\text{mL}$.
340 Secondary goat anti-mouse IgG1-488 Alexa-Fluor antibody (Invitrogen) was incubated at a
341 dilution of 1:250 for 1 hour. Slides were mounted with VectaShield containing DAPI (Vector
342 Laboratories) and visualized using Nikon A1R confocal on Eclipse T1 inverted microscope.
343 Images were analyzed with Fiji.

344

345 **Statistical analysis**

346 For quantitation of cell fusion in Fig. 1b, cells with 3 or more nuclei were considered syncytial
347 cells. The number of nuclei in syncytial cells and total number of nuclei were manually counted.
348 In Fig. 1c, the number of myosin⁺ myotubes (myosin structures with 3 or more nuclei) and GFP⁺

349 myosin⁺ myotubes were manually counted. To quantify fusion between myomaker⁺ myomerger⁺
350 GFP⁺ fibroblasts with either myomaker⁺ NLS-Tom⁺ or myomerger⁺ NLS-Tom⁺ fibroblasts
351 (Extended Data Fig. 3), we calculated the percentage of GFP⁺ NLS-Tom⁺ syncytial cells. The
352 differentiation index (Fig. 3c) was calculated as the percentage of nuclei in myosin⁺ cells, and
353 the fusion index (Fig. 3d) as the percentage of myosin⁺ cells with the indicated number of nuclei.
354 Quantitative data sets are presented as means ± SEM. For each quantitation, at least 3
355 independent experiments were performed in duplicate and 4-6 fields were randomly chosen for
356 imaging. Histological analysis of embryos was performed on 3-4 embryos per genotype per time
357 point. Multiple histological levels within each muscle were examined. The data were analyzed
358 using an unpaired Student's t-test with GraphPad Prism 6 software. A value of $P < 0.05$ was
359 considered statistically significant.

360

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365

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367 the data. Q.G., M.K., D.G.G., M.J.P., and V.K. performed experiments. D.P.M. wrote the
368 manuscript with assistance from all authors.

369

370 **Author Information** The authors declare no competing financial interests. Correspondence and
371 requests for materials should be addressed to D.P.M. (douglas.millay@cchmc.org).

372

373 **References**

374

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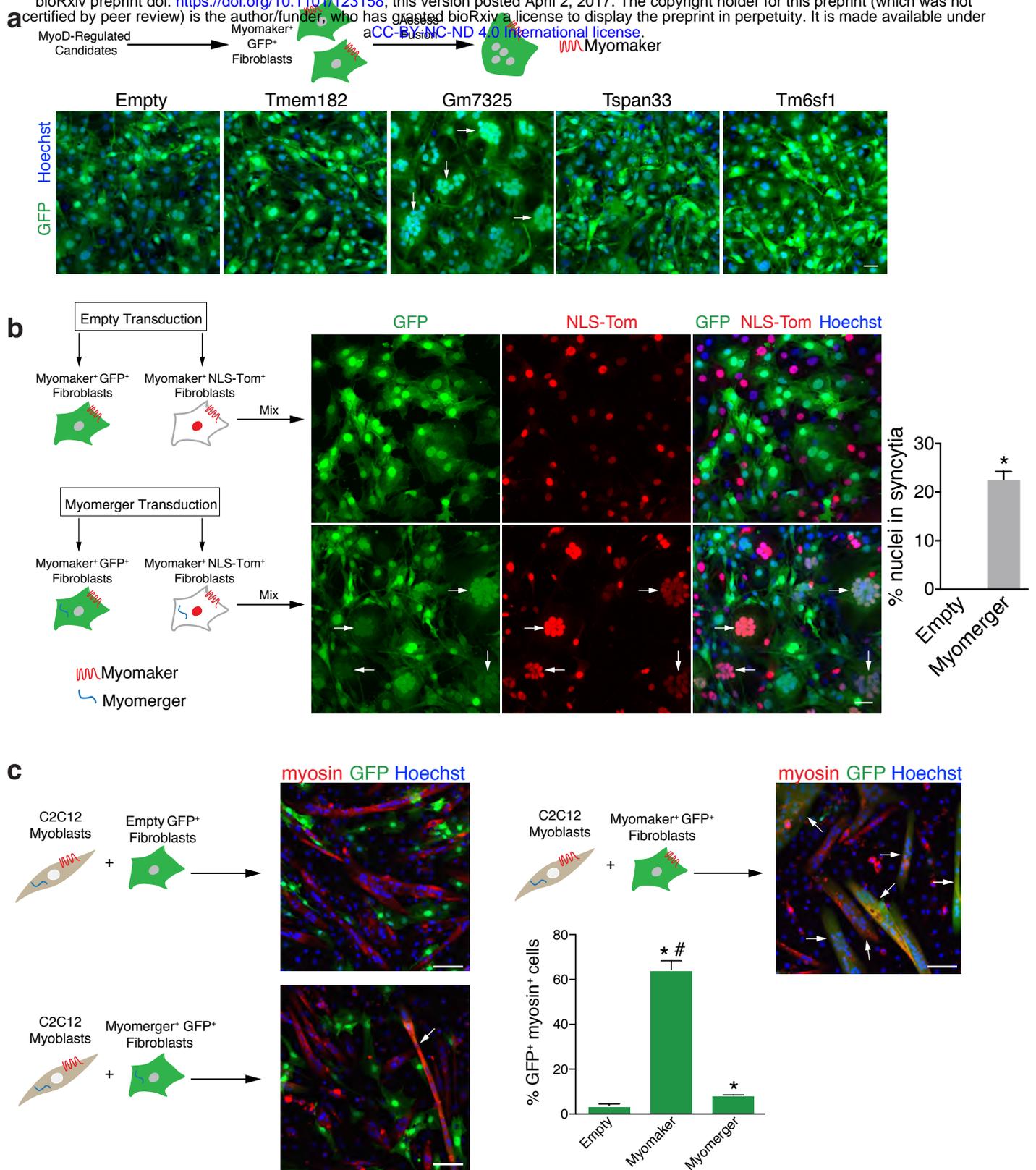


Figure 1. Induction of fibroblast fusion by myomerger. **a**, Schematic showing a screen for muscle genes that could activate fusion of GFP⁺ myomaker⁺ fibroblasts. Representative images of GFP⁺ cells and nuclei after expression of the indicated genes. Arrows depict cells with multiple nuclei. **b**, Illustration of cell mixing approach to show fusion between the populations of fibroblasts. Co-localization of GFP and NLS-TdTomato (NLS-Tom) in the nucleus represents fusion. Representative images demonstrate fusion of myomaker⁺ myomerger⁺ fibroblasts but not empty-infected myomaker⁺ fibroblasts. Arrows indicate fusion between GFP⁺ and NLS-Tom⁺ fibroblasts. The percentage of nuclei in syncytia after expression of empty or myomerger ($n=3$). **c**, Heterologous fusion experiment between C2C12 myoblasts and GFP⁺ fibroblasts infected with either empty, myomaker, or myomerger. Representative immunofluorescent images to visualize co-localization of myosin and GFP (arrows), indicating fusion. Quantification of the percentage of GFP⁺ myosin⁺ cells ($n=3$). Data are presented as mean \pm SEM. * $P<0.05$ compared to empty. # $P<0.05$ between myomaker and myomerger. Scale bars, 50 μ m (**a**, **b**), 100 μ m (**c**).

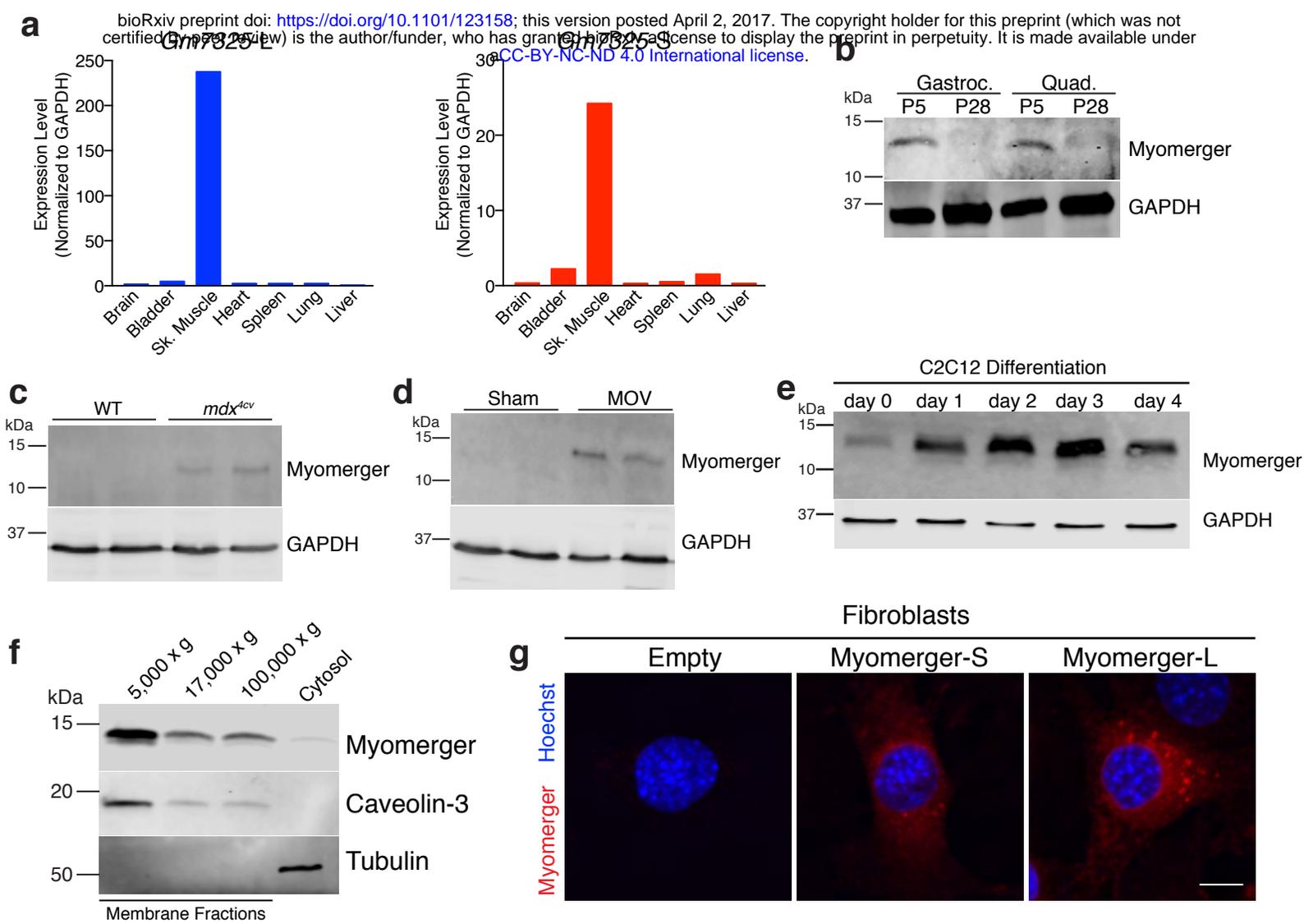


Figure 2. Muscle-specific expression and regulation of myomerger. **a**, qRT-PCR for both *Gm7325* long (L) and short (S) transcripts from various postnatal (P) day 5 tissues. **b-e**, Immunoblotting for myomerger comparing P5 muscle to P28 muscle (**b**), WT to *mdx^{4cv}* diaphragms (**c**), sham plantaris to mechanically overloaded (MOV) plantaris (**d**), and during C2C12 myoblast differentiation (**e**). GAPDH was used as a loading control. **f**, Fractionation of C2C12 lysates on day 2 of differentiation followed by immunoblotting. **g**, Representative immunostaining of fibroblasts infected with either empty, myomerger-short (S), or myomerger-long (L). Scale bar, 10 μ m.

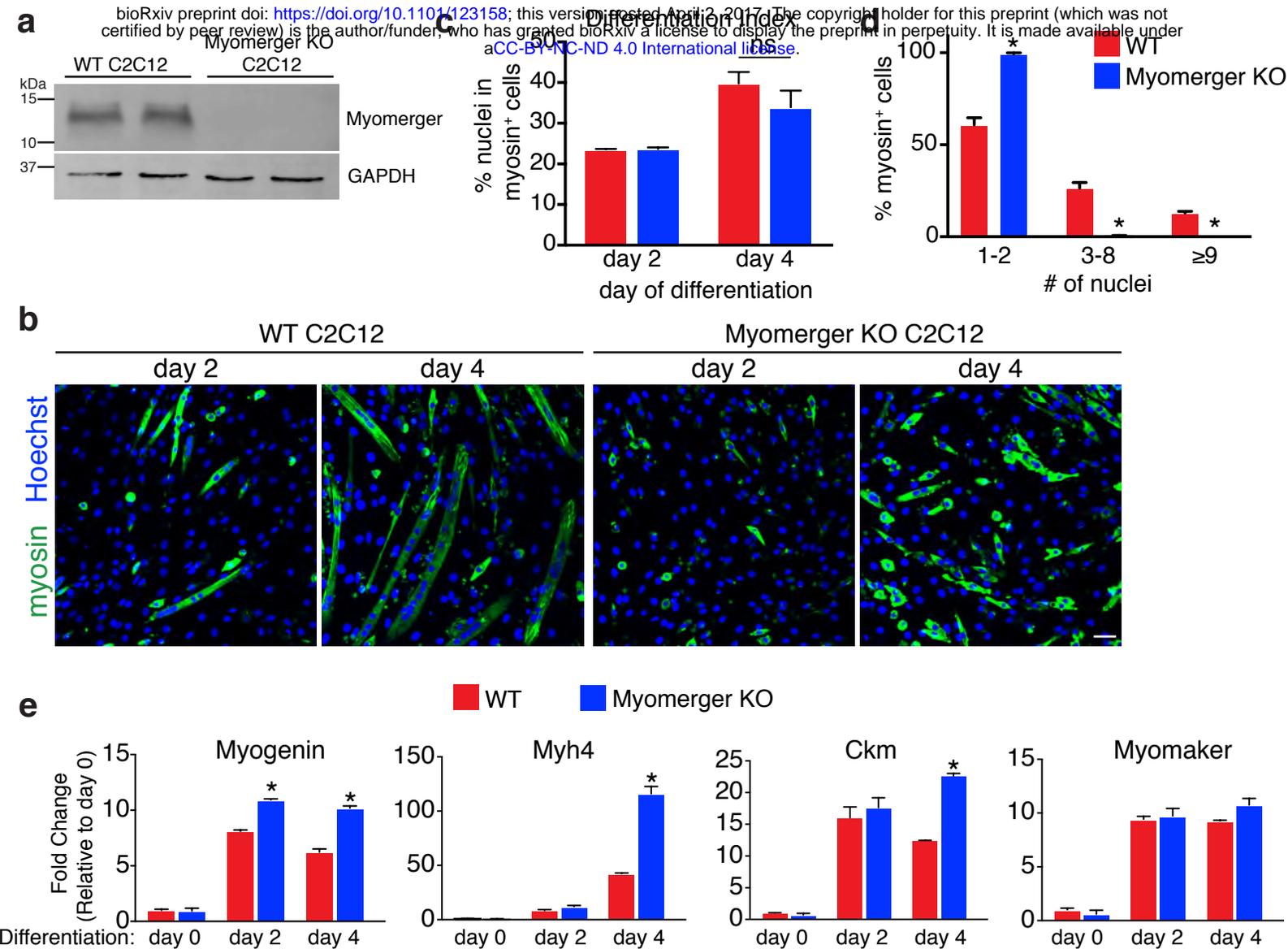


Figure 3. Requirement of myomerger for myoblast fusion *in vitro*. **a**, Immunoblotting for myomerger in WT and myomerger KO C2C12 cells on day 2 of differentiation. GAPDH was used as a loading control. **b**, Representative immunofluorescence images on day 2 and day 4 of differentiation for WT and myomerger KO C2C12 cells. Myomerger KO cells differentiate but fail to fuse. **c**, Quantification of the differentiation index, the percentage of nuclei in myosin⁺ cells ($n=4$). ns, not significant. **d**, The percentage of myosin⁺ cells that contain 1-2, 3-8, or ≥ 9 nuclei after 4 days of differentiation, as an indicator of fusogenicity ($n=3$). **e**, qRT-PCR for the indicated myogenic transcripts ($n=4$). Data are presented as mean \pm SEM. * $P < 0.05$ compared to WT. Scale bar, 50 μm .

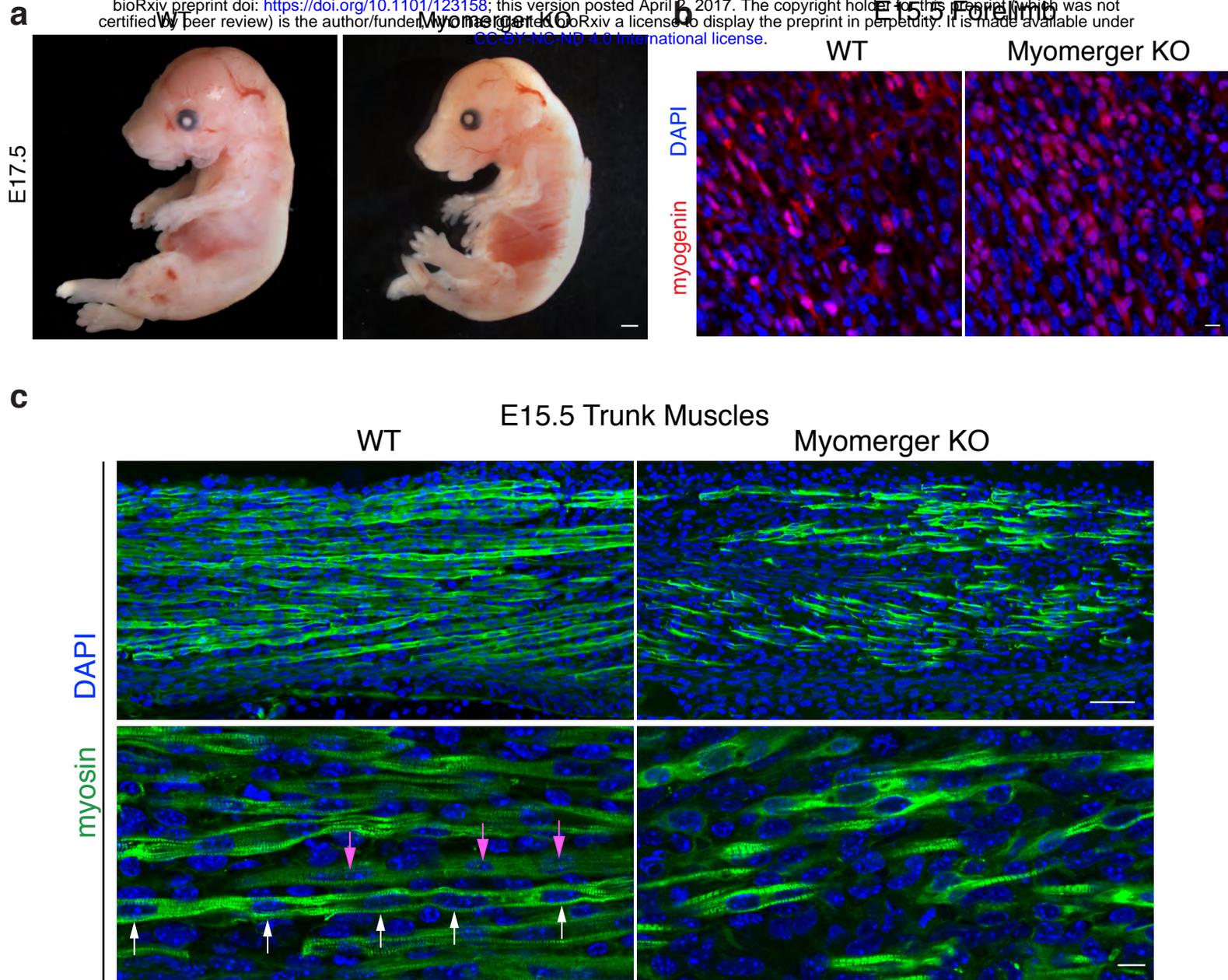
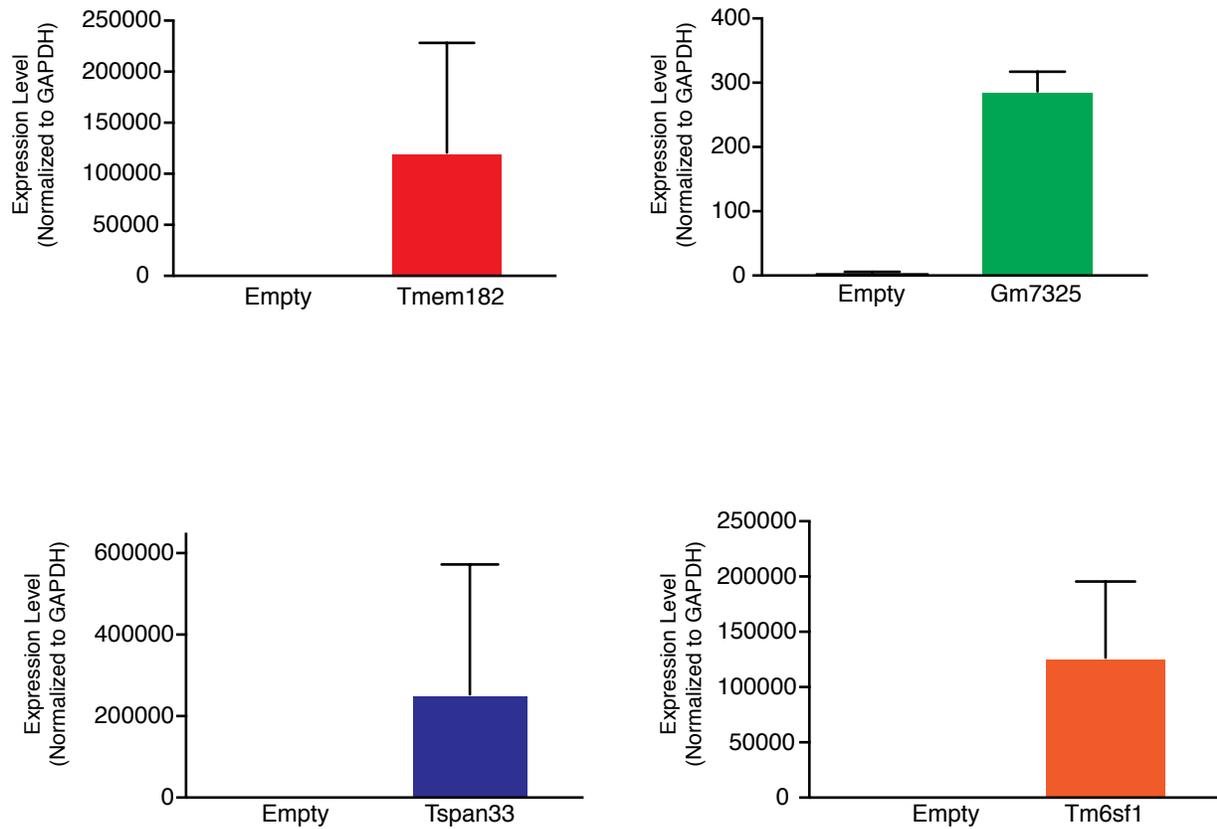
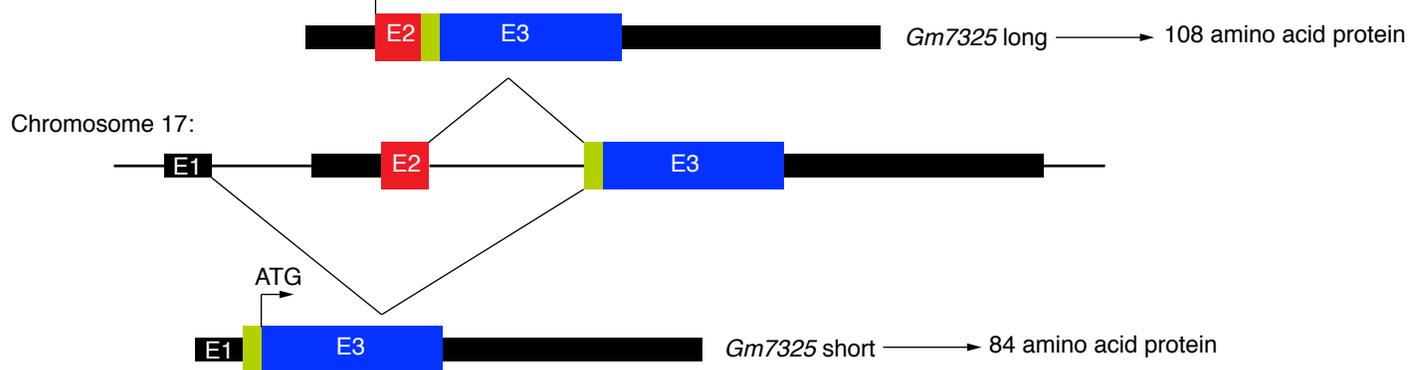


Figure 4. Myomerg is essential for myoblast fusion and muscle formation during embryonic development. **a**, Representative whole-mount images of WT and myomerg KO E17.5 embryos showing improper skeletal muscle formation in KO embryos ($n=4$). **b**, Immunofluorescence images for myogenin from WT and myomerg KO E15.5 forelimbs demonstrating that myomerg is not required for myogenic activation ($n=3$). **c**, Myosin immunofluorescence on the indicated E15.5 trunk muscles ($n=3$). Multi-nucleated myofibers (arrows of same color show nuclei within one myofiber) were observed in WT sections. Myomerg KO myocytes were myosin⁺ with sarcomeres but remained mono-nucleated. Scale bars, 1 mm (**a**), 50 μ m (**c**, top panels), 10 μ m (**b**, **c**, bottom panels).

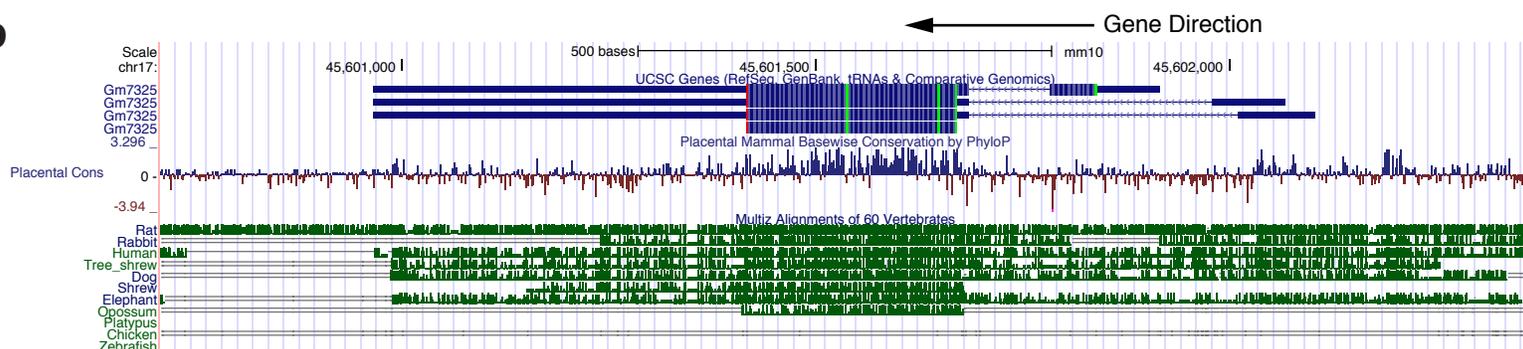


Extended Data Figure 1. Expression of MyoD-regulated genes in myomaker⁺ fibroblasts. qRT-PCR analysis for the indicated genes 72 hours after expression in fibroblasts. For *Gm7325*, we used primers specific for the long transcript.

a



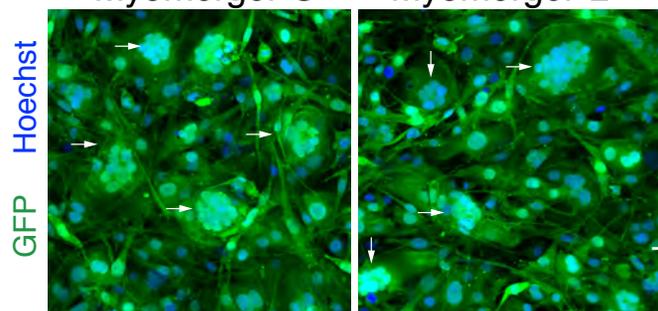
b



c

Myomaker⁺ 10T 1/2 Fibroblasts

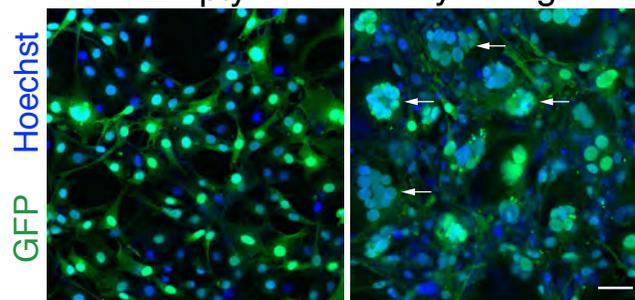
Myomerger-S Myomerger-L



d

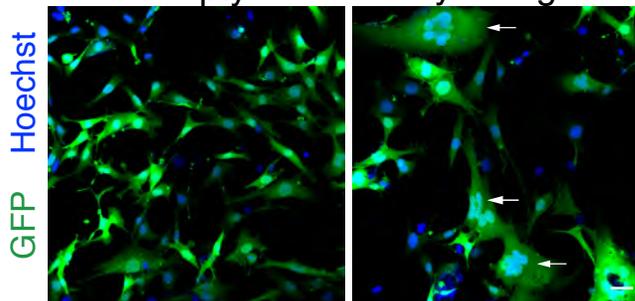
Myomaker⁺ 3T3 Fibroblasts

Empty Myomerger

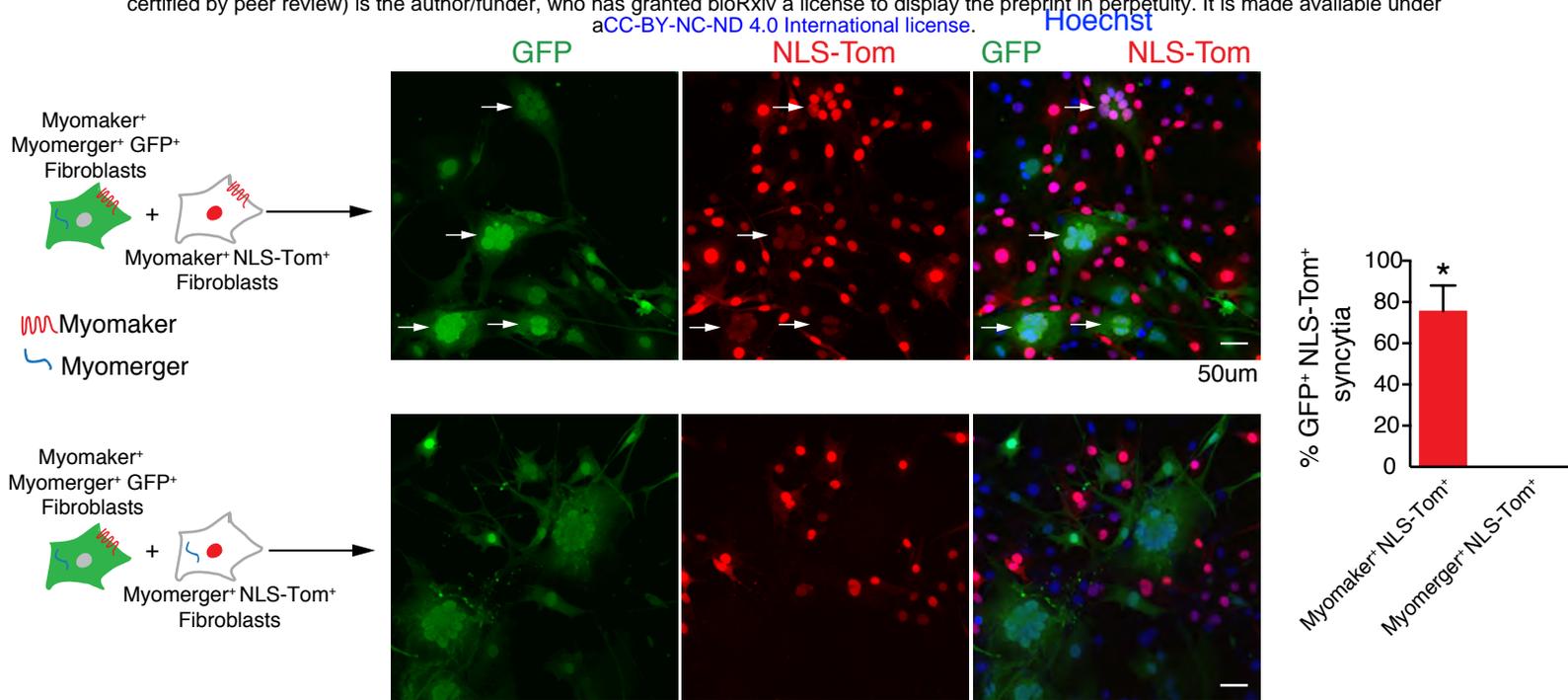


Myomaker⁺ Mesenchymal Stromal Cells

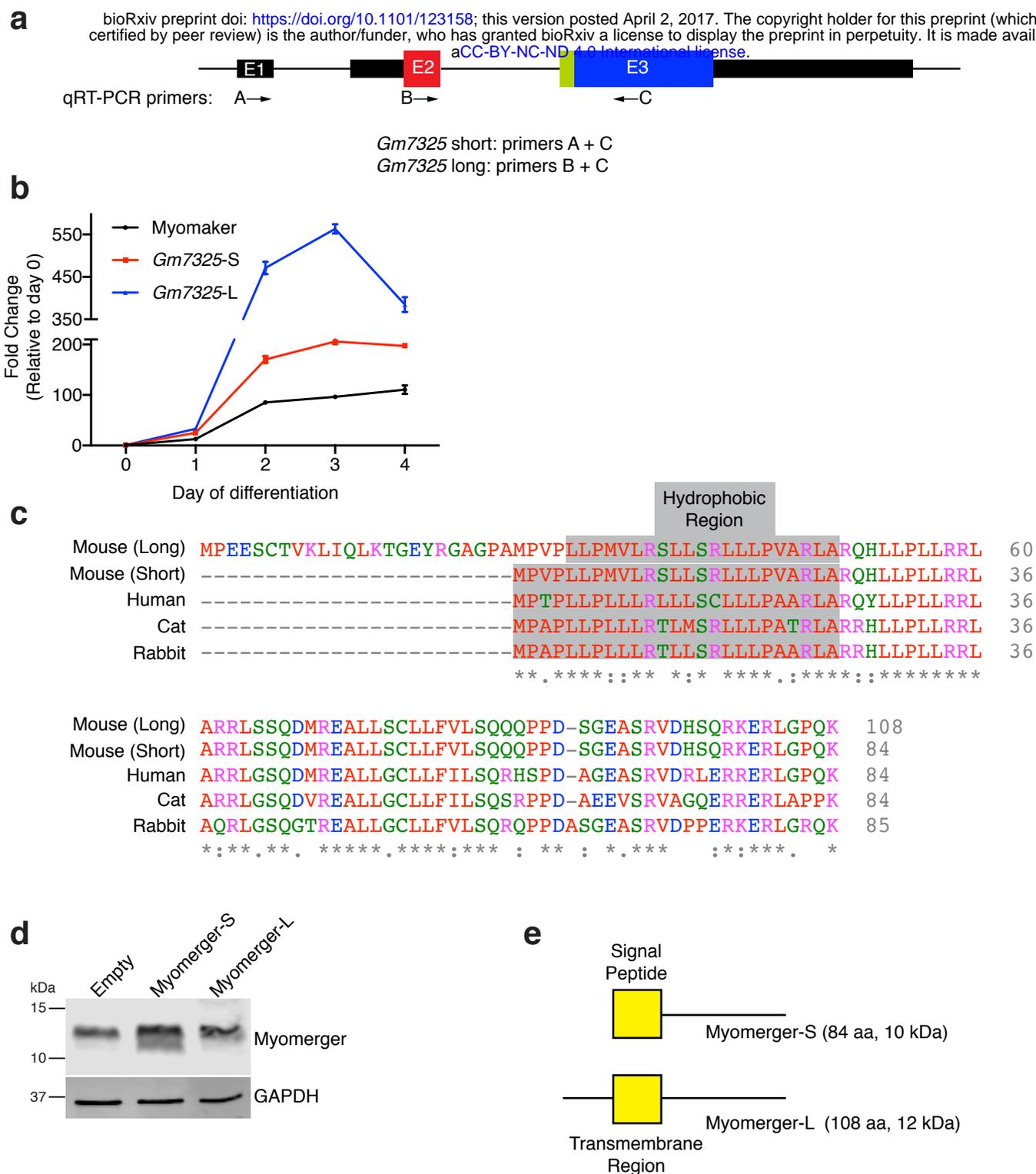
Empty Myomerger



Extended Data Figure 2. Genomic organization of murine *Gm7325* and the ability of both variants to induce fusion. **a**, Diagram showing the *Gm7325* locus on chromosome 17. The short transcript is generated by splicing of exon 1 (non-coding) with exon 3, leading to an 84 amino acid protein. The long transcript is produced by splicing of exon 2 with exon 3 and results in a 108 amino acid protein. **b**, UCSC genome browser track showing multiple transcripts and conservation across vertebrate species. The short transcript is highly conserved in multiple species, including human, but not present in zebrafish. The upstream exon that produces the longer transcript is not highly conserved. Note that this annotation displays the gene on the reverse strand. **c**, The short (S) or long (L) myomerger transcripts were expressed in myomaker⁺ 10T 1/2 fibroblasts and both induced fusion ($n=3$). **d**, Myomerger also induced fusion of myomaker⁺ NIH/3T3 fibroblasts ($n=3$) and myomaker⁺ mesenchymal stromal cells ($n=3$). Arrows indicate fusion. Scale bars, 50 μ m.



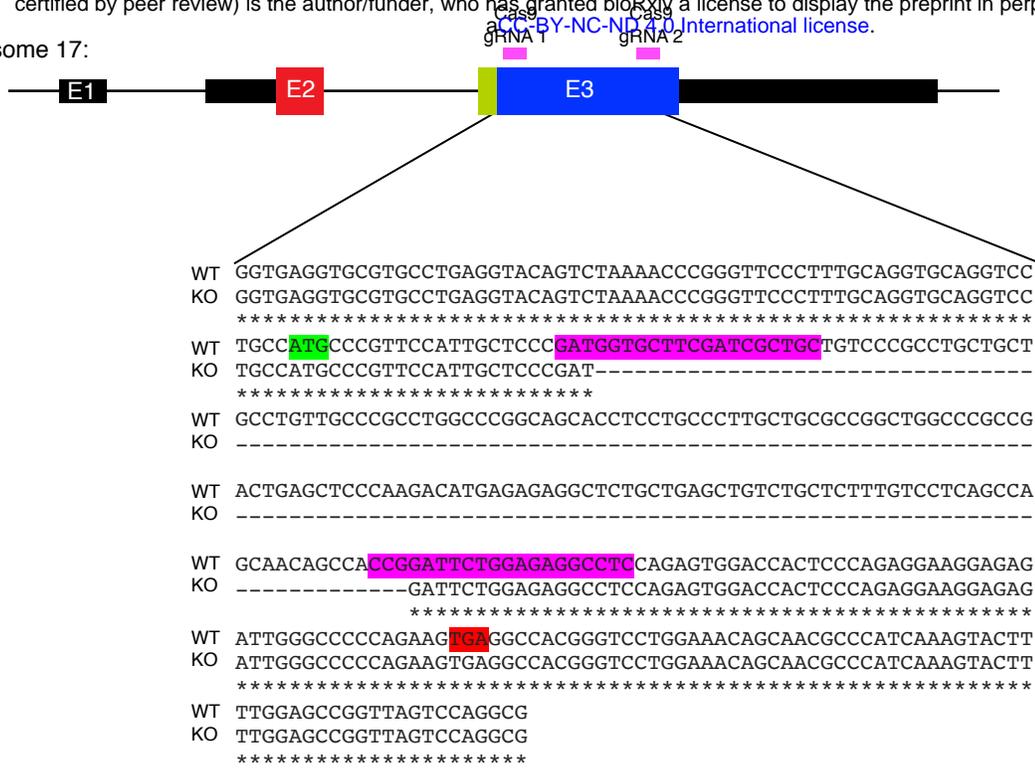
Extended Data Figure 3. Efficient fusion requires myomaker expression in both fusing cells but myomerger in one fusing cell. Diagram showing the cell mixing approach to assess fusion between the populations of fibroblasts. Co-localization of GFP and NLS-TdTomato (NLS-Tom) in the nucleus represents fusion (arrows). Representative images demonstrate fusion of myomaker+ myomerger+ GFP+ fibroblasts with myomaker+ NLS-Tom+ fibroblasts but not myomerger+ NLS-Tom+ fibroblasts. The percent of GFP+ NLS-Tom+ syncytia ($n=3$). Data are presented as mean \pm SEM. * $P<0.05$ compared to myomerger+ NLS-Tom+ fibroblasts. Scale bars, 50 μ m.



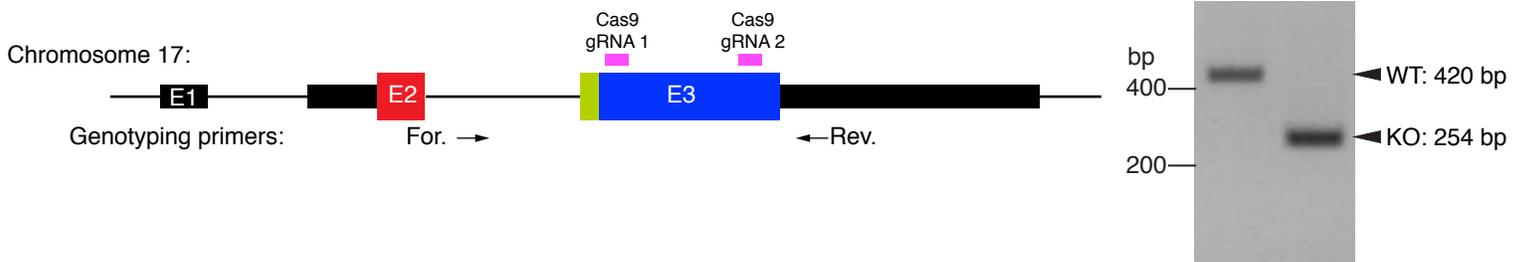
Extended Data Figure 4. Design of qRT-PCR primers and comparison of myomergers protein variants.

a, Schematic showing the location of primers to distinguish short and long transcripts. **b**, qRT-PCR for *Gm7325* transcript variants and myomaker in C2C12 cells on the indicated days of differentiation ($n=3$ for each time point). **c**, Sequence alignment of both mouse myomergers protein products with multiple mammalian orthologs using Clustal Omega. A potential hydrophobic region is highlighted in gray. **d**, Immunoblotting from C2C12 cells infected with either empty, myomergers-short (S), myomergers-long (L) on day 2 of differentiation. Myomergers migrates as a single band around 12 kDa when endogenously produced (empty). Over-expression of myomergers-S leads to an increase in the endogenous band and a lower band is also detected suggesting that myomergers transcripts may be subjected to intricate mRNA processing or post-translational modifications. **e**, Graphic showing the regions of myomergers-S and myomergers-L as predicted by SignalP and Phobius.

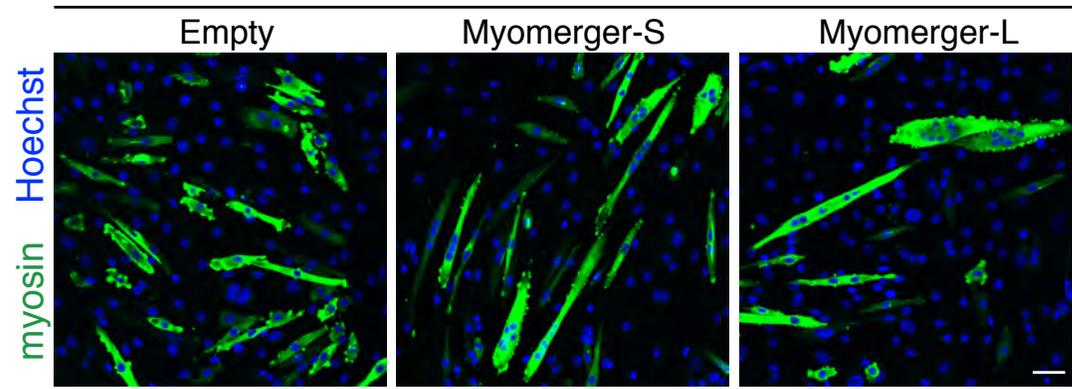
Chromosome 17:



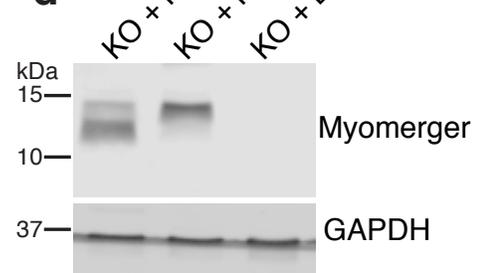
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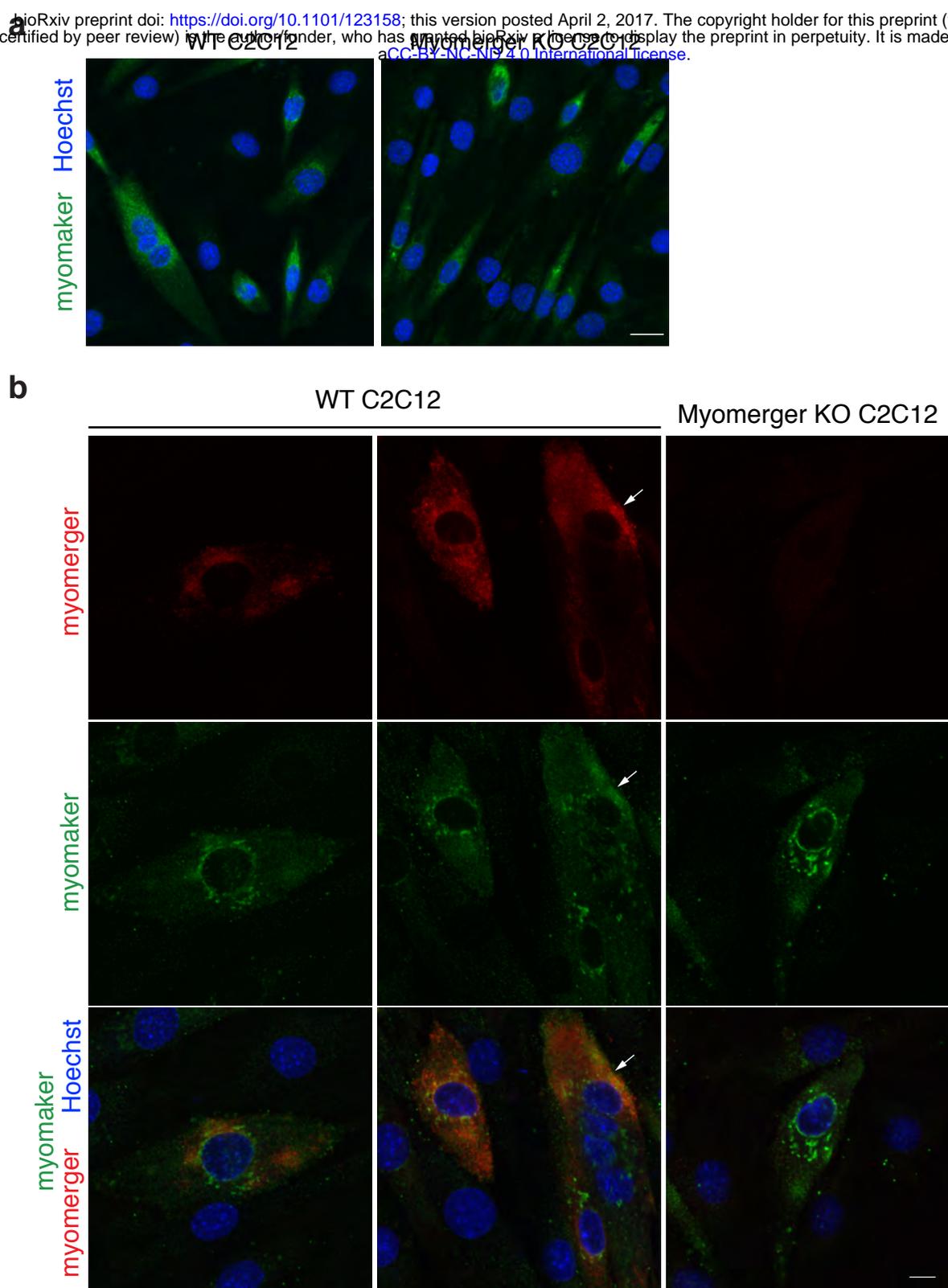
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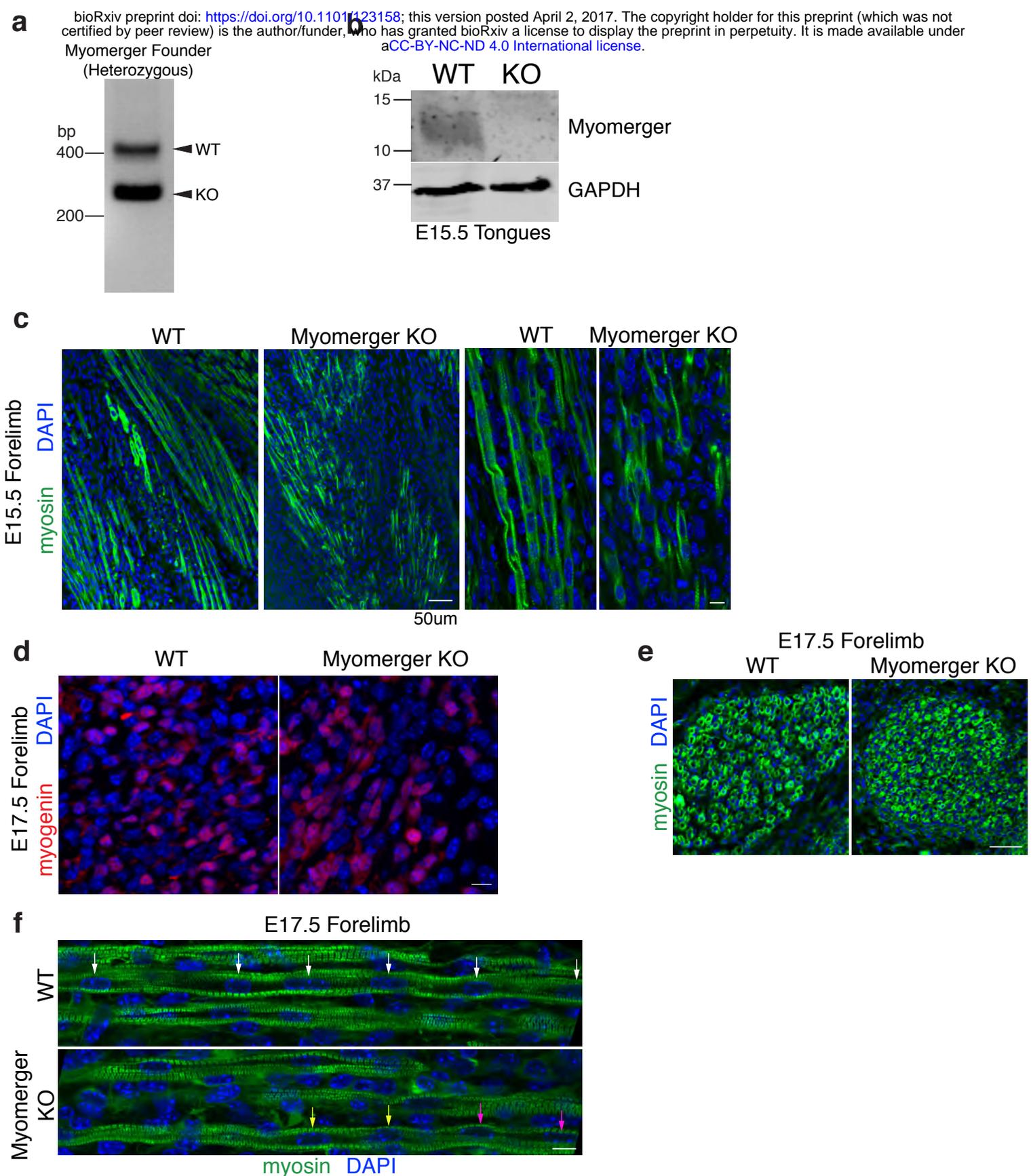
d



Extended Data Figure 5. CRISPR/Cas9 disruption of the *Gm7325* locus. **a**, Schematic showing the *Gm7325* locus and targeting of sgRNAs. **b**, Genotyping strategy for myomerger KO C2C12 cells. WT and KO PCR products were sequenced and the result is shown below the diagram in (a). The use of two sgRNAs results in reproducible cut sites leading to a 166 base pair deletion in both C2C12 cells and mice. The translational start site (ATG, green) for myomerger-S and stop site (TGA, red) for both myomerger-S and myomerger-L are noted. **c**, Myomerger KO C2C12 cells were infected with either empty, myomerger-S, or myomerger-L and induced to differentiate. Both myomerger-S and myomerger-L rescued the lack of fusion in myomerger KO cells. **d**, Immunoblotting for myomerger shows appropriate expression after transduction of myomerger KO cells. Scale bars, 50 μ m.



Extended Data Figure 6. Analysis of myomaker and myomerger co-localization. **a**, Representative immunofluorescence images from WT and myomerger KO C2C12 cells on day 2 of differentiation indicating that loss of myomerger does not alter myomaker expression or localization. **b**, Immunofluorescence for myomerger and myomaker on the indicated cells on day 2 of differentiation. These two fusion proteins exhibit different patterns, although modest co-localization (arrows) was observed. The functional significance of this potential co-localization is not currently known. Scale bars, 10 μm (**a**), 5 μm (**b**).



Extended Data Figure 7. Examination of myomergner KO muscle. **a**, Genotyping of the one founder harboring the *Gm7325* mutation generated through Cas9-mutagenesis. **b**, Immunoblotting on tongue lysates from WT and myomergner KO mice showing lack of myomergner in KO samples. GAPDH was used as a loading control. **c**, E15.5 forelimbs ($n=3$) immunostained with a myosin antibody demonstrates that myomergner KO myoblasts differentiate but are unable to fuse. **d-e**, E17.5 forelimbs from WT and myomergner KO mice were evaluated for myogenin and myosin expression, and multi-nucleation. Arrows of same color in **(f)** show nuclei within one myofiber. We observed myocytes in myomergner KO samples that contained two nuclei (arrows). Analysis of z-stack images (not shown) indicated that the nuclei labeled by the yellow and pink arrows are not within the same myofiber. Scale bars, 50 μm (**c**, left panels, **e**), 10 μm (**c**, right panels, **d**, **f**).