Knockout of zebrafish desmin genes does not cause skeletal muscle degeneration but alters calcium flux.

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Abstract

Desmin is a muscle-specific intermediate filament protein that has fundamental role in muscle structure and force transmission. Whereas human desmin protein is encoded by a single gene, two desmin paralogs (*desma* and *desmb*) exist in zebrafish. *Desma* and *desmb* show differential spatiotemporal expression during zebrafish embryonic and larval development, being similarly expressed in skeletal muscle until hatching, after which expression of *desmb* shifts to gut smooth muscle. We generated knockout (KO) mutant lines carrying loss-of-function mutations for each gene by using CRISPR/Cas9. *Desma*;*desmb* double mutants are viable and fertile, and lack obvious skeletal muscle, heart or intestinal defects. In contrast to morphants, knockout of each gene did

not cause any overt muscular phenotype, but did alter calcium flux in myofibres. These results point to a possible compensation mechanism in these mutant lines generated by targeting nonsense mutations to the first coding exon.

Introduction

Desmin is a type III intermediate filament protein that is specifically expressed in skeletal, cardiac and smooth muscles. In addition to their fundamental role in maintaining the structural integrity of the sarcomere, desmin intermediate filaments are involved in mechanotransduction and organelle positioning. Desminopathies belonging to myofibrillar myopathies are primarily characterized by abnormal protein aggregates in muscle and present as progressive skeletal myopathy and/or cardiomyopathy[1]. Although the involvement of smooth muscles is not widely reported, some patients suffer from smooth muscle dysfunction such as swallowing difficulties, intestinal pseudo-obstruction and respiratory insufficiency[2–4]. The majority of desmin mutations are associated with desmin aggregates; however, some mutations have been reported to not alter filament assembly and network integrity *in vivo* or *in vitro*[5,6]. KO of desmin in mice does not affect the viability and the development of muscles; however, muscle degeneration and cardiomyopathy are observed[7,8].

Zebrafish is a widely used model for neuromuscular disorders[9,10]. Besides the advantage of being the most abundant tissue in zebrafish; the gene profile, and the structural and histological features of mammalian skeletal muscle are highly preserved in zebrafish[11]. To date, two approaches have been used to study the effect of the loss of desmin in zebrafish. On one hand, it was shown that morpholino-mediated knockdown of desmin causes skeletal and cardiac muscle myopathy[12,13]. On the other hand, Ramspacher et al. studied a desmin mutant line (*desma*^{sa5}), generated by ENU mutagenesis, which causes a truncation mutation approximately half way along the molecule, that also presented with skeletal and cardiac muscle phenotype[14].

Gene duplications are common in zebrafish and most duplicated genes have similar functions to their human orthologs. Alternatively, paralogs can be expressed in different tissues or stages of development and have distinct functions to their human orthologs. Humans carry a single copy of the *DES* gene whereas two desmin genes (*desma* and *desmb*) are found in zebrafish[13]. Previous work has not established the tissue specific distribution and role of these two desmin paralogs.

Here, we report the spatiotemporal expression pattern of the two desmin paralogs in zebrafish, as well as the generation using CRISPR/Cas9 and characterization of putative null zebrafish mutant lines for each of the desmin paralogs. We show that *desma* and *desmb* are expressed in skeletal muscle until 72-hour post-fertilization (hpf). After 72 hpf, expression of *desmb* shifts to gut smooth muscle. Putative null mutation of each gene does not affect viability and adults do not develop any overt phenotype. However, altered calcium flux was observed in *desma*-KO myofibres. Surprisingly, *desma;desmb* double mutants also survive to adulthood with no sign of muscle defect. These results show that loss-of-function of desmin genes by mutations in the first coding exon result in a mild phenotype with no visible muscle degeneration but altered calcium flux, in contrast to morpholino-mediated knockdown models and the $desma^{sa5}$ allele.

RESULTS

Zebrafish desmin genes

Zebrafish have two *desmin* orthologs, *desma* (*desmin a*) and *desmb (desmin b)*, which are located on chromosome 9 and 6, respectively. *Desma* and *desmb* paralogs share 81% and 83% identity with human *DES* gene. Although a single transcript is known to be encoded by human *DES* gene, in ENSEMBL two isoforms are predicted to be transcribed from *desma*, *desma-1* mRNA coding for a 488 amino acid protein (predicted molecular weight of 55.7 kDa) and *desma-2* mRNA coding for a 473 amino acid protein (predicted molecular weight of 54.1 kDa). In *desma-2* transcript, exon 9 is skipped, corresponding to 15 amino acid located in the tail domain of Desma-1 protein (Supplementary Fig. S1). The 473 amino acid Desmb protein is predicted to have a molecular weight of 54.2 kDa. At the amino acid level, Desma-1, Desma-2 and Desmb show 80%, 82% and 83% similarity with human desmin protein.

Differential expression of desmin transcripts during zebrafish development

We analyzed the spatiotemporal expression patterns of *desma* and *desmb* genes during zebrafish development at several stages beginning from somitogenesis until 5 days post fertilization (dpf) by whole mount *in situ* hybridization (ISH). For detecting both *desma* transcripts (*desma-2* lacks exon 9), we synthesized an antisense probe by using a forward primer binding to exon 1 and a reverse primer binding to exon 10 (Supplementary Fig. S1). No specific staining was observed in embryos treated with sense probes for each gene (Supplementary Fig. S2). *Desma* was first

88 detected at 11 hpf in adaxial slow muscle precursor cells when the first somites form and remained
89 strongly expressed in somites at all examined stages (Fig. 1a). *Desma* was also expressed in the
90 developing heart from 35 hpf (Fig. 1b). Cryosections of 72 hpf embryos showed that *desma* was
91 distributed in the entire somite, including epaxial and hypaxial muscles (Fig. 1c). By 72 hpf, *desma*
92 was expressed in cranial muscles including external ocular muscles, opercular muscles and
93 mandibular muscles (Fig. 1d). At 96 hpf, transversal sections revealed *desma* expression at the
94 anterior intestine (Fig. 1d, upper panel). At 72, 96 and 120 hpf, expression of *desma* in pectoral
95 fin muscles was clearly visible (Fig. 1d). *Desmb* had an overlapping expression pattern to *desma*
96 from the beginning of somitogenesis until 72 hpf, including staining in trunk somites and heart
97 (Fig. 1a, b). At 72 hpf, somitic expression of *desmb* dramatically decreased with a residual
98 expression in the lateral edges of the myotome. By contrast, strong signal of *desmb* transcripts was
99 detected around the gut throughout the length of the intestine (Fig. 1a, c, e). Similar to *desma*,
100 *desmb* was also expressed in pectoral fin muscles, operculum muscles and mandibular muscles at
101 72 hpf (Fig. 1e). These results indicate that *desma* and *desmb* expression partially overlap during
102 zebrafish muscle development. As the gut develops, *desmb* expression shifts from somitic muscle
103 to gut smooth muscle.

104 **Generation of *desma* and *desmb* knockout zebrafish lines**

105 *Desma* and *desmb* mutant zebrafish lines were generated by using CRISPR/Cas9 genome editing.
106 The $desma^{kg97}$ line exhibited 2 bp deletion and 4 bp insertion in the first exon
107 (g.198_99delGAinsTGAT, NC_007120.7) leading to a frameshift at amino acid 45 and a
108 premature stop codon after four amino acids. In the $desmb^{kg156}$ mutant line, a 5 bp deletion in exon
109 1 (c.245_249delCTTAT, NM_001077452.1) resulted in a frameshift at amino acid 82 and
110 introduced a premature stop codon after three amino acids (Fig. 2a). Heterozygous mutants showed
111 no defect and homozygous mutant embryos and larvae developed relatively normally, at least for
112 the first few days (Fig. 2b-f).

113 **Expression of *desma* and *desmb* in knockout lines**

114 Mutations were predicted to trigger nonsense-mediated decay of mRNA or synthesis of short
115 truncated polypeptides. As expected, qRT-PCR revealed a significant decrease of *desma*
116 transcripts in $desma^{kg97}$ homozygous embryos (P=0.0119, Mann-Whitney U), while *desmb*

117 transcripts were significantly decreased in *desmb*^{kg156} homozygous embryos at 96 hpf compared to
118 wild type (WT) (P=0.0079, Mann-Whitney U) (Fig. 2b). Consistent with El-Brolosy et al.[15], weak
119 *desmb* or *desma* mRNA up-regulation may occur in the respective mutant embryos (Fig. 2b).
120 In order to investigate the potential compensatory effect of *desmin* paralog upregulation in
121 mutant embryos, expression patterns of *desmb* and *desma* transcripts were determined in *desma*^{kg97}
122 or *desmb*^{kg156} homozygous mutant embryos, respectively. In *desma*$^{kg97/kg97}$ embryos, the expression
123 pattern of *desmb* transcripts (Fig. 2c) was similar to that of wild-type embryos (Fig. 1a). Somitic
124 expression of *desmb* was observed in *desma*$^{kg97/kg97}$ at 48 hpf, and a shift towards gut expression
125 after 72 hpf. Beyond 72 hpf, no *desmb* staining was detected in somites of *desma*$^{kg97/kg97}$ embryos.
126 In *desmb*$^{kg156/kg156}$ embryos, *desma* was still strongly expressed in somites and visible in the
127 anterior intestine at 96 hpf (Fig. 2c). In order to confirm the loss-of-function of desmin in mutants
128 at the protein level, expression of Desma and Desmb in WT, *desma*^{kg97} or *desmb*^{kg156} homozygous
129 embryos at 96 hpf was determined by immunofluorescent staining using a desmin antibody
130 recognizing both proteins (Fig. 2d-f). In WT embryos, desmin staining was detected in both
131 somites and gut (Fig. 2d). As expected from ISH results, somitic expression was lost and gut was
132 preferentially stained in *desma*$^{kg97/kg97}$ mutants (Fig. 2f). In *desmb*$^{kg156/kg156}$ mutants, expression in
133 somites and anterior intestine were preserved while staining of the middle and posterior intestine
134 was lost (Fig. 2e). We conclude that mutation of each gene reduces the cognate mRNA and protein
135 at all stages examined and that compensation by up-regulation of the unmutated paralogous gene
136 is at best very weak.

137 **General larval characteristics of desmin mutants**

138 In order to evaluate the effects of the absence of *desma* or *desmb* on development, WT, *desma*^{kg97}
139 and *desmb*^{kg156} homozygous embryos/larvae were compared. No significant difference was
140 observed in the number of viable eggs between *desma*$^{kg97/kg97}$ and WT (P=0.6842, Mann-Whitney
141 U) or *desmb*$^{kg156/kg156}$ and WT (P=0.9654, Mann-Whitney U) (Fig. 3a). Hatching period (48-72
142 hpf[16]) is a critical process in embryonic development and reduction in the hatching rate could be
143 associated with reduced muscle function[17]. The time course showed that although different
144 hatching times were observed between groups, no significant difference was found between
145 hatching rates of mutant and WT embryos at 72 hpf with over 94% of embryos hatched

146 (*desma*$^{kg97/kg97}$ vs. WT, *P*=0.9902; *desmb*$^{kg156/kg156}$ vs WT, *P*=0.3186, repeated measures two-way
147 ANOVA, Bonferroni post *hoc* test) (Fig. 3b).
148 Similar mortality rates were observed with no significant difference between homozygous
149 mutants of *desma*^{kg97} or *desmb*^{kg156} and WT embryos (*desma*$^{kg97/kg97}$ vs. WT, *P*=0.1287;
150 *desmb*$^{kg156/kg156}$ vs. WT, *P*=0.6239, repeated measures two-way ANOVA) (Fig. 3d). Finally,
151 among surviving larvae, no statistically significant difference in body length was observed
152 between WT and mutant groups (*P*=0.2036 for WT vs. *desma*$^{kg97/kg97}$; *P*=0.1893 for WT vs.
153 *desmb*$^{kg156/kg156}$, Mann-Whitney U) (Fig. 3c).

154 **Mutant larvae show no neuromuscular defect**

155 Muscle fibre integrity and somite morphology length were investigated by staining muscle actin
156 with rhodamine phalloidin in 96 hpf embryos (Fig. 3e). At this stage, mainly *desma* is expressed
157 in somites. No muscle lesion or detachment of fibres was observed in mutants and no significant
158 difference was found in somitic length between mutants and WT (*P*=0.7912 for WT vs.
159 *desma*$^{kg97/kg97}$; *P*=0.4923 for WT vs. *desmb*$^{kg156/kg156}$, Mann-Whitney U) (Fig. 3f).
160 Touch-evoked response assay is a widely used method to assess neuromuscular function
161 in 48 hpf zebrafish embryos[18]. We performed motility experiments in a blinded manner on siblings
162 from three groups of heterozygous mutant in-crosses and genotyped them after. First, the average
163 escape time of siblings from *desma*$^{kg97/+}$ in-crosses was assessed and no significant difference was
164 found in *desma*^{kg97} heterozygous (*P*=0.122, Mann-Whitney U) or homozygous (*P*=0.5529, Mann-
165 Whitney U) mutants compared to WT (Fig. 3g). Similarly, no significant difference in the average
166 escape time was found in *desmb*^{kg156} heterozygous (*P*=0.3968, Mann-Whitney U) or homozygous
167 (*P*=0.6131, Mann-Whitney U) mutants compared to WT (Fig. 3h). Since both *desma* and *desmb*
168 are expressed in somites up to this stage (48 hpf) and might potentially compensate for the absence
169 of each other, we finally evaluated the effect of the absence of both proteins in siblings from
170 *desma*$^{kg97/+}$;*desmb*$^{kg156/kg156}$ double mutants. No significant difference in the average escape time
171 was found in homozygous double mutants (*desma*$^{kg97/kg97}$;*desmb*$^{kg156/kg156}$) compared to
172 *desma*$^{+/+}$;*desmb*$^{kg156/kg156}$ (*P*=0.3165, Mann-Whitney U) and in *desma*$^{kg97/+}$;*desmb*$^{kg156/kg156}$
173 embryos compared to *desma*$^{+/+}$;*desmb*$^{kg156/kg156}$ (*P* = 0.2334, Mann-Whitney U) (Fig. 3i).

174

175 **Mutant adult skeletal muscle shows no muscle degeneration**

176 Because no skeletal muscle phenotype was observed in mutant larvae, surviving one-year-old
177 adults were investigated for muscle defects. Some homozygous single and double mutants
178 survived until adulthood with no sign of behavioral defect. Western blot was performed using
179 myotomal skeletal muscle protein extracts isolated from adult fish (WT, homozgous and
180 heterozygous *desma*^{kg97} mutants, homozygous and heterozygous *desmb*^{kg156} mutants and
181 *desma*$^{kg97/kg97}$;*desmb*$^{kg156/kg156}$ double mutants). As *desma* was shown to be predominantly
182 expressed in somites from 72 to 120 hpf, the two bands detected in WT and *desmb*^{kg156} mutants
183 were predicted to correspond to Desma-1 (predicted molecular weight 55.7 kDa) and Desma-2
184 (predicted molecular weight 54.1 kDa) isoforms. In agreement with that, both bands were absent
185 in *desma*^{kg97} homozygous mutant and double mutants while still expressed in homozygous
186 *desmb*^{kg156} mutant (Fig. 4a). Nevertheless, histopathological examination of hematoxylin-eosin
187 stained skeletal muscle tissue sections revealed no pathological changes in homozygous single or
188 double mutants (Fig. 4b). General morphology of skeletal fibre shape and size, integrity of
189 sarcoplasm was normal. There was an absence of cytoplasmic aggregates, signs of degeneration
190 or regeneration, internal nuclei, inflammatory cell infiltration, increase in connective or adipose
191 tissue in interstitium (Fig. 4b). We conclude that fish entirely lacking wild-type desmin generate
192 and maintain functional skeletal muscle in the context of a zebrafish aquarium.

193 **Altered calcium flux in *desma* mutant fibres**

194 Although anatomically apparently wild-type, adult *desma*$^{kg97/kg97}$ mutants were physiologically
195 defective in calcium handling. To investigate amplitude and time course of calcium signals
196 released in response to a depolarizing voltage stimulus, individual fibres were dissected from WT
197 (N=8), *desma*^{kg97} (N=11) or *desmb*^{kg156} (N=8) homozygous mutant 1-year-old fish. Calcium flux
198 along fibres was monitored in the isolated fibres by Fluo-4 AM after four consecutive depolarizing
199 stimuli with the amplitude of the current pulse kept constant at 100 nA and the duration increased
200 by 10 ms at each pulse (Fig. 5a-b). Amplitudes of the calcium emission signals in the first (10 ms)
201 stimulus and fourth (40 ms) stimulus were compared between mutants and WT. To eliminate
202 variance induced by amplitude, offset and fibre size differences, amplitude values were baseline
203 corrected and divided by the diameter of each fibre. Amplitudes of the first or the fourth calcium
204 transient were significantly lower in *desma*$^{kg97/kg97}$ fibres compared to WT fibres (P=0.0008 for

205 first transient; P=0.0025 for fourth transient, Mann-Whitney U) (Fig. 5c-d). In contrast, no
206 significant difference was found in the amplitude of calcium flux between *desmb*$^{kg156/kg156}$ fibres
207 and WT fibres (P=0.0881 for first transient; P=0.6657 for fourth transient, Mann-Whitney U) (Fig.
208 5c-d).

209 Time course of the longest calcium transient (40 ms, fourth transient) has been analyzed
210 for comparing the waveform of the responses and dissecting each phase of the transient. Signals
211 have been baseline corrected and then normalized. Mean amplitude values as a function of time
212 were compared between *desma*$^{kg97/kg97}$ and WT or *desmb*$^{kg156/kg156}$ and WT. No significant
213 difference was found in the rising and the plateau phases of the recorded transients between the
214 experimental groups (Figure 5e). However, the decay phase was significantly faster in
215 *desma*$^{kg97/kg97}$ mutant fibres compared to WT (* indicates P<0.05, repeated measures ANOVA,
216 Bonferroni post *hoc* test). No significant difference was found in *desmb*$^{kg156/kg156}$ mutant fibres
217 compared to WT (Fig. 5e).

218 DISCUSSION

219 We demonstrated differential expression of *desma* and *desmb* paralogs during zebrafish
220 development. Although previous studies showed that *desma* is the main gene expressed in somites,
221 no study did clearly distinguish the spatial and temporal differences in the expression of the two
222 genes. We showed that until 72 hpf *desma* and *desmb* expression overlap but later in development,
223 their expression diverges. While *desmb* expression shifts from somites to gut, *desma* remains as
224 the predominantly expressed paralog in somites. Note that the residual *desmb* expression detected
225 in the lateral edges of the myotome might reflect a transient expression in new muscle fibres[19].
226 Reduction of Desmin protein in adult *desma* mutants confirms that Desma is the major desmin
227 protein in adult zebrafish skeletal muscle. Desmb appears the major desmin in smooth muscle, at
228 least in the gut. So zebrafish *desmb* mutants may be particularly useful for understanding the role
229 of desmin in smooth muscle without interference by additional phenotypes from skeletal muscle.
230 In future studies, it will be necessary to take into account this divergent expression when studying
231 the role of desmin in zebrafish.

232 Reduced *desma* or *desmb* mRNAs in their respective mutants suggest the mRNAs are
233 degraded by nonsense-mediated decay, such that only tiny amounts of the severely truncated
234 proteins would be produced. Such fragments lack the highly conserved α-helical central rod

235 domain responsible for filament formation and all known protein-protein interaction domains of
236 Desmin[20]. Intriguingly, no overt phenotype was observed in some double homozygous mutants.
237 In contrast to desmin morphants where both *desma* and *desmb* were targeted with morpholinos [13],
238 we found that *desma*^{kg97} or *desmb*^{kg156} alleles have no effect on hatching of eggs, mortality rate and
239 length of larvae. While motility is altered in 48 hpf desmin morphants, no significant alteration
240 was detected in *desma*^{sa5} embryos[13,21]. By reasoning that this could be due to a compensation by
241 *desmb*, which is still expressed in somites at 48 hpf, we performed motility assays in both single
242 mutant and double mutant embryos but found no additional neuromuscular defect in larvae mutant
243 in single or both desmin genes. At the histological level, no sign of muscle degeneration or
244 disorganisation of fibres was observed in *desma*^{kg97} or *desm*b^{kg156} mutant skeletal muscles in either
245 embryos and adults. In contrast, the desmin morphants and *desma*^{sa5} mutant muscles of embryos
246 were disorganized, with disruption of sarcomeres[13,14]. Our findings raise the possibility that a
247 haploinsufficient dominant effect in *desma*^{sa5} mutants may disrupt muscle; such an explanation
248 would no account for the morphant phenotype. We conclude that at least two further alleles of
249 *desma* may be needed to resolve these differences: firstly, a deletion allele that would
250 simultaneously prevent any Desma polypeptide synthesis and any RNA-triggered compensation
251 and, secondly, an allele lacking morpholino binding ability but nevertheless encoding wild-type
252 protein to prove that the morphant phenotype is due to *desma* binding.

253 We found that the amplitude of calcium signals released in response to a depolarizing
254 current stimulus applied to fibres was significantly lower in *desma*^{kg97} compared to WT. In
255 addition, while rising and plateau phases of calcium signals were similar between all groups, the
256 decay phase was shorter in *desma*^{kg97} mutants compared to WT. These results indicate that
257 mechanisms regulating cytosolic calcium are altered and fibres are not efficient in generating a
258 long lasting calcium increase in the absence of desmin. These results are concordant with calcium
259 flux defects observed in desmin mutant mice and hearts of *desma*^{sa5} embryos[14]. Our data support
260 the idea that desmin has a similar role in calcium conduction in skeletal muscle fibres and suggest
261 that some aspects of the *desma*^{sa5} phenotype may reflect loss of function of Desma.

262 Although not the focus of this study, it should be noted that heart and gastroinstestinal
263 development in mutant lines have not been extensively analyzed. No heart oedema such as
264 observed in desmin morphant or overt morphological abnormality in the gut have been observed
265 in *desma*^{kg97}, *desmb*^{kg156} or double mutant embryos and adults.

266 Many loss-of-function mutants not exhibiting an overt phenotype and failing to phenocopy
267 morphants have been previously reported[22–24]. El-Brolosy et al. proposed transcriptional
268 adaptation triggered by mutant RNA degradation as a mechanism to explain this discrepancy[15,25].
269 According to this model, decay of the mutant mRNA results in upregulation of related genes,
270 alleviating or suppressing the phenotype observed in morphants. Such adaptation could explain
271 the milder phenotype observed in mutants created in our study compared to desmin morphants.
272 However, it has been shown that *desma*^{sa5} allele results in a muscle phenotype similar to that of
273 morphants, even though the *desma*^{sa5} allele predicts an in-frame stop codon and no *desma* mRNA
274 was detected in mutants[14,21]. We also observe a severe reduction in *desma* mRNA in our *desma*^{kg97}
275 mutant. The specific mechanism leading to the absence of mRNA was not investigated either in
276 *desma*^{sa5} mutants or in the present study. It is possible that the *desma*^{sa5} allele is not as efficient as
277 *desma*^{kg97} allele in triggering an adaptation mechanism for yet unknown reasons. However, our
278 data suggest that the compensating gene in fish carrying our *desma* allele is not *desmb*, the only
279 gene known to have high and extensive homology to *desma*. It will be interesting to compare these
280 models in the contexts of mRNA decay and transcriptional adaptation. Alternatively, short
281 truncated polypeptides not detected in Western blot might play a role in this process before being
282 rapidly degraded. In conclusion, unraveling modifier genes and proteins that compensate for the
283 loss of desmin in our models will bring further insights in the role of desmin in muscle.

284 # METHODS

285 **Ethical approval and zebrafish maintenance**

286 All animal procedures were approved by the Hacettepe University Animal Experimentations Local
287 Ethics Board (2014/07-08). Adult zebrafish (AB) (*Danio rerio*) were kept in 14/10 hr light-dark
288 cycle at 28.5ºC. Adults are fed twice a day, with dry feed in morning and *Artemia spp.* in evening.
289 For spawning, male and female fish were placed in a breeding tank with a separator. Next morning
290 the separator was removed, viable eggs were collected and rinsed in E3 medium (5 mM NaCl, 0.17
291 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 10^{-5}% Methylene Blue). Embryos were kept in E3
292 medium for five days and placed into adult tanks containing system water. Embryos were fed five
293 times a day with dry feed and Artemia.

294 **Whole mount *in situ* hybridization**

295 Embryos were fixed in 4% PFA in PBS (w/v) overnight at 4°C, dehydrated in 50% and %100
296 methanol washes and stored at -20°C at least overnight before use. Probe templates were amplified
297 from cDNA isolated from 96 hpf wild-type embryos. Reverse primers for antisense probe
298 contained T3 promoter sequence while forward primers for sense probes contained T7 promoter
299 sequence. Primers sequences can be found as Supplementary Table S1 online. Digoxigenin-
300 labelled probes were generated using T3 or T7 RNA polymerases. Whole mount *in situ* mRNA
301 hybridization was performed as described[26]. Embryos were photographed as on Zeiss Axiophot
302 with Axiocam (Carl Zeiss, Oberkochen, Germany) using Openlab software (Agilent, Santa Clara,
303 CA, USA).

304 **Generation of mutant zebrafish lines**

305 CRISPR-Cas9 genome editing was performed as described in Fin et al[24]. CRISPR-Cas9 sgRNAs
306 showing minimal off-target sites were designed by using the online software ZiFiT Targeter.
307 Oligos encoding sgRNAs targeting the first exon of *desma* (GRCz11, Chr9: 7539113-7539132,
308 GGTCACCTCGTAAACTCTGG) and *desmb* (GRCz11, Chr6: 13891417-13891437,
309 GGCTATACTCGCTCTTATGG) were cloned into pDR274 plasmid (Addgene). sgRNAs were
310 synthesized using T7 RiboMAX Large Scale RNA production system (Promega) following the
311 manufacturer's protocol. Transcribed sgRNAs were purified using sodium acetate and ethanol
312 precipitation and quantified on Qubit fluorometer. Cas9 mRNA was transcribed from pCS2-Cas9
313 plasmid using mMessage mMachine SP6 Kit (Ambion) and purified by lithium chloride
314 precipitation. One-cell stage embryos were injected with 2 nl containing 80-200 pg of sgRNA and
315 100 pg of Cas9 mRNA. At 48 hpf, ten embryos were analysed for mosaicism at targeted loci using
316 high resolution melt analysis. Injected embryos were raised to adulthood and back-crossed to
317 identify transmitted mutations in F1 progeny. Homozygotes for each gene were generated by in-
318 cross of F2 heterozygotes. Primers sequences used for genotyping by Sanger sequencing can be
319 found as Supplementary Table S1 online.

320 **Quantitative real-time PCR**

321 Total RNA was isolated from a pool of 20 mechanically homogenized embryos at 96 hpf for each
322 sample (n=8) using TRItidy G (AppliChem) and 500 ng of cDNA was synthesized using
323 QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturer's protocol. Primer

324 sequences for *desma* (covering both *desma-1* and *desma-2*), *desmb* and *actb1* can be found as
325 Supplementary Table S1 online. qPCR was performed in triplicates using SensiFAST SYBR No-
326 ROX Kit (Bioline) in Rotor-Gene 6000 (Corbett Life Science). *Desma* and *desmb* expression
327 levels were normalized to *actb1* expression, mutant expression levels were calculated relative to
328 wild-type using ΔΔCt method[27].

329 **Protein Extraction and Immunoblotting**

330 For protein extraction, 1-year-old fish were euthanized using overdose of tricaine (MS222, 300
331 mg/l). Skeletal muscle tissue was immediately dissected and fresh-frozen in liquid nitrogen. Tissue
332 was pulverized and homogenized in blending buffer (16 M Tris HCl, 200 M EDTA, 20% SDS,
333 1X protease inhibitor cocktail, volume adjusted to 5 ml with dH_2O) by sonication on ice. Lysates
334 were then centrifuged and supernatant transferred in a fresh tube. Total protein concentrations were
335 determined by bicinchoninic acid Protein Assay (Pierce, 23225). Equal amounts of protein (40 µg)
336 were loaded on a 13% polyacrylamide gel and transferred onto a nitrocellulose membrane using
337 semi-dry transfer, blocked for one hour at room temperature (%5 nonfat dried milk in %0,2 TBS-
338 T) and probed with anti-desmin antibody (1:1000; Sigma, D8281) overnight at 4ºC or anti-lamin
339 B1 antibody (Abcam, ab90169) followed by appropriate HRP-conjugated secondary antibody.
340 Chemiluminescence detection was done by using SuperSignal™ West Femto Maximum Sensivity
341 substrate (Thermo Scientific, 34095) in GeneGnome device.

342 **Whole-mount Immunofluorescence**

343 96 hpf embryos were fixed in %4 PFA overnight at +4ºC and washed with PBTx (%0.01 Triton
344 X-100 in PBS). Embryos were permeabilized in 50 µg/ml Proteinase K for 2 hours and re-fixed in
345 2% PFA for 20 minutes at room temperature. Embryos were blocked in %5 BSA for 2 h at room
346 temperature and incubated with anti-desmin antibody (1:20; Sigma, D8281) for two overnights.
347 After two overnight incubations with Alexa-Fluor 488 conjugated secondary antibody, embryos
348 were mounted with DAPI onto glass bottom dishes for imaging with Zeiss LSM Pascal laser
349 scanning confocal microscope.

350 **Larval Phenotype and Morphology**

351 After successful mating, embryos were collected, counted and recorded for the following rates.
352 Hatching rate was reported as cumulative percentage of hatched embryos at 24 hpf, 48 hpf and 72

353 hpf. Mortality rate was expressed as the percentage of death embryos for 5 dpf. For body length
354 measurements, at least 16 embryos at 96 hpf were mounted, photographed (The Imaging Source,
355 DFK 41AU02) and measured from the mouth tip to the tail base using ImageJ (Version 1.49u).
356 Values for body length were presented as mean body length in cm. All the experiments except
357 body length measurements (three times) were performed at least eight times.

358 **Phalloidin Staining**

359 Phalloidin staining was performed on 96 hpf embryos (N=8) as described[28]. Following fixation in
360 4% PFA and permeabilization with 50 µg/ml Proteinase K, embryos were incubated in Alexa-
361 Fluor 488 conjugated Phalloidin (Invitrogen, A12379) overnight at +4ºC. Embryos were then
362 mounted onto glass bottom dishes and photographed with Zeiss LSM Pascal laser scanning
363 confocal microscope. Somite lengths were measured by using ImageJ (Version 1.49u).

364 **Hematoxylin Eosin Staining**

365 Adult zebrafish were freshly frozen in methyl-butanol cooled down in liquid nitrogen and then
366 taken into O.C.T compound for sectioning. Tissue sections were incubated in hematoxylin for 5
367 minutes, 1% acid-alcohol solution for 1 minute, 1% ammoniac for 1 minute, eosin solution for 3
368 minutes respectively by washing with distilled water in between steps. Finally, sections were
369 washed with alcohol and xylene, and mounted. Sections were scanned with Nikon Eclipse E-400
370 microscope and photographed by DXM 1200F digital camera.

371 **Motility Assay**

372 For motility assay 48 hpf embryos from in-crosses of *desma*$^{kg97/+}$, *desmb*$^{kg156/+}$ or
373 *desma*$^{kg97/+}$;*desmb*$^{kg156/kg156}$ adults were used. Motility assays were performed by previously
374 described protocol[18]. A petri dish was placed on a transparent sheet with concentric circles 5 mm
375 apart. One embryo at a time was positioned in the centre of the dish and stimulated using a
376 dissection needle. The escape response of the embryo was recorded using a high-speed camera
377 (Huawei, Mate 10 Lite, 16 MP, 120 fps). The movements of the embryo—midpoint between the
378 two eyes—were tracked using a custom software that works based on template matching
379 algorithm. The elapsed time between the last frame before touch stimulus and the first frame after
380 the body of the fish contacts the 10 mm circle was computed from the digitized embryo movements

381 with a resolution of 8.3 ms (120 fps). Experiments were performed blindly and after video
382 accusation, DNA was extracted from each embryo for genotyping.

383 **Calcium Flux**

384 1-year-old fish were euthanized using overdose of tricaine (MS222, 300 mg/l). After removal of
385 the skin, tissue samples were incubated into 5 mg/ml collagenase solution in PBS (without Ca^{2+}
386 and Mg^{2+}) at 28.5 °C for 20 minutes on a shaker at 50 rpm. Samples have been triturated gently
387 under dissecting microscope in order to dissociate muscle fibres from the tissue components until
388 rode-shaped muscle fibres were obtained. Dissociated fibres were incubated in DMEM containing
389 10% FBS and 1% antibiotic-antimycotic overnight at 28.5 °C and 5% CO_2 environment. On the
390 following day, fibres were incubated in 2.5 μM Fluo-4 AM for 20 minutes in 5% CO_2. Under a
391 laser scanning confocal microscope (Zeiss LSM Pascal), glass microelectrodes (filled with 3M
392 KCl) were inserted into muscle fibres in a current clamp mode[29]. A depolarizing current stimulus
393 was delivered through the microelectrode to excite the fibre and evoke a calcium signal. The
394 evoked calcium-specific fluorescent emission signal was recorded in "Line Scan" mode of
395 confocal microscope, supplying information about a line cross sectioning the muscle fibre at a high
396 frequency in tandem with the electrical recordings. Data were analyzed in MATLAB (The
397 MathWorks, Inc). The stimulus waveform, which consisted of four consecutive pulses increasing
398 in duration, was used in all the experiments. The amplitude of the current pulse was kept constant
399 at 100 nA while the durations were 10 ms, 20 ms, 30 ms, 40 ms respectively. Experiments were
400 done by using three WT, three *desma*$^{kg97/kg97}$ and two *desmb*$^{kg156/kg156}$ fish and at least eight fibres
401 from each group were recorded.

402 ## Statistics

403 Data were statistically analyzed by GraphPad Prism 8 (GraphPad Software Inc.) by nonparametric
404 Mann-Whitney U test (Two-tailed) when two groups were compared. The results were considered
405 significant when $P<0.05$. For analysis of time course experiments (hatching rate, mortality rate
406 and time course calcium flux), repeated measures two-way ANOVA was used and Bonferroni post
407 *hoc* test was used for multiple comparisons. All error bars were presented as mean±SEM and bars
408 were represented as median.
409

Acknowledgements

This work was supported by The Scientific and Technological Research Council of Turkey, Project no. 214S174 to P.R.D.

Competing interests

The author(s) declare no competing interests.

Author Contributions

P.R.D. designed, P.R.D. and S.M.H. supervised the study. G.K.K. wrote the paper, E.K.M. made contribution to writing the paper and assembled the figures. G.K.K., E.K.M, C.K., S.U., B.S., B.E., I.U., N.D., B.T. and N.P. performed the experiments and/or analyzed the results. All authors interpreted the data and edited the manuscript.

Data Availability

Supporting information is available in Supplementary files and further information is available from the corresponding author upon request.

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499

500 ## Figure Legends

501 **Fig. 1. Whole mount *in situ* mRNA hybridisation of embryos at the indicated stages for**
502 **antisense probes to *desma* and *desmb*.** (**a**) Lateral views are anterior to top, dorsal to left for 13-
503 22 hpf; anterior to left except 11 hpf which is a dorsal view. 48-120 hpf whole mounts are anterior
504 to left, dorsal to top. Scale bar: 250 µm. (**b**) Arrows indicate heart in frontal view for left panel
505 (*desma*) and ventral view for right panel (*desmb*) of 35 hpf embryos. (**c**) Transversal sections where
506 dorsal is top. (**d**) Upper left panel is a lateral view at 96 hpf, scale bar: 250 µm. Dashed line
507 represents the position of the transversal section in upper right panel where dorsal is top, scale bar:
508 100 µm. Lower panels are lateral views of zebrafish heads at 72 and 120 hpf treated with *desma*
509 probe. Scale bar: 100 µm. (**e**) Left panel is a ventral view with anterior at top. Right panels are
510 lateral views. Ai, anterior intestine; eom, extraocular muscles; fast, fast muscles; fm, pectoral fin
511 muscles; hm, hypaxial muscles; mdm, mandibular muscles; om, opercular muscles; slow, slow
512 muscles; som, somites. Scale bar: 250 µm.

513 **Fig. 2. Generation of *desma* and *desmb* knockout lines. (a**) Alignments and chromatograms of
514 wild-type DNA sequences with mutant alleles, and predicted mutant polypeptide sequences. In
515 DNA sequences, yellow highlights gRNA target sequence, hyphens show deleted bases, inserted
516 bases are indicated in red font. In protein sequences, the first residue affected by the frameshift is
517 indicated in red font, asterisks represent early stop codons. (**b**) Quantitative real-time PCR results
518 showing the expression of *desma* mRNA (upper graphic) and *desmb* mRNA (lower graphic) in
519 wild-type and homozygous mutant 96 hpf embryos (N=5). A significant decrease of *desma*
520 transcripts in $desma^{kg97}$ homozygous embryos (P=0.0119, Mann-Whitney U) was observed while
521 *desmb* transcripts were significantly decreased in $desmb^{kg156}$ homozygous embryos at 96 hpf
522 compared to WT (P=0.0079, Mann-Whitney U). (**c**) Left panel shows whole mount *in situ* mRNA
523 hybridisation of $desma^{kg97}$ homozygous embryos at the indicated stages for antisense probes to
524 *desmb*. Right panel shows whole mount *in situ* mRNA hybridisation of $desmb^{kg156}$ homozygous
525 embryos at the indicated stages for antisense probes to *desma.* Scale bar: 250 µm. Ai, anterior
526 intestine; som, somites. (**d-f**) Whole mount immunofluorescence staining of desmin with an anti-
527 desmin polyclonal antibody (Sigma, D8281) recognizing both Desma and Desmb, in wild-type
528 and mutant 96 hpf embryos. Scale bar: 500 µm. Boxes with dashed lines delineate areas that were

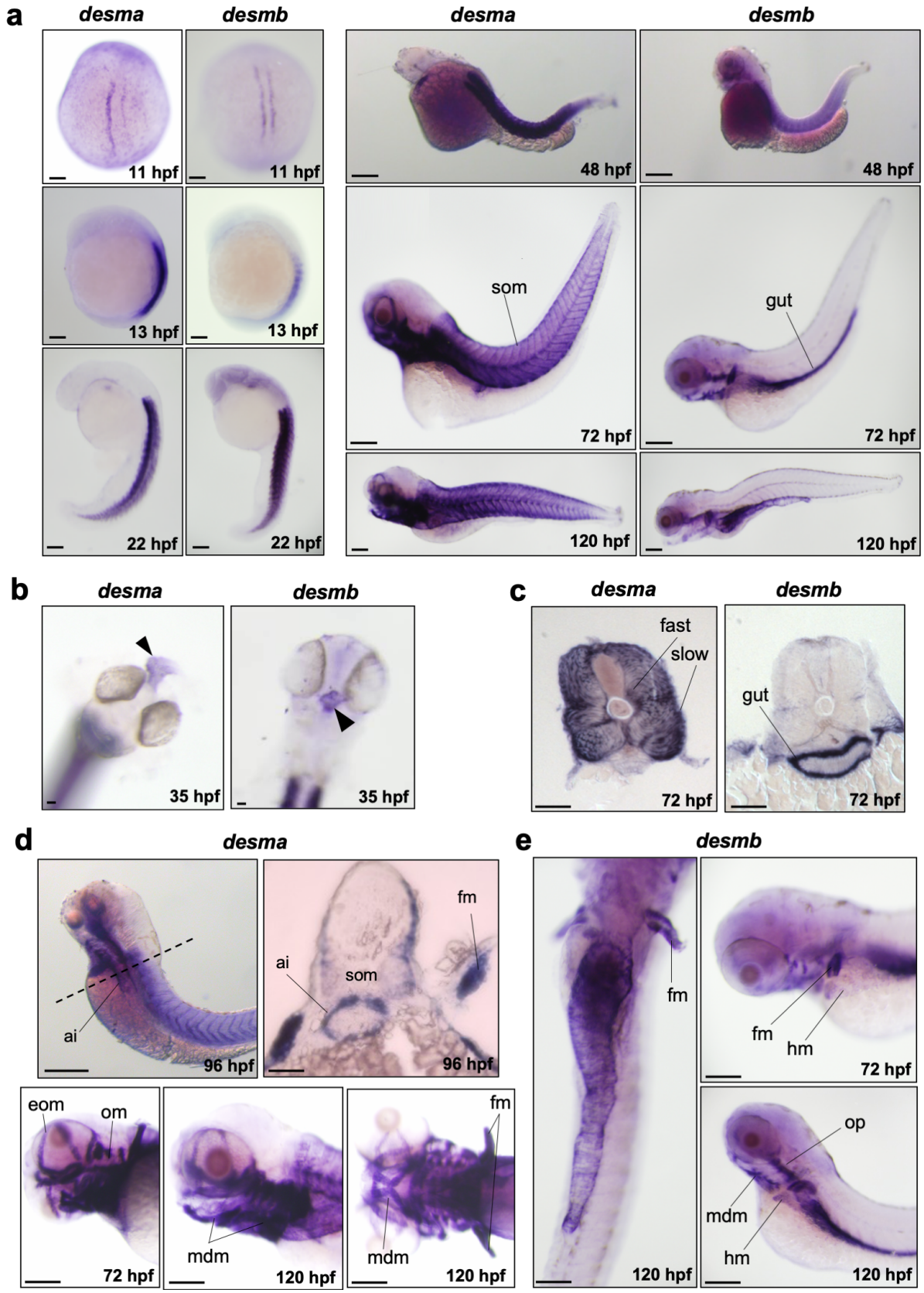
529 zoomed and focused on intestine. Pictures showing DAPI staining of each embryo are included at
530 their bottom right. (**d**) Upper panel is lateral view of a WT embryo with anterior to left, dorsal to
531 top. Lower panel is ventral view with anterior to left, arrowheads indicate the gut. (**e**) Upper panels
532 are lateral views of *desmb*$^{kg156/kg156}$ embryo with anterior to left, dorsal to top. Lower left panel
533 shows anterior intestine (orange arrowheads), lower right panel shows middle and posterior
534 intestine (white arrowheads). (**f**) Lateral view of *desma*$^{kg97/kg97}$ with anterior to left, dorsal to top.

535 **Fig. 3. Evaluation of neuromuscular defect in mutant larvae.** (**a)** The number of viable embryos
536 after successful mating of WT fish (n=10) compared to homozygous *desma*^{kg97} (n=10) (*P*=0.6842,
537 Mann-Whitney U) or homozygous *desmb*^{kg156} (n=8) (*P*=0.9654, Mann-Whitney U) fish. (**b)**
538 Comparison of hatching rate between homozygous mutants and WT 24, 48 and 72 hpf embryos
539 At 72 hpf, no significant difference in the hatching rate was found between mutants and WT
540 (*desma*$^{kg97/kg97}$ vs. WT, *P*=0.9902; *desmb*$^{kg156/kg156}$ vs WT, *P*=0.3186, repeated measures two-way
541 ANOVA, Bonferroni post *hoc* test) (**c)** Body length of 96 hpf WT (N=18) and homozygous
542 *desma*^{kg97} (N=25) or homozygous *desmb*^{kg156} (N=16) mutant embryos (*P*=0.2036 for WT vs.
543 *desma*$^{kg97/kg97}$; *P*=0.1893 for WT vs. *desmb*$^{kg156/kg156}$, Mann-Whitney U). (**d)** Cumulative mortality
544 rate from 1 to 5 dpf homozygous *desma*^{kg97} mutants (N=1247) compared to WT (N=2460)
545 (*P*=0.1287, repeated measures two-way ANOVA, Bonferroni post *hoc* test) and homozygous
546 *desmb*^{kg156} mutants (N=2615) compared to WT (*P*=0.6239, repeated measures two-way ANOVA,
547 Bonferroni post *hoc* test). **e.** Optical sections of the mid-trunk region of WT and homozygous
548 mutants 96 hpf embryos stained with phalloidin (N=8). Scale bars: 50 µm. (**f)** Somite lengths (µm,
549 N=8) were measured from optical sections of WT and mutant embryos stained with phalloidin
550 (*P*=0.7912 for WT vs. *desma*$^{kg97/kg97}$; *P*=0.4923 for WT vs. *desmb*$^{kg156/kg156}$, Mann-Whitney U). (**g-**
551 **i)** Touch-evoked escape time (ms) of 48 hpf embryos. Motility experiments were performed blind
552 on siblings from (**g**) *desma*$^{kg97/+}$, (**h**) *desmb*$^{kg156/+}$ or (**i**) *desma*$^{kg97/+}$;*desmb*$^{kg156/kg156}$ in-crosses
553 followed by *post hoc* genotyping. g. Heterozygous *desma*^{kg97} embryos (N=23) were compared to
554 WT (N=9) (P=0.122, Mann-Whitney U) and *desma*$^{kg97/kg97}$ (N=15) compared to WT (P=0.5529,
555 Mann-Whitney U). h. Heterozygous *desmb*^{kg156} embryos (N=6) were compared to WT (N=38)
556 (P=0.3968, Mann-Whitney U) and *desmb*$^{kg156/kg156}$ (N=6) compared to WT (P=0.6131, Mann-
557 Whitney U). i. Homozygous double mutants (N=8) were compared to *desma*$^{+/+}$;*desmb*$^{kg156/kg156}$
558 (n=15) (*P*=0.3165, Mann-Whitney U) and *desma*$^{kg97/+}$;*desmb*$^{kg156/kg156}$ embryos (n=34) compared
559 to *desma*$^{+/+}$;*desmb*$^{kg156/kg156}$ (*P*=0.2334, Mann-Whitney U).

560 **Figure 4**. **Desmin protein expression and histological examination of adult skeletal muscle.**
561 **(a)** Immunoblotting of skeletal muscle protein extracts of WT, *desma*$^{kg97/kg97}$, *desma*$^{kg97/+}$,
562 *desmb*$^{kg156/kg156}$, *desmb*$^{kg156/+}$ and *desma*$^{kg97/kg97}$*;desmb*$^{kg156/kg156}$ double mutant fish, showing
563 expression of putative Desma-1 (55.7 kDa) and Desma-2 (54.1 kDa) isoforms. Lamin B1 was
564 labeled as loading control. (**b)** Transversal cryosections of mid-trunk somites from WT and
565 homozygous single and double mutant adults stained with hematoxylin eosin showing no
566 pathological changes. Scale bar: 250 µm.

567 **Fig. 5. Calcium flux in isolated fibres.** (**a**) Calcium flux along fibres was monitored in vivo by
568 Fluo-4 AM after four consecutive depolarizing stimuli. Top panel, membrane potential responses
569 to current stimulus pulses with varying durations of 10, 20, 30, 40 ms with the amplitude of the
570 current pulse kept constant at 100 mV and the duration increased by 10 ms at each pulse. Middle
571 panel, evoked calcium transients in line scan mode (*tx*) to current stimuli. Bottom panel, integrated
572 emission signal as a function of time for each experimental group. (**b**) Membrane potential
573 response to the first stimulus of 10 ms (top) or to the fourth stimulus of 40 ms. (**c**) Baseline
574 corrected amplitude divided by fibre diameter (ΔF/µm) of the calcium emission signals during the
575 first (10 ms) stimulus and fourth (40 ms) stimulus were compared between mutants and WT. (**e**)
576 Time course analysis of the baseline corrected and normalized amplitude of the calcium transient
577 from the longest stimulus (40 ms) was represented as mean amplitude values as a function of time.
578 Homozygous *desma*^{kg97} fibres (N=11, red curve) or homozygous *desmb*^{kg156} fibres (N=8, orange
579 curve) were compared to WT (N=8, blue curve). (* indicates time points where $P<0.05$, repeated
580 measures two-way ANOVA, Bonferroni post *hoc* test).

Figure 1.


a
desma
desmb
desma
desmb
11 hpf
11 hpf
48 hpf
48 hpf
13 hpf
13 hpf
som
gut
22 hpf
22 hpf
72 hpf
72 hpf
120 hpf
120 hpf
b
desma
desmb
35 hpf
35 hpf
c
desma
desmb
fast
slow
gut
72 hpf
72 hpf
d
desma
ai
ai
som
fm
96 hpf
96 hpf
eom
om
mdm
mdm
fm
72 hpf
120 hpf
120 hpf
e
desmb
fm
fm
hm
op
mdm
hm
120 hpf
72 hpf
120 hpf

581

Figure 2.

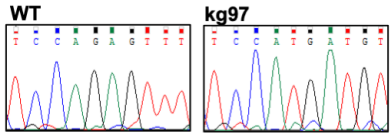
a

desma

```
WT    CCTCAGGTTCCTCTGGCTCCTCAAGACTGACCTCCAGAGTTTACGAGGTGACCAAGAG
kg97  CCTCAGGTTCCTCTGGCTCCTCAAGACTGACCTCCA--TGATGTTTACGAGGTGACCA   -2+4
```

Protein sequence

```
WT    MSTKYSASAESASSYRRTFGSGLGSSIFAGHGSSGSSGSSRLTSRVYEVTKSSASPHF
kg97  MSTKYSASAESASSYRRTFGSGLGSSIFAGHGSSGSSGSSRLTSMMFTR*
```

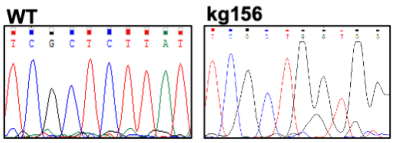


desmb

```
WT    CTCCTACAGCATGCCCAACTTGTCTGCAGGCTATACTCGCTCTTATGGTGGGATGGGT
kg156 CTCCTACAGCATGCCCAACTTGTCTGCAGGCTATACTCGCT-----GGTGGGATGGGT   -5
```

Protein sequence

```
WT    MSHSYATSSSSSYRRMFGGPGYMTP...PGFSGYRASSYSMPNLSAGYTRSYGGMGE
kg156 MSHSYATSSSSSYRRMFGGPGYMTP...PGFSGYRASSYSMPNLSAGYTRWWDG*
```



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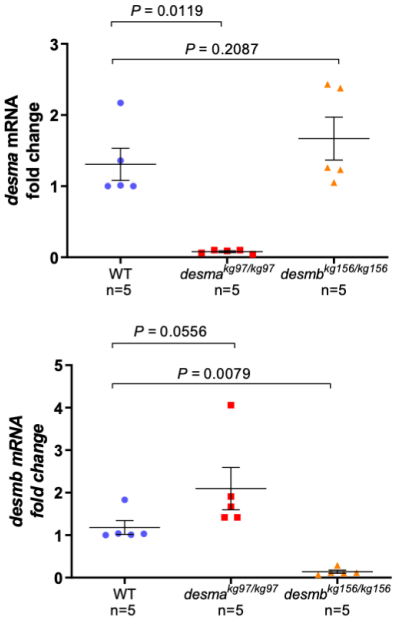
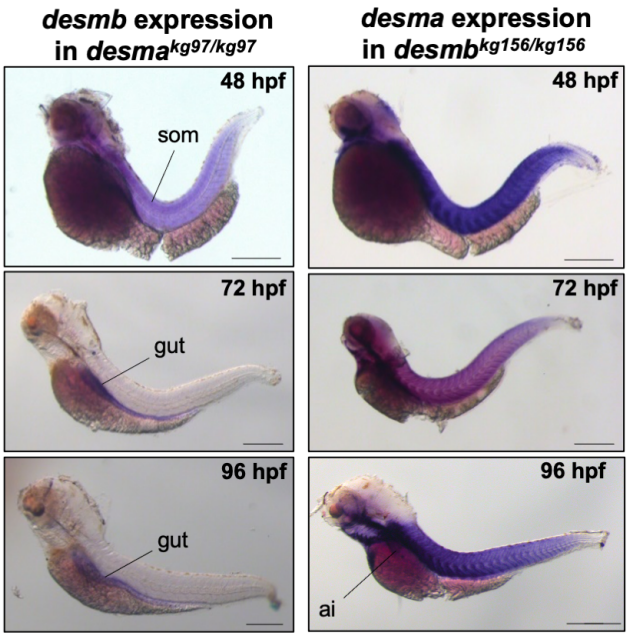


c



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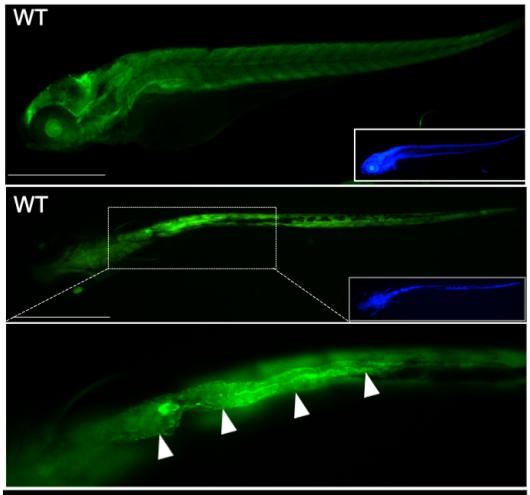

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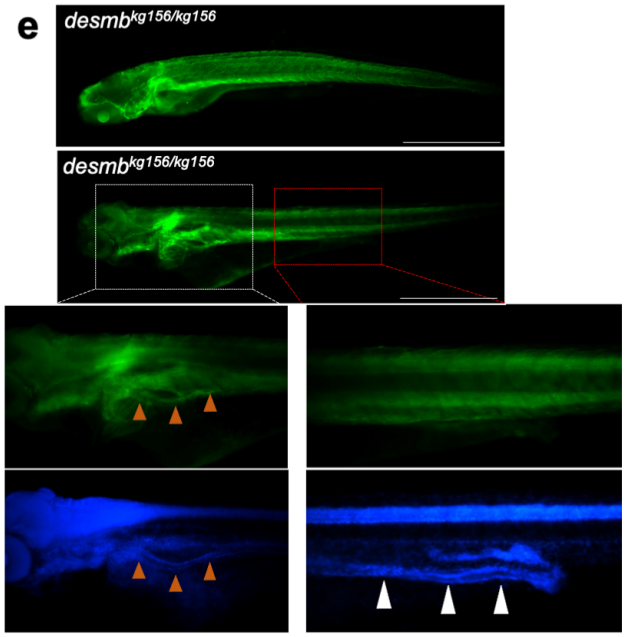
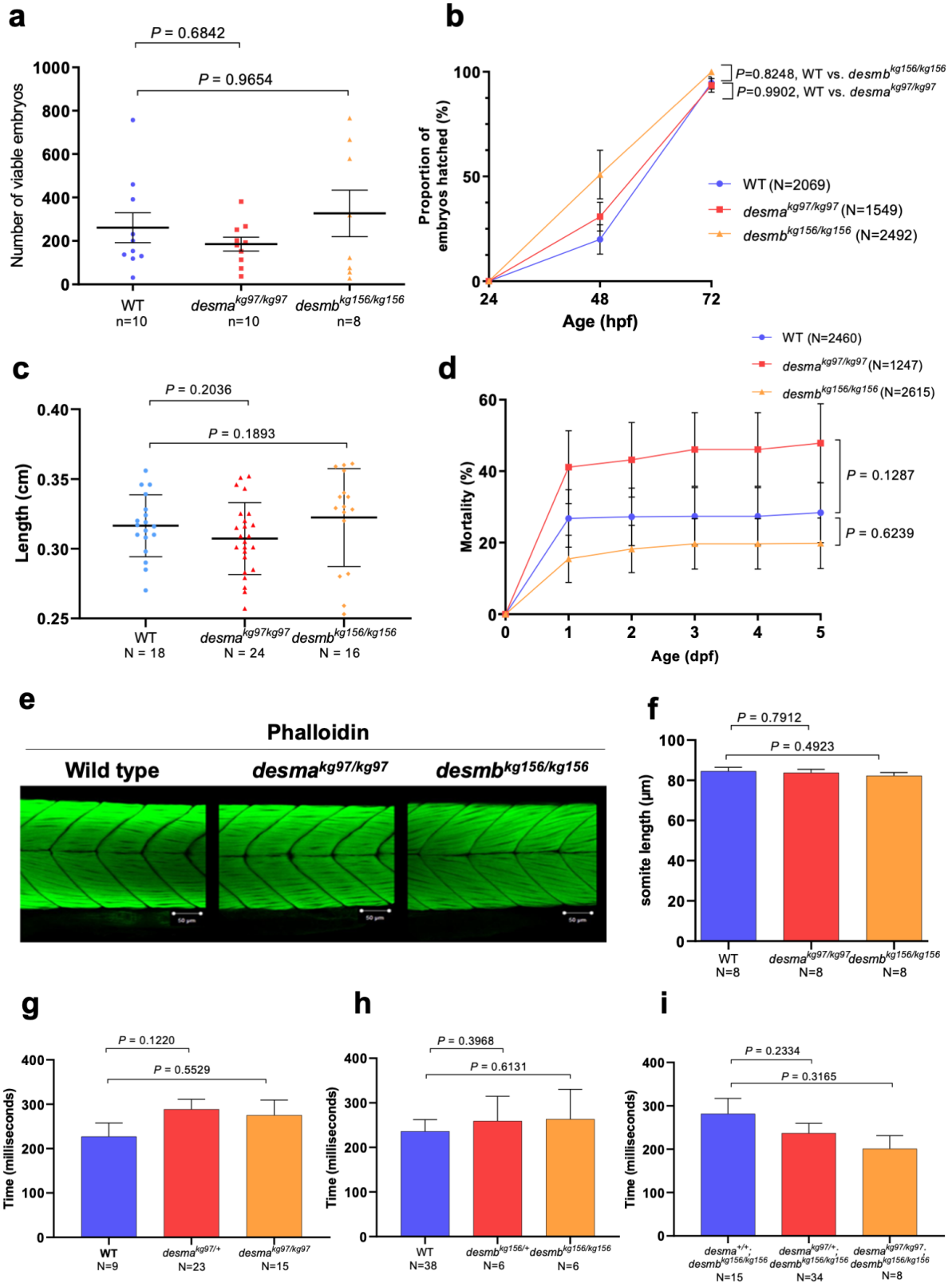


f

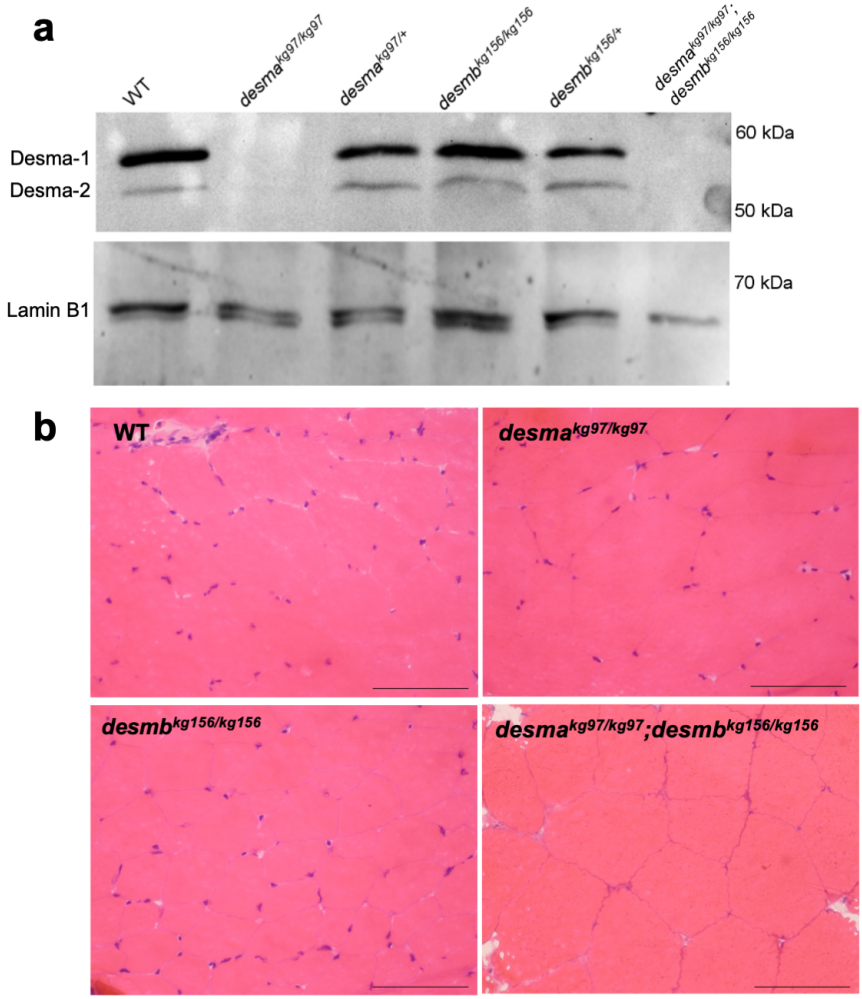
582

Figure 3.


a
P = 0.6842
P = 0.9654
Number of viable embryos
1000
800
600
400
200
0
WT n=10
desma kg97/kg97 n=10
desmb kg156/kg156 n=8
b
P=0.8248, WT vs. desmb kg156/kg156
P=0.9902, WT vs. desma kg97/kg97
Proportion of embryos hatched (%)
100
50
0
24
48
72
Age (hpf)
WT (N=2069)
desma kg97/kg97 (N=1549)
desmb kg156/kg156 (N=2492)
c
P = 0.2036
P = 0.1893
Length (cm)
0.40
0.35
0.30
0.25
WT N = 18
desma kg97kg97 N = 24
desmb kg156/kg156 N = 16
d
WT (N=2460)
desma kg97/kg97 (N=1247)
desmb kg156/kg156 (N=2615)
Mortality (%)
60
40
20
0
P = 0.1287
P = 0.6239
0
1
2
3
4
5
Age (dpf)
e
Phalloidin
Wild type
desma kg97/kg97
desmb kg156/kg156
f
P = 0.7912
P = 0.4923
somite length (µm)
100
80
60
40
20
0
WT N=8
desma kg97/kg97 N=8
desmb kg156/kg156 N=8
g
P = 0.1220
P = 0.5529
Time (milliseconds)
400
300
200
100
0
WT N=9
desma kg97/+ N=23
desma kg97/kg97 N=15
h
P = 0.3968
P = 0.6131
Time (milliseconds)
400
300
200
100
0
WT N=38
desmb kg156/+ N=6
desmb kg156/kg156 N=6
i
P = 0.2334
P = 0.3165
Time (milliseconds)
400
300
200
100
0
desma +/+; desmb kg156/kg156 N=15
desma kg97/+; desmb kg156/kg156 N=34
desma kg97/kg97; desmb kg156/kg156 N=8

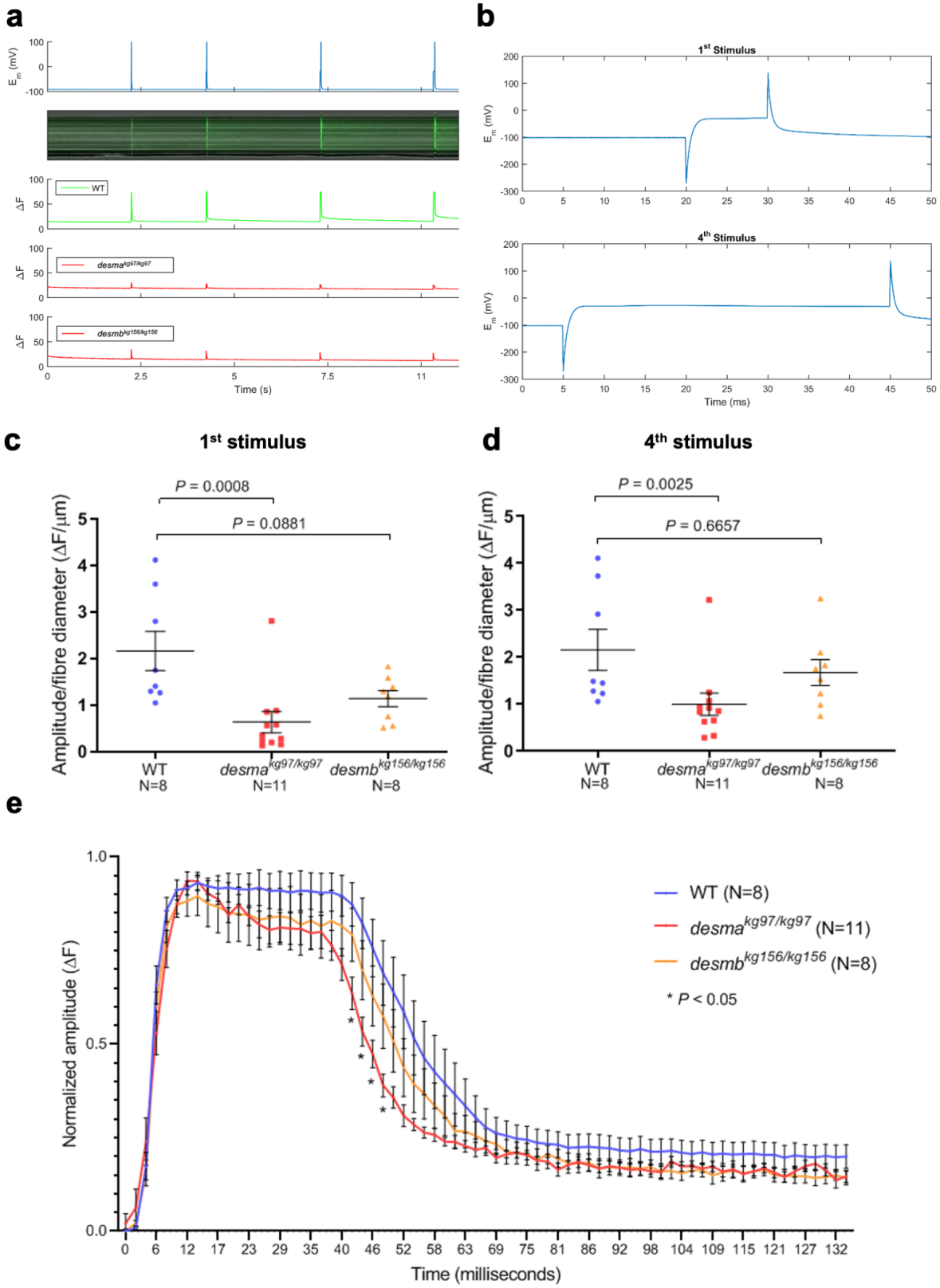
583

Figure 4.


a
WT
desma kg97/kg97
desma kg97/+
desmb kg156/kg156
desmb kg156/+
desma kg97/kg97 ; desmb kg156/kg156
Desma-1
Desma-2
60 kDa
50 kDa
Lamin B1
70 kDa
b
WT
desma kg97/kg97
desmb kg156/kg156
desma kg97/kg97 ;desmb kg156/kg156

584

Figure 5.


a
b
1st Stimulus
4th Stimulus
Time (s)
Time (ms)
c
1st stimulus
d
4th stimulus
P = 0.0008
P = 0.0881
P = 0.0025
P = 0.6657
Amplitude/fibre diameter (ΔF/μm)
WT N=8
desmakg97/kg97 N=11
desmbkg156/kg156 N=8
e
Normalized amplitude (ΔF)
Time (milliseconds)
WT (N=8)
desmakg97/kg97 (N=11)
desmbkg156/kg156 (N=8)
* P < 0.05

585