

1 **Early life lipid overload in Native American myopathy is**
2 **phenocopied by *stac3* knock out in zebrafish**

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22

23 **Abstract**

24 Understanding the early stages of human congenital myopathies is critical for proposing
25 strategies for improving skeletal muscle performance by the functional integrity of cytoskeleton.
26 SH3 and cysteine-rich domain 3 (Stac3) is a protein involved in nutrient sensing, and is an
27 essential component of the excitation-contraction (EC) coupling machinery for Ca^{2+} releasing. A
28 mutation in *STAC3* causes debilitating Native American myopathy (NAM) in humans, and loss of
29 this gene in mice and zebrafish resulted in death in early life. Previously, NAM patients
30 demonstrated increased lipids in skeletal muscle biopsy. However, elevated neutral lipids could
31 alter muscle function in NAM disease via EC coupling apparatus is yet undiscovered in early
32 development.

33 Here, using a CRISPR/Cas9 induced *stac3* knockout (KO) zebrafish model, we determined that
34 loss of *stac3* led to muscle weakness, as evidenced by delayed larval hatching. We observed
35 decreased whole-body Ca^{2+} level at 5 days post-fertilization (dpf) and defects in the skeletal
36 muscle cytoskeleton, i.e., F-actin and slow muscle fibers at 5 and 7 dpf. Homozygous larvae
37 exhibited elevated neutral lipid levels at 5 dpf, which persisted beyond 7 dpf. Myogenesis
38 regulators such as *myoD* and *myf5*, were significantly altered in *stac3*^{-/-} larvae at 5 dpf, thus a
39 progressive death of the KO larva by 11 dpf.

40 In summary, the presented findings suggest that *stac3*^{-/-} can serve as a non-mammalian model
41 to identify lipid-lowering molecules for refining muscle function in NAM patients.

42

43 **Keywords:** Native American myopathy, zebrafish, cytoskeleton of muscle, muscle weakness,
44 neutral lipids.

45

46 **Introduction**

47 The musculoskeletal system provides a unique framework for bone, skeletal muscle, and
48 connective tissue for enabling mobility and withstanding mechanical load generated in daily
49 activities of human life. Skeletal muscle (~40% in the human body mass) serves to provide
50 anatomical support for the bones/internal organs, to produce contractions and to store energy for
51 locomotion, all essential structures and processes for the survival of an organism¹. Defects in the
52 genetic makeup or metabolic activity of skeletal muscle result in dystrophy, myopathy, cachexia
53 and/or sarcopenia. Among these muscle diseases, myopathies have often been reported as
54 heterogeneous entities that include channelopathies, inflammatory, metabolic, mitochondrial, and
55 myotoxic diseases, which affect 1.62 in 100,000 individuals of all ages across the globe^{2, 3, 4, 5}.

56 Muscle weakness is one of the key hallmarks of congenital myopathies (1 in 26,000)⁶, while its
57 further consequences are escalated fibrosis, fat infiltration, and degenerating myofibers⁷. The
58 cytoskeleton of muscle tissue is composed of filamentous actin (F-actin) and myosin. Of late, it
59 was shown that the structural position of fast muscle cell fusion was guided by slow muscle fibers
60 in myotome formation⁸. In vertebrates, slow muscle fibers originate from somitic mesoderm as
61 one of the embryonic lineages; these embryonic lineages further differentiate into the first muscle
62 fiber types to perform longer contractions by firing action potential^{8, 9, 10}. In early life, human
63 myopathies are usually associated with accumulation of disorganized actin, α -actinin and myosin,
64 and failure of myofibrillar assembly in fast-twitch muscle fibers¹¹.

65 Historically, Native American myopathy (NAM) was identified in the Lumbee Indian infant
66 population of North Carolina, USA. The genetic predisposition to NAM was determined to be
67 caused by homozygosity for a variant G>C in exon 10 of *STAC3*, a gene highly expressed in
68 skeletal muscle. The *STAC3* protein contains a Src homology 3 (SH3) and cysteine-rich C1
69 domains. Clinically, other genetic mutations in *STAC3* result in congenital anomalies, such as

70 cleft palate, micrognathia, *talipes equinus* (club foot), and arthrogryposis also found in NAM
71 patients. Besides these skeletal abnormalities, NAM patients demonstrate increased levels of
72 neutral lipids in muscle biopsy and it was reported that one-third of patients (36%) of disease
73 afflicted individuals die before the age of 18 years^{12, 13}. Of late, it was suggested that C1 domain
74 of STAC3 is likely to bind the lipids¹⁴. Multiple studies have focused on investigating the regulation
75 of *STAC3* in NAM. Among all, *Stac3* knockout mice had a higher percentage of type I (oxidative)
76 muscle fibers and exhibited poor musculoskeletal performance¹⁵. Transcriptional regulation of
77 *Stac3* suggests that nutrient balance is a prerequisite for cytoskeletal integrity and myogenesis
78 via energy metabolism¹⁶. However, the genetic etiology and pathophysiology of slow skeletal
79 muscle weakness in NAM remain largely elusive.

80 Conventionally, lipids play a significant role in energy homeostasis, cellular structure and cellular
81 signaling, while lipid composition is crucial for ion channel activity and maintenance of membrane
82 receptor conformation^{17, 18}. Oftentimes, alterations in lipid metabolism account for metabolic
83 muscle diseases¹⁹. Importantly, storage of complex lipid molecules or glycogen content in skeletal
84 muscle can modify the calcium (Ca^{2+}) release at excitation-contraction (EC) coupling apparatus
85 on sarcoplasmic reticulum (SR). Interestingly, calcium-sensing dihydropyridine receptor (DHPR)
86 and calcium-releasing ryanodine receptor (RYR1) are under the control of STAC3 for intracellular
87 functions including muscle contractions^{20, 21}. These physiological actions involving the EC
88 apparatus underscore the absolute necessity of STAC3 for Ca^{2+} and nutrients homeostasis.

89 One decade ago, the first *stac3* knockout study in zebrafish revealed that a mutation in the *stac3*
90 gene of zebrafish (ZF) larvae impeded muscle function through EC coupling-driven defects in the
91 motor system in early life. STAC3 regulates the amount and stability of DHPR and/or RYR1 at
92 skeletal muscle triads by protein trafficking²². Markedly, the nutrient load (neutral lipids)
93 accelerates degeneration of filamentous actin (F-actin) and slow muscle myosin (Smyhc1) fibers,

94 which exemplify harmful behaviour of lipids in the maturation of skeletal muscle fibers. Thus,
95 identification of genetically-driven metabolic peculiarities, i.e., in lipid composition, may be vital
96 for preventing the loss of muscle fibers. Such disease-associated fatty acids may serve as
97 biomarkers for the early diagnosis of the disease or primary clinical indicators of treatment
98 success of NAM patients.

99 Of late, human myopathies have been modeled by knocking-out disease-causing genes using
100 CRISPR/Cas9 technology. In parallel, ZF serve as remarkable teleost animal model for skeletal
101 muscle research²³ and as a non-mammalian model for myopathies. Inherently, ZF acquired
102 multiple advantages, including high fecundity, external fertilization, and quick transformation of
103 one-to-multiple somite stages of the larva, which could provide substantial clinical and
104 histopathological information on the muscle disease^{24, 25}. The current study generated *stac3*^{-/-}
105 zebrafish using CRISPR/Cas9 technology. Remarkably, the *stac3*^{-/-} fish resemble human
106 phenotypes of Native American myopathy with increased neutral lipids level in the larvae. This
107 metabolic disbalance was further evident in structural and functional defects in zebrafish, which
108 seemingly drive failure of *stac3*^{-/-} larvae's musculature and their early demise.

109 **Methods**

110 **Animal husbandry**

111 Danio rerio zebrafish strains were maintained at 28 °C, under a 14:10 light: dark cycle and their
112 offspring was propagated until 6 dpf in system water containing 0.1% methylene blue (embryo
113 water) and then transferred to the system (nursery) or kept in the Petri dishes sans methylene
114 blue, for beginning of feeding. All animal experiments were conducted in accordance with the
115 Faculty of Medicine's Zebrafish Facility, Bar-Ilan University, Israel, Institutional Animal Care and
116 Ethical Committee (IACUC) guidelines. The protocol was approved by the committee (protocol
117 #53-08-2020).

118 **Generation of *stac3*^{-/-} by CRISPR/Cas9 system**

119 The strategy for establishing a stable zebrafish knockout followed a published CRISPR/Cas9
120 protocol²⁶. In brief, the guide RNA (gRNA) was designed to target sequence 5'-
121 AGTTCTGTGACGTCTGCGCACGG- 3' in exon 4 of the *stac3* gene. The *Fsp1* restriction enzyme
122 (New England BioLabs, Cat#R0135S) was utilized to distinguish between mutant, wild type and
123 heterozygous fish (Supplementary materials and methods). At the one-cell stage (30-45 minutes
124 post-fertilization), wild type zebrafish embryos of AB strain (F0) were injected with a mixture of
125 gRNA, crRNA, tracer RNA and Cas9 protein (Abcam, Cat#224892), at total amount of 300 µg/µL,
126 using a pneumatic Pico Pump (WPI, Worcester, MA, USA), and embryos were transferred into
127 the incubator.

128 **Genotyping and sequencing**

129 After 24 hours, DNA was extracted from of subset of pooled gRNA mix-injected embryos. A
130 polymerase chain reaction (PCR) was carried with forward primer: 5'-
131 GTGTTTCAACGTTAGTTCTGCTG-3' and reverse primer: 5'-
132 TGGCAAGAACAGTTACCTTAACA-3'. The reactions were performed using two programs: (A)
133 DNA was denatured at 94 °C for 3 seconds, annealed at 63 °C for 3 seconds and extended at 72
134 °C for 5 cycles, 3 seconds each. (B) DNA was denatured at 94 °C for 3 seconds, annealed at 60
135 °C for 3 seconds and extended at 72 °C for 37 cycles, 3 seconds each, followed by final extension
136 at 72 °C, for 3 minutes. Products of PCR amplification and restriction digestion were examined
137 on a 2% agarose gel. The 10-base-pair (bp) deletion was verified by DNA sequencing (Hy-lab,
138 Rehovot, Israel and Macrogen, Amsterdam, Netherlands).

139 For generation of a stable knockout, CRISPR/Cas9-targeted larvae were grown in a 1-liter (L)
140 tank for 35 days and then maintained in 3-L tank in a system water. At 2 months post-fertilization
141 (mpf), fish were genotyped to identify a founder that carried a mutation. Germline transmission of
142 a mutation in F1-progeny was deciphered by crossing the adult founders (F0) to WT. To acquire

143 the 10 bp deletion in F2-progeny, adults (F1) were out-crossed to WT. To minimize an off-target
144 effect, adult fish carrying the 10 bp deletion (F2) were further outcrossed to WT. The *stac3^{+/+}*,
145 *stac3⁺⁻*, and *stac3^{-/-}* siblings were obtained by intercrossing *stac3⁺⁻* F3 adult parents.

146 **Calculating hatching percentage of *stac3^{-/-}* zebrafish larvae**

147 Mass spawning of adult *stac3⁺⁻* parents produced all three genotypes: *stac3^{+/+}*, *stac3⁺⁻* and *stac3^{-/-}*
148 according to the Mendelian ratio. After spawning, embryos were collected and grown in a Petri
149 plate containing embryo water. During the course of somite to muscle fiber transition, the number
150 of hatched and unhatched larvae was recorded at 3, 4 and 5 dpf, and delayed hatching of the
151 larvae was documented by bright field light microscope imaging, while their genotypes were
152 verified by DNA sequencing of pooled sample (n=10) for each group. Delayed hatching
153 observations and analyses were conducted on at least three independent clutches of parent's
154 spawning. We saw skeletal peculiarities early on, thus we identified larvae as "normal" (*stac3* wild
155 type and heterozygous siblings) and "deformed" (*stac3* knockout) for the ensuing study.

156 As a rule, larvae were examined under the light microscope every 24 hours. The total number of
157 embryos produced by *stac3⁺⁻* parents was recorded. Dead larvae at (4-11 dpf) were extracted
158 from Petri plates and genotyped. In all experiments, larvae were euthanized in 0.4% methane
159 sulfonate (Sigma-Aldrich, MS-222) with subsequent immersion in ice-cold water for 15 minutes.

160 **Whole-body Ca²⁺ measurement during larval development**

161 Total body calcium level was determined for zebrafish larvae groups, i.e. *stac3^{+/+}* and *stac3⁺⁻*:
162 n=90 and *stac3^{-/-}*: n=60). After euthanasia, larvae were washed gently with deionized water and
163 dried for 1 hour at 65 °C. Then, samples were digested with 80-90 µL 1M Tris-Cl (pH 8.0), at 95
164 °C, overnight. Digested samples were centrifuged at 13,000 rpm for 5 minutes and the
165 supernatant was collected in 1.5 mL Eppendorf tube. Calcium amount was detected using the
166 Colorimetric Calcium Assay Kit (Abcam, Cat#102505), according to the manufacturer's

167 instructions. Optical density of each sample was measured at 575 nm with absorption
168 spectrometry (Tecan-Plate Reader-Spark Cyto, DKSH technology, Bangkok, Thailand).

169 **RNA extraction**

170 We extracted RNA from *stac3* wild type and heterozygous siblings and *stac3* knockout for gene
171 expression analysis at 4 and 5 dpf. After euthanasia, pooled (n=10) larvae were homogenized
172 with a pestle and motor mixer (Thomas Scientific, Swedesboro, NJ, USA, Cat#47747-370) in 350
173 μ L Trizol (Sigma-Aldrich). RNA was extracted in biological triplicates, using the Direct-Zol RNA
174 kit (Zymo Research, Tustin, CA, USA).

175 **Real-time quantitative PCR (RT-qPCR)**

176 cDNA (500-1000 ng) was synthesized using the Takara PrimeScript Kit (Takara, Mountain View,
177 CA, USA). Gene expression was quantified using the PowerUp SYBR Green Master Mix and a
178 ViiA™7 Dx qPCR Instrument (Thermo Fisher Scientific, Waltham, MA, USA, Cat#4453534). The
179 target gene expression (TableS1) was normalized to the endogenous control *rpl32* (ribosomal
180 protein I32). For each expression analysis, a non-template control (NTC) was included along with
181 technical triplicates. Delta-delta-threshold cycle (DDCT) values were plotted on the graph as
182 relative gene expression.

183 **Whole-mount staining of zebrafish larvae**

184 At 5 and 7 dpf, *stac3*^{+/+} and *stac3*^{+/−}: n=15 and *stac3*^{−/−}: n=15 larvae were fixed in 4%
185 paraformaldehyde (PFA, Sigma-Aldrich) either for 1 hour at RT or overnight at 4 °C. Staining was
186 performed using a published protocol²⁷. Slow muscle fibers were stained with the anti-Myhc (F59,
187 Santa Cruz Biotechnology, Dallas, TX, USA, Cat#sc-32732) antibody (1:1000 dilution). F-actin
188 fibers were stained with Phalloidin (iFluor 488, Cat#Ab176753) (1:1000 dilution), followed by
189 secondary antibody (Sigma-Aldrich).

190

191 **Neutral lipid visualization by Oil Red O (ORO) staining**

192 ORO (Sigma Aldrich, Cat#0-0625) powder 0.05 g was dissolved in 10 mL isopropanol and then
193 incubated for 10 minutes at RT. The ORO working solution was prepared by adding three parts
194 of filtered ORO stock to the two parts of distilled water. Muscle section-containing slides were air-
195 dried for 2-3 hours at RT, washed with 1x PBS for 2 minutes and air-dried again for 2-3 hours.
196 The slides were incubated in 500 μ L ORO working solution for 30 seconds, then washed with tap
197 water and air-dried for another 3 hours at RT, before mounting them in 100% glycerol.

198 **Muscle histology**

199 **Embedding of zebrafish larva**

200 At 5 dpf, larvae were fixed in 4% PFA for 3-6 days, at 4 °C, and washed 3x with PBS for 15
201 minutes. Then, they were soaked in 10% sucrose for 48 hours at RT, and then embedded with
202 Tissue Tek (Sakura, Torrance, CA, USA) in 12x12 cm embedding molds (PEEL-A-WAY;
203 Cat#70181). The embedding molds were frozen in liquid nitrogen for about 2-3 hours, and then
204 stored in aluminum foil, at -20 °C. Muscle sections were cut serially to 20 μ M thickness with a
205 cryostat (Leica CM1950), and sections were collected on 26X76 mm/1-1.2 mm adhesion
206 microscope slides (BAR-NAOR Ltd., Tel Aviv, Israel, Cat#BN93080C).

207 **Hematoxylin and eosin staining**

208 Paraffin-based horizontal muscle sections were dehydrated 3x in xylene for 2 minutes, and then
209 dehydrated in 100% alcohol, before being washed with 80% alcohol and tap water. Slides were
210 then stained with both hematoxylin and eosin solution using the LEICA AUTOSTAINER XL at the
211 Department of the Pathology, Ziv Hospital, Safed, Israel.

212 **Locomotion of *stac3*^{-/-} larvae**

213 To quantify locomotion function of *stac3* KO, *stac3*^{+/+} and *stac3*^{-/-} (n=48) and *stac3*^{-/-} (n=22) larvae
214 were grouped at 5 dpf. Tracking was performed with a DanioVision system (Noldus Information
215 technology, Wageningen, NL). For habituation, larvae were individually placed before the test, in

216 96-well-plates containing 200 μ L embryo water (A1-D12: *stac3^{+/+}* and *stac3⁺⁻* and E1-F10: *stac3^{-/-}*) and incubated for 30 minutes at 28 °C. The locomotion tracking strategy included 15 minutes
217 light on, 5 minutes light off, 5 minutes light on. Total distance moved by larvae was averaged for
218 every one-minute represented by a dot on the graph under each (light/dark) condition. After the
219 test, larvae genotype was confirmed using the *FSPI* enzyme digestion.
220

221 **Senescence-associated (SA) β -galactosidase staining**

222 To assess the effect of senescence on early life muscle fiber growth and development in zebrafish,
223 larvae at 6 dpf were classified as normal or deformed and fixed in 4% PFA, at 4 °C, overnight.
224 Larvae were washed with phosphate buffered saline (PBS pH 7.4, followed by PBS pH 6.0), 3x
225 for 10 minutes each. Next, larvae were incubated with 1% X-Gal solution [in PBS pH 6.0: 1 mg of
226 5-bromo-4-chloro-3-indolyl beta-D-galactosidase (X-Gal)] per mL, 5 mM K₃Fe[CN]₆, 5 mM
227 K₄Fe[CN]₆, 150 mM NaCl and 2 mM MgCl₂ (Sigma-Aldrich) at 37 °C, overnight²⁸. Images were
228 acquired with a bright field microscope.

229 **Skeletal muscle defects observed by birefringence assay in zebrafish larvae**

230 *stac3^{+/+}* and *stac3⁺⁻* control siblings and *stac3^{-/-}* larvae were anesthetized on 6 dpf and positioned
231 to allow a dorso-ventral view, in 3% methylcellulose. Muscle fiber architecture was assessed, as
232 described in the published protocol²⁹ at the Faculty of Marine Sciences, Ruppin Academic Center,
233 Mikhmoret, Israel.

234 **Caffeine treatment of zebrafish larvae**

235 At 5 and 7 dpf, *stac3^{+/+}* and *stac3⁺⁻* control siblings and *stac3^{-/-}* larvae (sample sizes from 25 to
236 30 animals per group), were subjected to caffeine treatment (Sigma-Aldrich, Cat#C0750). Stock
237 caffeine 1M solution was prepared in double distilled water, and a working concentration of 5mM
238 caffeine (v/v) was subsequently diluted in 1L system water. Each petri dish contained either 30mL
239 of working solution of caffeine or embryo water as no-treatment control solution. Survival of the

240 larvae was monitored for 4 days (96 hours), and each day dead larvae were visually recorded by
241 using the light microscope (then discarded).

242 **Image processing**

243 Embryo hatching, developmental defects, and whole-mount Oil Red O staining of neutral lipids
244 were imaged using a Leica microscope (M165 FC). Muscle histology imaging was performed with
245 an automated upright slide-scanning microscope (Axio Scan.Z1, Carl Zeiss Microscopy,
246 Oberkochen, Germany) with a 20x/0.95 objective at z-planes of 0.5mm. Fluorescent images were
247 taken with upright microscope (Apotom.2, Carl Zeiss, Jena, Germany).

248 **Data analysis**

249 Most of the experimental data were analyzed with Prism (version 9, GraphPad Software, Inc., La
250 Jolla, CA, USA) and some data sets were processed in Excel. All graphs present means \pm
251 standard deviation values. Two independent groups were compared using the student's t-test.
252 Larvae locomotor activity was analyzed by one-way ANOVA. Survival rate of knockout fish was
253 determined by Prism, using the Log-rank test. In all the analyses, statistical significance was
254 defined as a $p < 0.05$.

255 **Results**

256 **1. Generation of CRISPR/Cas9 based *stac3* knockout line in zebrafish to study NAM 257 disease process**

258 Loss of STAC3 leads to severe skeletal abnormalities in both mice and zebrafish, although a
259 mechanism by which *stac3* mutants die at an early age^{22, 15} is unknown. Further, morbidity in NAM
260 patients had associated with increased lipid levels in skeletal muscle. To study the relation
261 between early muscle fibers development and lipid metabolism, CRISPR/Cas9 system was
262 applied to induce a global knockout of exon 4 of *stac3*. A 10-base-pair deletion in ZF was
263 generated, which led to a frame shift which created a stop codon at V78 and produced a truncated

264 protein (80 aa total length) (Fig1A, B). The targeted knockout in *stac3* gene was confirmed by
265 both *Fsp1* restriction enzyme digestion and sequencing of genomic DNA (Fig1C and FigS1A, B).
266 Adult *stac3* heterozygous (*stac3*^{+/}) parents were then inter-crossed to obtain homozygous
267 (knockout) *stac3*^{-/-} offspring. Further, *stac3*^{+/} progeny were “blindly” characterized as we saw
268 congruence with skeletal phenotypes at the early development of the homozygous larvae. ZF
269 larvae from 2-3 independent clutches were then selected based on their morphology, - either
270 normal (healthy; *stac3*^{+/} and *stac3*^{+/}) or deformed (*stac3*^{-/-}) - for functional characterization of
271 skeletal muscle tissue at the early life. All unhatched larvae were genotypically confirmed as *stac3*
272 knockouts (FigS2A-C). At 3 days post-fertilization (dpf), *stac3*^{-/-} larvae exhibited multiple
273 congenital skeletal muscle defects, such as bending at head, trunk, and tail regions, and were
274 distinct from *stac3*^{+/} and *stac3*^{+/} control siblings (FigS1C). RT-qPCR analysis showed that the
275 relative gene expression of *stac3* was significantly (p<0.05) downregulated in knockout compared
276 to *stac3*^{+/} and *stac3*^{+/} control siblings at 5 dpf (Fig1D). These findings align with previous
277 observations in *stac3* mutant mice and ZF; we therefore utilized *stac3*^{-/-} zebrafish for identifying
278 genetically-driven metabolic regulators, which could be potentially associated with human muscle
279 disease (congenital myopathies) pathology.

280 **2. *stac3* knockout constrains embryo hatching by altered Ca²⁺ level via *ryr1a* receptor in
281 zebrafish**

282 The *Stac3* gene plays a vital role in calcium homeostasis by modulating calcium-sensing
283 dihydropyridine (DHPR) receptor and calcium-releasing ryanodine (RYR1) receptor at the
284 sarcoplasmic reticulum²⁰. We presumed that a mutation in *stac3* gene causes muscle weakness
285 by altering physiological calcium level in zebrafish at early life. Intercrosses of adult *stac3*^{+/}-
286 parents produced all three genotypes: *stac3*^{+/}, *stac3*^{+/} and *stac3*^{-/-} larvae; the proportion of these
287 genotypes complied with the Mendelian ratio. On 4 dpf, there was a significant (p<0.05) delay in
288 hatching of *stac3* knockout larvae (12%) compared to wild type and heterozygous control siblings

289 (76.7%) (Fig2A, B). Under physiological conditions, muscle function (contraction) begins at 17
290 hours post-fertilization in ZF, such that, by 2 dpf, larvae are completely shed their chorion through
291 rapid contractions²². Therefore, we sought to determine whether mutation in *stac3* alters lipid
292 metabolism, resulting in damage to muscle fiber functional organization, consequently promoting
293 the early death of ZF larvae.

294 Indeed, we found that *stac3*^{-/-} larvae carrying 10 bp deletion mutation died within the chorion at 4
295 dpf, which was not seen in their controls siblings (FigS2A), suggesting that severity of declined
296 muscle function (muscle weakness) gradually escalated. Moreover, genotypes of hatched and
297 unhatched larvae were verified by DNA sequencing of a pooled sample (n=10) from each group
298 (FigS2B, C). To determine whether low Ca²⁺ constrain hatching of ZF larvae, the progeny of
299 *stac3*^{+/+} parents was categorized into contrasting phenotypic groups for measuring whole body
300 Ca²⁺ levels. Whole-body Ca²⁺ levels were compared between hatched vs. unhatched and normal
301 vs. deformed larvae. As anticipated, total-body calcium levels were significantly lower (p<0.05;
302 Fig2C) in *stac3* knockout in comparison to *stac3*^{+/+} and *stac3*^{+/+} (control) siblings at the early
303 muscle fibers growth and development. In parallel, we found that the relative gene expression of
304 ryanodine receptor 1a (*ryr1a*) was significantly (p=0.0171; Fig2D) elevated in *stac3*^{-/-} larvae
305 compared to their controls. These results suggest that a mutation in *stac3* leads to a calcium
306 deficiency via *ryr1a* receptor, which impedes skeletal muscle function, as demonstrated by
307 delayed embryo hatching and declined locomotion (Fig7A, B and Fig9).

308 **3. F-actin inadequacy and slow muscle fiber organization in *stac3*^{-/-} larvae**

309 *stac3* knockdown has been shown to affect myofibrillar protein assembly¹⁶; we sought to
310 determine whether *stac3* knockout can affect the cytoskeleton of skeletal muscle i.e., F-actin and
311 slow myosin wirings. In humans, BA-D5 antibody was known to recognize one of the myosin MHC
312 (cardiac β) isoforms³⁰. Therefore, we performed slow muscle (anti-Myhc; F59) staining for
313 visualization of all myosin isoforms in knockout *stac3*, heterozygous and wild type siblings at the

314 age of 5 and 7 dpf. Consequently, disorganized slow muscle fibers and a deficit in conventional
315 organization of individual fibers were identified in trunk and tail regions of *stac3*^{-/-} larvae compared
316 to *stac3*^{+/+} and *stac3*^{+/-} siblings at 5 dpf, this was more prominent with larger gaps at somite
317 boundaries at 7 dpf (Fig3A-C and FigS3A and FigS4A, C panel). Furthermore, whole-body F-
318 actin fibers were reduced in the *stac3* KO in contrast to *stac3*^{+/+} and *stac3*^{+/-} siblings (Fig3D and
319 FigS3B). The myosin structural organization is essential for energy production of skeletal muscle
320 for contractions³¹. In comparison to *stac3*^{+/+} and *stac3*^{+/-} control siblings, *stac3*^{-/-} larvae showed a
321 significant reduction in relative expression of both *srebf1* (p=0.0025) and its down-stream target
322 acetyl co-enzyme-A (p=0.004), that are involved in lipogenesis (FigS3D). Therefore altered
323 cytoskeleton architecture of skeletal muscle could impair metabolic processing of nutrients (lipids)
324 in *stac3* KO.

325 **4. Expression of myogenic regulators is dysregulated in *stac3*^{-/-} larva**

326 *STAC3* is highly expressed in skeletal muscle tissue. Therefore, we sought to check whether the
327 fragility of the musculoskeletal system in early life of the *stac3*^{-/-} is dictated by impaired
328 myogenesis, relative gene expression of myogenesis markers was evaluated. The lineage of
329 skeletal muscle in the embryonic stage is determined by myogenic regulatory factors such as
330 *MYOD*, *MYF5*, and *MYOG*³². Similarly, RT-qPCR analyses identified significant upregulation of
331 *myoD* (p=0.001) alongside downregulation of *myf5* (p=0.03) and unaltered expression of *myoG*
332 in *stac3*^{-/-} larvae as compared to wild type and heterozygous controls at 5 dpf (Fig4A-C).
333 Genetically, *MYOD* and *MYF5* are *bona fide* for myoblast fusion by maintaining the functional
334 organization of actin and myosin fibers, while *MYOG* involves in the terminal differentiation of
335 muscle cells³³. Disorganized slow muscle fibers with a reduced amount of F-actin were found at
336 5 and 7 dpf in *stac3*^{-/-} larvae (Fig3). However, gene expression of skeletal muscle markers such
337 as *sox6*, slow muscle myosin (*smyhc1*), and troponin (*tnnca1*) were unchanged in *stac3*^{-/-}
338 compared to the *stac3*^{+/+} and *stac3*^{+/-} groups at 5 dpf (FigS3C). To our notice, it was shown that

339 *MyoD*^{-/-} mice are viable and fertile while contrastingly double knockout of *MyoD* and *Myf5* had led
340 to a perinatal death³⁴. Further, we show that unaltered *myoG* expression of *stac3*^{-/-} larvae
341 indicates defective signals that could commence in myogenesis before the formation of complete
342 skeletal muscle fiber (Fig4D). Furthermore, abnormal histopathological features and
343 compromised myogenesis were reported in myopathies³⁵. Altogether, our results suggest that
344 both *myf5* and *myoD* might have compensatory mechanisms of action in *stac3*^{-/-} larvae, to secure
345 cytoskeleton integrity by actin and myosin fibers formation.

346 **5. Distinct lipid metabolism in *stac3*^{-/-} fish at the early life**

347 Besides in adipose and liver tissues, excess neutral lipids are often stored in skeletal muscle
348 tissue, a hallmark of lipid storage myopathy. An increase in lipid droplets was revealed by electron
349 microscope analysis of NAM patient skeletal tissue samples^{12, 14}. Therefore, we sought to
350 determine whether lipid metabolism is altered in *stac3*^{-/-} zebrafish and associated with impaired
351 muscle fiber functional organization. At multiple developmental stages, *stac3* knockout and wild
352 type and heterozygous siblings were stained with Oil Red O dye (ORO) for lipid visualization. In
353 early life, zebrafish larvae utilize transported maternal lipids as the energy source for their active
354 metabolic functions^{36, 37}. Correspondingly, no differences in whole-body lipids of hatched (*stac3*^{+/+}
355 and *stac3*⁺⁻) compared to unhatched (*stac3*^{-/-}) larvae were observed on 4 dpf (Fig5A-A'). Notably,
356 elevated neutral lipids were primarily observed in the yolk sac region of knockout larvae compared
357 to *stac3*^{+/+} and *stac3*⁺⁻ control siblings ($p=0.0023$; Fig5B-B' and E) at 5 dpf, which corroborates
358 the increase in lipid droplets measured in NAM patients^{12, 38}. Impairments in genetic and metabolic
359 process can affect lipid synthesis, transportation, utilization, and degradation in multiple cell and
360 tissue types, and including skeletal muscle. We therefore further visualized neutral lipids of larvae
361 by whole-mount ORO staining on the day of exogenous feeding (7 dpf) and, in knockout larva,
362 found levels identical to those shown at 5 dpf (Fig5B-B' and Fig5C-C', D), suggesting that *stac3*
363 ^{-/-} maintained surplus lipids, while in control groups these levels go down (FigS4B-B'). Of note,

364 studies¹⁶ reported that *stac3* is involved in nutrition sensing, while its C1 domain binds to lipids¹⁴.
365 Hence, we speculate that in presence of the *stac3* genetic background wild type and
366 heterozygous siblings are capable of sensing nutrients for generating energy for performing
367 enhanced muscle contractions, and survival of the larva (Fig7A, B and Fig9).

368 **6. Metabolic dysfunction promotes death of *stac3* KO fish**

369 The impact of accumulated lipids in NAM and pathophysiology of skeletal muscle tissue affected
370 by this disease are unknown. However, detrimental effects on cytoskeletal elements and on their
371 interactions in muscle tissue can lead to myopathies in humans³⁹. We next asked whether a delay
372 in embryo hatching, reduction in F-actin and alteration in slow muscle fibers organization weaken
373 musculoskeletal system in *stac3* knockout. To confirm whether 10bp deletion mutation alters the
374 structural integrity of *stac3*^{-/-} skeletal muscle, birefringence analysis of *stac3*^{-/-} found a patchy
375 pattern of muscle fibers with dense black spots, whereas *stac3*^{+/+} and *stac3*⁺⁻ control siblings had
376 rich on muscle fibers with bright chevron structure on 6 dpf (Fig6A and TableS3). In this study,
377 knockout larvae died gradually by 5 dpf, with obvious skeletal muscle defects; out of the expected
378 by Mendelian ratio for *stac3* knockouts (25%) we found only ~18.24% of knockout larvae survived
379 until 7 dpf (TableS2). To understand the significance of genetic regulation of *stac3* in nutrients
380 processing and its contribution to survival of zebrafish, we further maintained these surviving
381 knockout larvae in an incubator and changed embryo water after feeding with nursery food,
382 suggesting that *stac3* KO carries a systemic metabolic dysfunction manifested by increased
383 neutral lipids (Fig5B, C panels). To determine whether high neutral lipid (nutrients load) levels
384 can induce lethal lipotoxicity in *stac3* knockouts, we performed cellular senescence assay at the
385 early development of zebrafish larvae²⁸. As expected, our senescence-associated beta-gal probe
386 (SA- β -gal) was strongly noticeable at the yolk sac region of the *stac3*^{-/-} compared to *stac3*^{+/+} and
387 *stac3*⁺⁻ control siblings at 6 dpf (Fig6B, C and FigS5B, C panels). Furthermore, fasting of *stac3*
388 KO larvae revealed a trend of improved survival between 7 and 10 dpf ($p<0.0001$; FigS6). These

389 findings align with the lipid (ORO) staining observations and suggest that deletion of 10 bp in the
390 *stac3* gene accelerates an early-age-specific senescence in cells, culminating in metabolic failure
391 of the zebrafish larva (p<0.0001; Fig6D).

392 **7. Caffeine treatment improves survival of the *stac3*^{-/-} larvae at 5 but not 7 dpf**

393 We sought to determine whether caffeine could be an effective therapeutic molecule to a
394 betterment of congenital myopathies due to its key mechanistic biochemical actions across
395 muscle and other tissues⁴⁰. Previous studies revealed that caffeine treatment had significantly
396 enhanced muscle power with the improvement of Ca²⁺ release, increased fatty acid utilization,
397 and decrease in the mortality of mice by antioxidant functions in brain^{20, 41, 42}. We monitored the
398 growth and survival of *stac3*^{+/+} and *stac3*^{-/-} (non-deformed) control siblings and *stac3*^{-/-} larvae with
399 and without 5mM caffeine treatment at two different time points. After 5mM caffeine treatment for
400 96 hours, we discovered an improvement in the survival of the *stac3*^{-/-} larvae compared to
401 untreated *stac3*^{-/-} control group at 5 dpf (Fig8A). Moreover, the difference of overall survival of the
402 *stac3*^{+/+} and *stac3*^{-/-} control siblings was significantly (p<0.0001) increased compared to untreated
403 and treated deformed animals 5 dpf. In the *stac3*^{-/-} zebrafish larvae at 7 dpf, we found that over
404 96 hours the *stac3*^{-/-} larvae were dying much faster (p<0.0001) than their untreated *stac3*^{-/-} and
405 non-deformed control siblings (Fig8B). In contrast to 5 dpf, at 7 dpf deformed larvae did not show
406 any improvement in their survival percentage after 5mM caffeine treatment compared untreated
407 *stac3*^{-/-} (deformed) and treated *stac3*^{+/+} and *stac3*^{-/-} animals. In summary, the mechanistic
408 regulation of *stac3* by caffeine is important during muscle fibers' development at the early life (5
409 dpf), while caffeine regulation could be inhibited at the start of external feeding - in 7 dpf *stac3*^{-/-}
410 zebrafish.

411

412

413 **Discussion**

414 In CRISPR/Cas9-generated *stac3* zebrafish mutants, we noticed that out of the expected
415 Mendelian ratio of 25% only 18.24% *stac3* KO larvae survived till the age of 11 dpf. Additionally,
416 functional and morphological observations of *stac3*^{-/-} larvae suggested muscle paralysis and
417 skeletal muscle deterioration in the early life of *stac3*^{-/-}. The present study thus explored the
418 phenotypic basis for early life mortality of *stac3*^{-/-} zebrafish and found that *stac3* is critical for early
419 life lipid balance in ZF. Knocking-out this gene in zebrafish led to a delay in embryo hatching and
420 to skeletal muscle defects, and eventually, to a gradual decline in survival at age 4-11 dpf. Under
421 physiological conditions, muscle function (contraction) begins at 17 hours post-fertilization in ZF,
422 such that, by 2 dpf, larvae should completely shed their chorion through rapid contractions. Of
423 note, a proportion of *stac3*^{-/-} larvae died within the chorion (~4 dpf), which is implying the existence
424 of a paralyzed muscle function during early development. In CRISPR/Cas9 system-induced
425 global knockout, we saw gross phenotypes early on, thus we identified larvae as normal (*stac3*
426 wild type and heterozygous siblings) and deformed (*stac3* knockout) in the entire study. Earlier
427 studies found altered ratios of slow and fast muscle fibers in *Stac3* KO mice, possibly reflecting
428 impaired muscle metabolism^{15, 43}. Zebrafish carrying a missense mutation of the *stac3* gene were
429 shown to have EC coupling alteration-instigated swimming impairments, which preceded larval
430 death²².

431 To improve the survival of the larvae beyond the age of 11 dpf, we applied special conditions (i.e.
432 nursery feeding and replenishing with a fresh system water) to these 18.24% *stac3* KO larvae
433 that survived, along with *stac3*^{+/+} and *stac3*^{-/-} control siblings. We thus discovered that between
434 7-11 dpf, expedited mortality was observed in *stac3*^{-/-} fish only. Furthermore, fasting revealed a
435 trend in better survival of fasted vs. fed *stac3* KO larvae between 7 and 10 dpf (FigS6). It could
436 be that nutrient-dependent transcriptional regulation in *stac3* KO zebrafish failed in coping with
437 exogenous feeding. We further sought to measure the expression of myogenic regulators and

438 lipogenesis synthesis genes to correlate with metabolic activities involved in developing muscle
439 fiber architecture. We found that loss of *stac3* significantly upregulated *myoD* and
440 downregulated *myf5* expression, while *myoG* gene expression was unaltered, whereas *srebf1*
441 and acetyl co-enzyme were significantly down-regulated, which suggests that myogenesis can
442 be independently controlled by *myoD* and *myf5* genes^{44, 45}. Congruently, *myoG* mutant larvae
443 showed no effect on muscle phenotypes⁴⁵. Of note, in chicken development, distinct diet
444 conditions associated with increased mRNA levels of myogenic regulators (*Myf5*, *MyoD*, and
445 *Myf4*) controlled the growth of skeletal muscle^{46, 47, 48}. Recently, the expression of *MYF5* was
446 reported to be significantly altered in human congenital myopathy⁴⁹. In sum, transcription of the
447 myogenic regulatory factors *myoD* and *myf5*, which encode proteins critical for fine-tuning of
448 musculoskeletal health in zebrafish, is dependent on *stac3*.

449 In humans, clinical features of congenital myopathies include muscle weakness, increased
450 intramuscular fat and muscle fiber degeneration⁵⁰. Histologically and molecularly, congenital
451 myopathies are categorized into sub-entities based on the affected protein (e.g. desmin,
452 dystrophin, collagen) and lipid contents in skeletal muscle tissue^{51, 52, 53}. Of note, NAM patients
453 demonstrate muscle weakness and increased lipid levels^{12, 54}. In our analysis of the gross
454 appearance and organization of *stac3*^{-/-} larvae muscle fibers, *stac3* was found to play a pivotal
455 role in establishing F-actin and slow muscle fiber functional coordination to generate strength and
456 execute active contractions. Despite *Stac3* nutrient-sensing activity, its potential functional role
457 via lipid metabolism has barely been studied in animal models for NAM. Our studies found that
458 deletion of 10 base pairs in *stac3* had no impact on neutral lipid levels in 4 dpf *stac3*^{-/-} fish. Yet,
459 higher levels of neutral lipids were measured in *stac3*^{-/-} compared to *stac3*^{+/+} and *stac3*⁺⁻ control
460 larvae on 5 dpf and maintained through 7 dpf. Taken together, these findings suggest that *stac3*
461 acts as an age-specific metabolic regulator in the transformation from unhatched to hatched
462 zebrafish larvae, by controlling lipid (energy) utilization in early life. Five dpf is an ideal age for

463 investigating skeletal muscle formation while the effect of metabolic changes on muscle fibers
464 organization can be examined at 7 pf in zebrafish. We observed that the damaged organization
465 of F-actin and slow muscle fibers could play a role in the progression of myopathy. Due to a
466 significant role in energy processing, utilization, and generating strength in humans, we suggest
467 that improvement of slow muscle fiber integrity could delay the progression of muscle diseases
468 (congenital myopathies)⁵⁵.

469 According to the literature^{56, 57}, the yolk is considered a reservoir of metabolically active nutrients
470 needed for the embryonic growth and development of an organism. Around 3 dpf, yolk sac
471 contains vascular network with compartments delimited by veins, while reduction of yolk sac is
472 seen as embryo utilizes it at 4 dpf^{37, 58}. Inherently, zebrafish larvae utilize maternally acquired
473 lipids as an energy source for the routine biological functions until the age of ~5 dpf, while the
474 larva transforms into adult zebrafish with rapid development of musculoskeletal system^{36, 59}. The
475 yolk syncytial layer (YSL) is important for the delivery of lipids during the larval growth and
476 development⁵⁸. With functional activity in early-life stages and nutrients' demand in an organism,
477 lipids are essential for formation of organ and tissue types e.g. heart, liver, intestine, pancreas,
478 and other vascularized regions, including skeletal muscle. Recently, it was reported that
479 intramuscular adipose tissue is increased in dystrophy and neuromuscular disease patients
480 compared with control individuals⁶⁰; it now is evident that NAM's etiology is similar. Altogether,
481 the persistence of altered metabolic condition, which can deteriorate muscle function in NAM, is
482 under-appreciated. We found that *stac3* KO larvae retained neutral lipids, mostly at the yolk sac
483 region, even at the age when the exogenous feeding becomes necessary (7 dpf). At this age,
484 neutral lipids were less visible in *stac3^{+/+}* and *stac3⁺⁻* control groups. Consequently, we speculate
485 that the intact *stac3* enables nutrient sensing for growth of the musculoskeletal system, resulting
486 in rapid muscle contractions, and larvae survival. Loss of *stac3* manifests by accumulation of
487 neutral lipids, which is detrimental in the early growth stages of zebrafish larvae⁶¹. Storage of

488 neutral lipids might contribute to a systemic metabolic dysfunction by lipotoxicity/senescence
489 effect on cytoskeleton organization of the larvae. Of interest, the family of STAC proteins has
490 been associated with nuclear factor- κ B and C/EBP in controlling senescence in the course of
491 muscle cell proliferation and differentiation¹⁶. We found that senescence-associated beta-gal
492 staining was accentuated in the yolk sac region of *stac3*^{-/-} at 6 dpf. Taken together, at early or
493 late-embryonic stages, skeletal muscle cells might encounter lipotoxicity-induced stress, which
494 triggers their dormant state through cellular senescence³¹. Prolonged accumulation of lipids via
495 lipotoxicity in *stac3* knockout zebrafish could further contribute to unfavorable conditions (signals)
496 that cause tissue (i.e. muscle) deterioration and larval death. Comprehensive studies will be
497 required to understand lipid metabolism-driven vertebrate developmental transformations in early
498 ZF life.

499 Physiologically, STAC3 oversees the biochemical relationship between calcium sensing (DHPR)
500 and calcium-releasing (RYR1) receptors at the sarcoplasmic reticulum in skeletal muscle^{20, 43},
501 with calcium homeostasis being essential for active contractions, tissue formation, maturation,
502 and regeneration⁶². In the present work, *stac3*^{-/-} larvae were found to have lower concentrations
503 of calcium and upregulation of *rry1a* in an early patterning of skeletal muscle fibers. These findings
504 compelled us to draw a working hypothesis that escalated lipid levels can directly reduce muscle
505 contractions, as manifested by (a) a delay of embryo hatching due to muscle weakness, (b)
506 impaired Ca^{2+} release via *rry1a*, and (c) damaged organization of F-actin and slow muscle fibers
507 (Fig9). In sum, these *stac3* KO phenotypes suggest paralyzed muscle function manifested as a
508 significantly reduced locomotion on 5 dpf. Recent case studies from Turkey⁶³, France⁶⁴ and
509 Russia⁶⁵ suggested that pathophysiology of myopathy progression is comparable to NAM disease
510 phenotypic outcome in humans. 36% of individuals afflicted with Native American myopathy
511 (NAM) died by the age of 18 years¹². We hypothesized that caffeine could be an effective
512 therapeutic molecule to a betterment of congenital myopathies due to its key mechanistic

513 biochemical actions across muscle and other tissues^{20, 41, 42}. Indeed, our findings suggest that
514 there is increased trend in the survival of the 5mM caffeine treatment in deformed *stac3* larvae at
515 5 dpf, while 5mM caffeine treatment revealed rather an opposite effect on *stac3*^{-/-} at 7 dpf.
516 Hypothetically, the biochemical action of caffeine could be inhibited at the age of external feeding
517 should start, which is ~7 dpf. Thus, we postulate that our *stac3* knockout zebrafish, established
518 for NAM is a lipid-related human congenital myopathy (lipid storage myopathy). Altogether,
519 exploring early life's genetic mechanisms using *stac3*^{-/-} zebrafish model are frontiers for
520 repressing muscle fiber degeneration and refining muscle function maintaining lipid homeostasis
521 in NAM disease during the course of muscle fibers formation.

522 Animal models are the gold standard for gaining pre-clinical insights into various diseases, due
523 to their anatomical and morphological resemblance to humans⁶⁶. Of late, modeling of human
524 myopathies is achieved by knocking out disease-causing genes by CRISPR/Cas9 technology⁶⁷.
525 The current work demonstrated that *stac3*^{-/-} fish may serve as a potential model for identifying
526 lipid-based biomarkers or small molecules for the early diagnosis or treatment of NAM.
527 Intramuscular adipose tissue is increased in dystrophy and neuromuscular disease patients
528 compared with control individuals. We suggest that STAC3 has beneficial effects on muscle
529 metabolism by preventing the damage triggered by genetic or metabolic dysfunctions due to
530 abnormally accumulated lipids.

531 **Acknowledgements**

532 We are grateful to Dr. Chen Shochat for her helpful advice in CRISPR. The authors would like to
533 thank Dr. Sergio Szvalb's lab at the Department of the Pathology, Ziv Hospital, Safed, Israel, for
534 the muscle histology. We extend our gratitude to Dr. Daya's lab for allowing us to perform the
535 birefringence assay at the Faculty of Marine Sciences, Ruppin Academic Center, Mikhmoret,
536 Israel.

537

538 **Conflict of interest**

539 The authors declare no conflict of interest.

540 **Data availability**

541 Data generated or utilized in this study can be found in online data repository source.

542 **Funding**

543 Supported by grant ISF-1121/19 from Israel Science Foundation and ISF-2058/21 from China-
544 Israel Cooperation.

545 **Supplementary materials**

546 The supplementary materials and figures related to this article can be accessed through online
547 version of the manuscript.

548 **Author Contributions**

549 RD: Conceptualization, investigation, data curation, data analysis, writing original draft,
550 manuscript review and editing, DK: Conceptualization, data curation, funding acquisition, project
551 administration, supervision, manuscript review and editing. HFZ: investigation, data curation, data
552 analysis and manuscript review and editing. All the authors contributed to the article and approved
553 the version submitted for the publication.

554 **References**

- 555 1. McLeod, M., Breen, L., Hamilton, D.L., and Philp, A. Live strong and prosper: the importance
556 of skeletal muscle strength for healthy ageing.
- 557 2. Gineste, C., and Laporte, J. (2023). Therapeutic approaches in different congenital myopathies.
558 Current Opinion in Pharmacology 68, 102328.
- 559 3. Huang, K., Bi, F., and Huan, Y. (2021). A systematic review and meta-analysis of the
560 prevalence of congenital myopathy. Frontiers in neurology, 1959.
- 561 4. Gineste, C., and Laporte, J. Therapeutic approaches in different congenital myopathies.

562 5. Tolchin, D., Yeager, J.P., Prasad, P., Dorrani, N., Russi, A.S., Martinez-Agosto, J.A., Haseeb,
563 A., Angelozzi, M., Santen, G.W.E., Ruivenkamp, C., et al. De Novo SOX6 Variants Cause
564 a Neurodevelopmental Syndrome Associated with ADHD, Craniosynostosis, and
565 Osteochondromas.

566 6. Claeys, K.G. (2020). Congenital myopathies: an update. *Developmental Medicine & Child
567 Neurology* 62, 297-302.

568 7. Ogasawara, M., and Nishino, I. (2023). A review of major causative genes in congenital
569 myopathies. *Journal of Human Genetics* 68, 215-225.

570 8. Mendieta-Serrano, M.A., Dhar, S., Ng, B.H., Narayanan, R., Lee, J.J.Y., Ong, H.T., Toh, P.J.Y.,
571 Röllin, A., Roy, S., and Saunders, T.E. (2022). Slow muscles guide fast myocyte fusion to
572 ensure robust myotome formation despite the high spatiotemporal stochasticity of fusion
573 events. *Developmental Cell* 57, 2095-2110.e2095.

574 9. Zempo, B., Yamamoto, Y., Williams, T., and Ono, F. Synaptic silencing of fast muscle is
575 compensated by rewired innervation of slow muscle. *Science Advances* 6, eaax8382.

576 10. Jackson, H.E., Ono, Y., Wang, X., Elworthy, S., Cunliffe, V.T., and Ingham, P.W. (2015). The
577 role of Sox6 in zebrafish muscle fiber type specification. *Skeletal Muscle* 5, 2.

578 11. Li, S., Wen, H., and Du, S. Defective sarcomere organization and reduced larval locomotion
579 and fish survival in slow muscle heavy chain 1 (smyhc1) mutants.

580 12. Stamm, D.S., Aylsworth, A.S., Stajich, J.M., Kahler, S.G., Thorne, L.B., Speer, M.C., and
581 Powell, C.M. (2008). Native American myopathy: Congenital myopathy with cleft palate,
582 skeletal anomalies, and susceptibility to malignant hyperthermia. *American Journal of
583 Medical Genetics Part A* 146A, 1832-1841.

584 13. Miller, D.M., Daly, C., Aboelsaod, E.M., Gardner, L., Hobson, S.J., Riasat, K., Shepherd, S.,
585 Robinson, R.L., Bilmen, J.G., Gupta, P.K., et al. Genetic epidemiology of malignant
586 hyperthermia in the UK.

587 14. Rufenach, B., and Van Petegem, F. Structure and function of STAC proteins: Calcium channel
588 modulators and critical components of muscle excitation-contraction coupling.

589 15. Cong, X., Doering, J., Mazala, D.A., Chin, E.R., Grange, R.W., and Jiang, H. (2016). The SH3
590 and cysteine-rich domain 3 (Stac3) gene is important to growth, fiber composition, and
591 calcium release from the sarcoplasmic reticulum in postnatal skeletal muscle. *Skeletal*
592 *muscle* 6, 1-17.

593 16. Bower, N.I., de la Serrana Dg Fau - Cole, N.J., Cole Nj Fau - Hollway, G.E., Hollway Ge Fau
594 - Lee, H.-T., Lee Ht Fau - Assinder, S., Assinder S Fau - Johnston, I.A., and Johnston, I.A.
595 Stac3 is required for myotube formation and myogenic differentiation in vertebrate skeletal
596 muscle.

597 17. Saini-Chohan, H.K., Mitchell Rw Fau - Vaz, F.M., Vaz Fm Fau - Zelinski, T., Zelinski T Fau -
598 Hatch, G.M., and Hatch, G.M. Delineating the role of alterations in lipid metabolism to the
599 pathogenesis of inherited skeletal and cardiac muscle disorders: Thematic Review Series:
600 Genetics of Human Lipid Diseases.

601 18. Seyssel, K., Alligier, M., Meugnier, E., Chanseaume, E., Loizon, E., Canto, C., Disse, E.,
602 Lambert-Porcheron, S., Brozek, J., Blond, E., et al. (2014). Regulation of Energy
603 Metabolism and Mitochondrial Function in Skeletal Muscle During Lipid Overfeeding in
604 Healthy Men. *The Journal of Clinical Endocrinology & Metabolism* 99, E1254-E1262.

605 19. Shimano, H., and Sato, R. (2017). SREBP-regulated lipid metabolism: convergent physiology
606 — divergent pathophysiology. *Nature Reviews Endocrinology* 13, 710-730.

607 20. Linsley, J.W., Hsu, I.-U., Groom, L., Yarotskyy, V., Lavorato, M., Horstick, E.J., Linsley, D.,
608 Wang, W., Franzini-Armstrong, C., and Dirksen, R.T. (2017). Congenital myopathy results
609 from misregulation of a muscle Ca²⁺ channel by mutant Stac3. *Proceedings of the*
610 *National Academy of Sciences* 114, E228-E236.

611 21. Rufenach, B., Christy, D., Flucher, B.E., Bui, J.M., Gsponer, J., Campiglio, M., and Van
612 Petegem, F. Multiple Sequence Variants in STAC3 Affect Interactions with Ca(V)1.1 and
613 Excitation-Contraction Coupling.

614 22. Horstick, E.J., Linsley, J.W., Dowling, J.J., Hauser, M.A., McDonald, K.K., Ashley-Koch, A.,
615 Saint-Amant, L., Satisch, A., Cui, W.W., and Zhou, W. (2013). Stac3 is a component of the
616 excitation–contraction coupling machinery and mutated in Native American myopathy.
617 Nature communications 4, 1952.

618 23. Berger, J., and Currie, P.D. Zebrafish models flex their muscles to shed light on muscular
619 dystrophies.

620 24. Sztal, T.E., Zhao, M., Williams, C., Oorschot, V., Parslow, A.C., Giousoh, A., Yuen, M., Hall,
621 T.E., Costin, A., Ramm, G., et al. (2015). Zebrafish models for nemaline myopathy reveal
622 a spectrum of nemaline bodies contributing to reduced muscle function. Acta
623 Neuropathologica 130, 389-406.

624 25. Daya, A., Donaka, R., and Karasik, D. (2020). Zebrafish models of sarcopenia. Disease
625 Models & Mechanisms 13, dmm042689.

626 26. Mesika, A., Nadav, G., Shochat, C., Kalfon, L., Jackson, K., Khalailah, A., Karasik, D., and
627 Falik-Zaccai, T.C. (2022). NGLY1 Deficiency Zebrafish Model Manifests Abnormalities of
628 the Nervous and Musculoskeletal Systems. Frontiers in Cell and Developmental Biology
629 10.

630 27. Li, H., Xu, J., Bian, Y.-H., Rotllant, P., Shen, T., Chu, W., Zhang, J., Schneider, M., and Du,
631 S.J. (2011). Smyd1b_tv1, a Key Regulator of Sarcomere Assembly, Is Localized on the M-
632 Line of Skeletal Muscle Fibers. PLOS ONE 6, e28524.

633 28. Kishi, S., Bayliss, P.E., Uchiyama, J., Koshimizu, E., Qi, J., Nanjappa, P., Imamura, S., Islam,
634 A., Neuberg, D., Amsterdam, A., et al. (2008). The Identification of Zebrafish Mutants
635 Showing Alterations in Senescence-Associated Biomarkers. PLOS Genetics 4, e1000152.

636 29. Livne, H., Avital, T., Ruppo, S., Harazi, A., Mitrani-Rosenbaum, S., and Daya, A. (2022).
637 Generation and characterization of a novel gene Knockout Model in Zebrafish. *Frontiers in*
638 *Cell and Developmental Biology* 10.

639 30. Sokoloff, A.J., Yang, B., Li, H., and Burkholder, T.J. (2007). Immunohistochemical
640 characterization of slow and fast myosin heavy chain composition of muscle fibres in the
641 styloglossus muscle of the human and macaque (*Macaca rhesus*). *Arch Oral Biol* 52, 533-
642 543.

643 31. Stewart, M.A., Franks-Skiba, K., Chen, S., and Cooke, R. (2010). Myosin ATP turnover rate
644 is a mechanism involved in thermogenesis in resting skeletal muscle fibers. *Proc Natl Acad*
645 *Sci U S A* 107, 430-435.

646 32. Yamamoto, M., Legendre, N.P., Biswas, A.A., Lawton, A., Yamamoto, S., Tajbakhsh, S.,
647 Kardon, G., and Goldhamer, D.J. Loss of MyoD and Myf5 in Skeletal Muscle Stem Cells
648 Results in Altered Myogenic Programming and Failed Regeneration.

649 33. Cao, Y., Kumar Rm Fau - Penn, B.H., Penn Bh Fau - Berkes, C.A., Berkes Ca Fau -
650 Kooperberg, C., Kooperberg C Fau - Boyer, L.A., Boyer La Fau - Young, R.A., Young Ra
651 Fau - Tapscott, S.J., and Tapscott, S.J. Global and gene-specific analyses show distinct
652 roles for Myod and Myog at a common set of promoters.

653 34. Reinholt, B.M., Ge, X., Cong, X., Gerrard, D.E., and Jiang, H. (2013). Stac3 is a novel
654 regulator of skeletal muscle development in mice. *PLoS one* 8, e62760.

655 35. Shi, H., Verma, M., Zhang, L., Dong, C., Flavell, R.A., and Bennett, A.M. (2013). Improved
656 regenerative myogenesis and muscular dystrophy in mice lacking Mkp5. *The Journal of*
657 *Clinical Investigation* 123, 2064-2077.

658 36. Quinlivan, V.H., and Farber, S.A. (2017). Lipid Uptake, Metabolism, and Transport in the
659 Larval Zebrafish. *Frontiers in Endocrinology* 8.

660 37. Jiang, K., Pichol-Thievend, C., Neufeld, Z., and Francois, M. Assessment of heterogeneity in
661 collective endothelial cell behavior with multicolor clonal cell tracking to predict
662 arteriovenous remodeling.

663 38. Stamm, D.S., Aylsworth As Fau - Stajich, J.M., Stajich Jm Fau - Kahler, S.G., Kahler Sg Fau
664 - Thorne, L.B., Thorne Lb Fau - Speer, M.C., Speer Mc Fau - Powell, C.M., and Powell,
665 C.M. Native American myopathy: congenital myopathy with cleft palate, skeletal
666 anomalies, and susceptibility to malignant hyperthermia.

667 39. Kepser, L.-J., Damar, F., De Cicco, T., Chaponnier, C., Prószyński, T.J., Pagenstecher, A.,
668 and Rust, M.B. (2019). CAP2 deficiency delays myofibril actin cytoskeleton differentiation
669 and disturbs skeletal muscle architecture and function. *Proceedings of the National
670 Academy of Sciences* 116, 8397-8402.

671 40. Barcelos, R.P., Lima, F.D., Carvalho, N.R., Bresciani, G., and Royes, L.F. Caffeine effects on
672 systemic metabolism, oxidative-inflammatory pathways, and exercise performance.

673 41. Tallis, J., James Rs Fau - Cox, V.M., Cox Vm Fau - Duncan, M.J., and Duncan, M.J. The
674 effect of physiological concentrations of caffeine on the power output of maximally and
675 submaximally stimulated mouse EDL (fast) and soleus (slow) muscle.

676 42. Gonçalves, D.F., Tassi, C.C., Amaral, G.P., Stefanello, S.T., Dalla Corte, C.L., Soares, F.A.,
677 Posser, T., Franco, J.L., and Carvalho, N.R. Effects of caffeine on brain antioxidant status
678 and mitochondrial respiration in acetaminophen-intoxicated mice.

679 43. Nelson, B.R., Wu, F., Liu, Y., Anderson, D.M., McAnally, J., Lin, W., Cannon, S.C., Bassel-
680 Duby, R., and Olson, E.N. (2013). Skeletal muscle-specific T-tubule protein STAC3
681 mediates voltage-induced Ca²⁺ release and contractility. *Proceedings of the National
682 Academy of Sciences* 110, 11881-11886.

683 44. Conerly, M.L., Yao, Z., Zhong, J.W., Groudine, M., and Tapscott, S.J. Distinct Activities of
684 Myf5 and MyoD Indicate Separate Roles in Skeletal Muscle Lineage Specification and
685 Differentiation.

686 45. Ganassi, M., Badodi, S., Wanders, K., Zammit, P.S., and Hughes, S.M. (2020). Myogenin is
687 an essential regulator of adult myofibre growth and muscle stem cell homeostasis. *eLife* 9,
688 e60445.

689 46. Mok, G.F., Mohammed, R.H., and Sweetman, D. Expression of myogenic regulatory factors
690 in chicken embryos during somite and limb development.

691 47. Malila, Y., Thanatsang, K.V., Sanpinit, P., Arayamethakorn, S., Soglia, F., Zappaterra, M.,
692 Bordini, M., Sirri, F., Rungrassamee, W., Davoli, R., et al. (2022). Differential expression
693 patterns of genes associated with metabolisms, muscle growth and repair in Pectoralis
694 major muscles of fast- and medium-growing chickens. *PLOS ONE* 17, e0275160.

695 48. Wen, C., Jiang, X., Ding, L., Wang, T., and Zhou, Y. (2017). Effects of dietary methionine on
696 breast muscle growth, myogenic gene expression and IGF-I signaling in fast- and slow-
697 growing broilers. *Scientific Reports* 7, 1924.

698 49. Coppens, S., Barnard, A.M., Puusepp, S., Pajusalu, S., Õunap, K., Vargas-Franco, D., Bruels,
699 C.C., Donkervoort, S., Pais, L., Chao, K.R., et al. (2021). A form of muscular dystrophy
700 associated with pathogenic variants in JAG2. *The American Journal of Human Genetics*
701 108, 840-856.

702 50. Jungbluth, H., Ochala, J., Treves, S., and Gautel, M. (2017). Current and future therapeutic
703 approaches to the congenital myopathies. *Seminars in Cell & Developmental Biology* 64,
704 191-200.

705 51. Schessl, J., Taratuto, A.L., Sewry, C., Battini, R., Chin, S.S., Maiti, B., Dubrovsky, A.L., Erro,
706 M.G., Espada, G., Robertella, M., et al. (2009). Clinical, histological and genetic

characterization of reducing body myopathy caused by mutations in FHL1. *Brain* 132, 452-464.

52. Böhm, J., Vasli, N., Malfatti, E., Le Gras, S., Feger, C., Jost, B., Monnier, N., Brocard, J., Karasoy, H., Gérard, M., et al. (2013). An Integrated Diagnosis Strategy for Congenital Myopathies. *PLOS ONE* 8, e67527.

53. Cassandrini, D., Trovato, R., Rubegni, A., Lenzi, S., Fiorillo, C., Baldacci, J., Minetti, C., Astrea, G., Bruno, C., Santorelli, F.M., et al. (2017). Congenital myopathies: clinical phenotypes and new diagnostic tools. *Italian Journal of Pediatrics* 43, 101.

54. Stamm, D., Powell, C., Stajich, J., Zismann, V., Stephan, D., Chesnut, B., Aylsworth, A., Kahler, S., Deak, K., and Gilbert, J. (2008). Novel congenital myopathy locus identified in Native American Indians at 12q13. 13-14.1. *Neurology* 71, 1764-1769.

55. Talbot, J., and Maves, L. Skeletal muscle fiber type: using insights from muscle developmental biology to dissect targets for susceptibility and resistance to muscle disease.

56. Miyares, R.L., de Rezende, V.B., and Farber, S.A. Zebrafish yolk lipid processing: a tractable tool for the study of vertebrate lipid transport and metabolism.

57. Fraher, D., Sanigorski, A., Mellett, Natalie A., Meikle, Peter J., Sinclair, Andrew J., and Gibert, Y. (2016). Zebrafish Embryonic Lipidomic Analysis Reveals that the Yolk Cell Is Metabolically Active in Processing Lipid. *Cell Reports* 14, 1317-1329.

58. Goi, M., and Childs, S.J. (2016). Patterning mechanisms of the sub-intestinal venous plexus in zebrafish. *Developmental Biology* 409, 114-128.

59. Edinburgh, R.M., Bradley, H.E., Abdullah, N.-F., Robinson, S.L., Chrzanowski-Smith, O.J., Walhin, J.-P., Joanisse, S., Manolopoulos, K.N., Philp, A., Hengist, A., et al. (2020). Lipid Metabolism Links Nutrient-Exercise Timing to Insulin Sensitivity in Men Classified as Overweight or Obese. *The Journal of Clinical Endocrinology & Metabolism* 105, 660-676.

731 60. Goodpaster, B.H., Bergman, B.C., Brennan, A.M., and Sparks, L.M. (2022). Intermuscular
732 adipose tissue in metabolic disease. *Nature Reviews Endocrinology*.

733 61. Yoganantharjah, P., Byreddy Ar Fau - Fraher, D., Fraher D Fau - Puri, M., Puri M Fau - Gibert,
734 Y., and Gibert, Y. Rapid quantification of neutral lipids and triglycerides during zebrafish
735 embryogenesis.

736 62. Sinha, S., Elbaz-Alon, Y., and Avinoam, O. (2022). Ca²⁺ as a coordinator of skeletal muscle
737 differentiation, fusion and contraction. *The FEBS Journal* 289, 6531-6542.

738 63. Telegrafi, A, Webb, B, Robbins, S.M., Speck-Martins, C.E., FitzPatrick, D., Fleming, L.A,
739 Redett, R., Dufke, A., Houge, G, van Harssel, J.J.T., et al. Identification of STAC3 variants
740 in non-Native American families with overlapping features of Carey-Fineman-Ziter
741 syndrome and Moebius syndrome.

742 64. Gromand, M., Gueguen, P., Pervillé, A., Ferroul, F., Morel, G., Harouna, A., Doray, B.,
743 Urtizberea, J.A., Alessandri, J.-L., and Robin, S. (2022). STAC3 related congenital
744 myopathy: A case series of seven Comorian patients. *European Journal of Medical
745 Genetics* 65, 104598.

746 65. Murtazina, A., Demina, N., Chausova, P., Shchagina, O., Borovikov, A., and Dadali, E. (2022).
747 The First Russian Patient with Native American Myopathy. In *Genes*.

748 66. Patton, E.E., Zon, L.I., and Langenau, D.M. (2021). Zebrafish disease models in drug
749 discovery: from preclinical modelling to clinical trials. *Nature Reviews Drug Discovery* 20,
750 611-628.

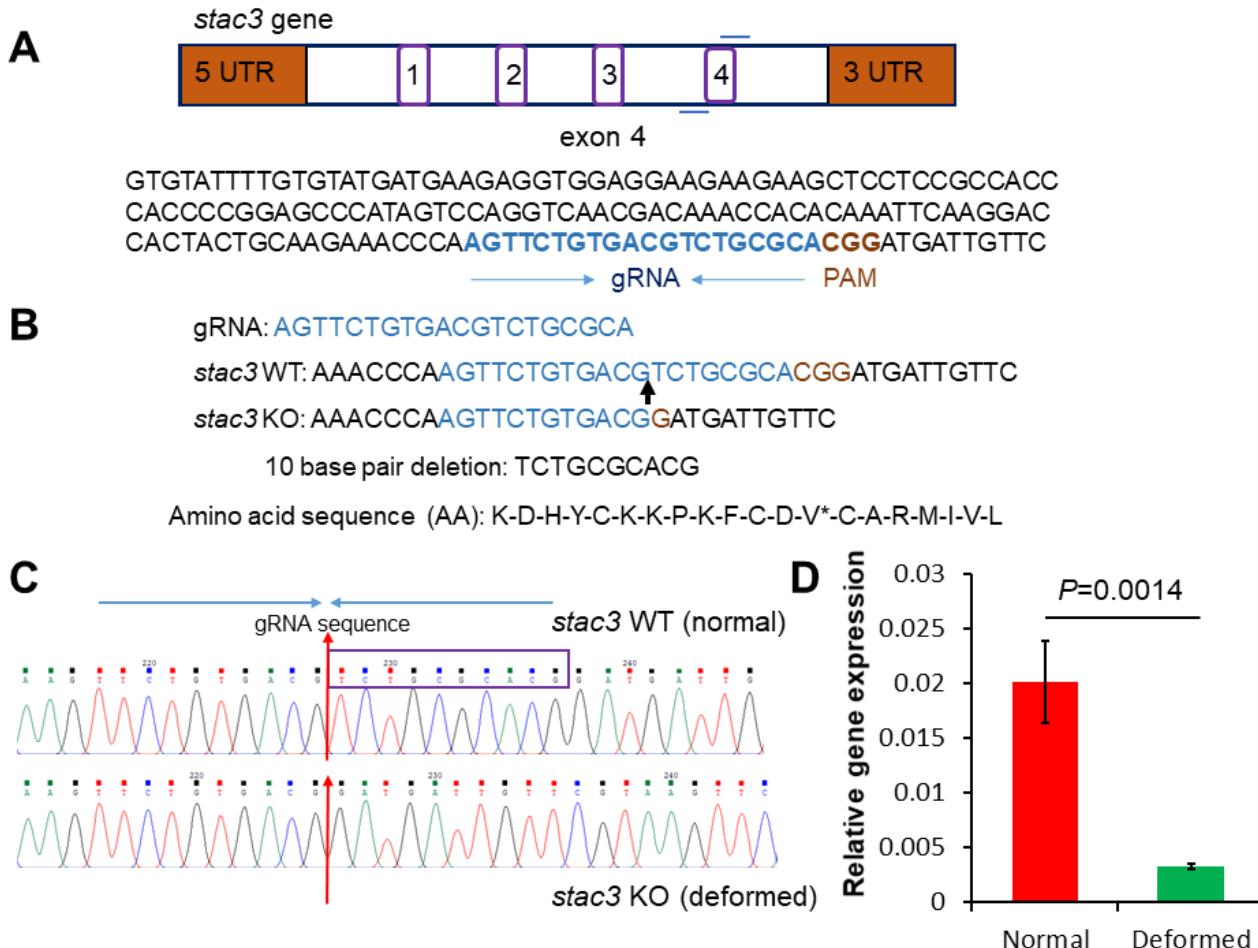
751 67. Espinosa, K.G., Geissah, S., Groom, L., Volpatti, J., Scott, I, Dirksen, R.T., Zhao, M, and
752 Dowling, J. Characterization of a novel zebrafish model of SPEG-related centronuclear
753 myopathy. *LID - 10.1242/dmm.049437*.

754

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Figure legends

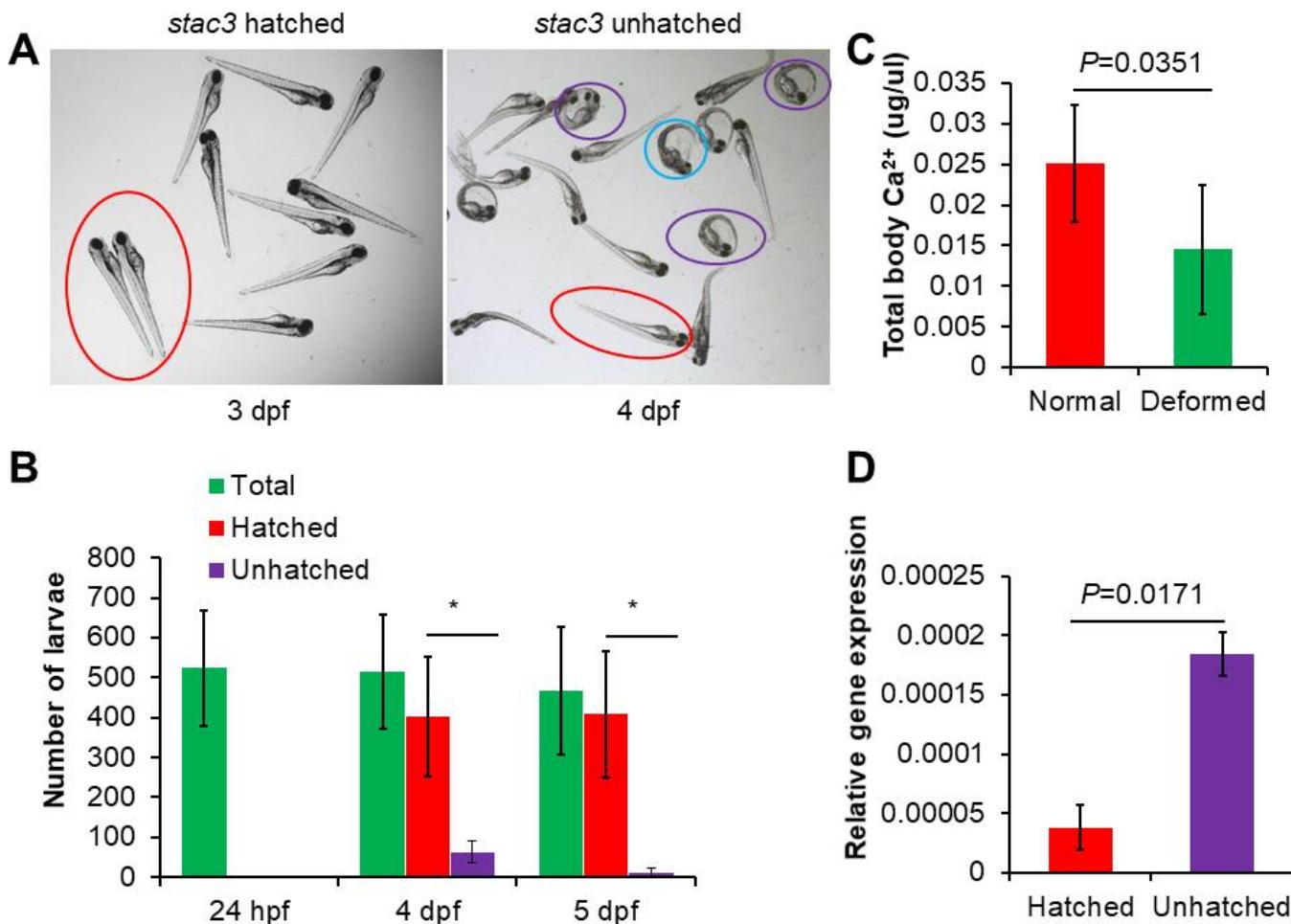
756 1. Deletion of 10 base pairs (bp) of *stac3* gene in zebrafish



757

758 (A, B). Schematic presentation of the *stac3* gene and nucleotide sequence of exon 4 region, which
759 is marked for both guide RNA and PAM sequences, amino acids predicted using Ensembl.org.
760 The stop codon at V78 is shown with asterisk. (C). DNA sequencing of the *stac3* wild type siblings
761 (top), knockout (bottom). The deleted sequence TCTGCGCACG is marked by a violet box, red
762 arrows indicate the starting position of the knockout. (D). RT-PCR analysis showed significant
763 downregulation of *stac3* expression in deformed larvae (knockout) compared to wild type and
764 heterozygous siblings (5 dpf). Data are presented as mean \pm standard deviation. T-Test, *p=
765 0.0014.

766 2. Delayed hatching of *stac3*^{-/-} (KO) zebrafish larvae

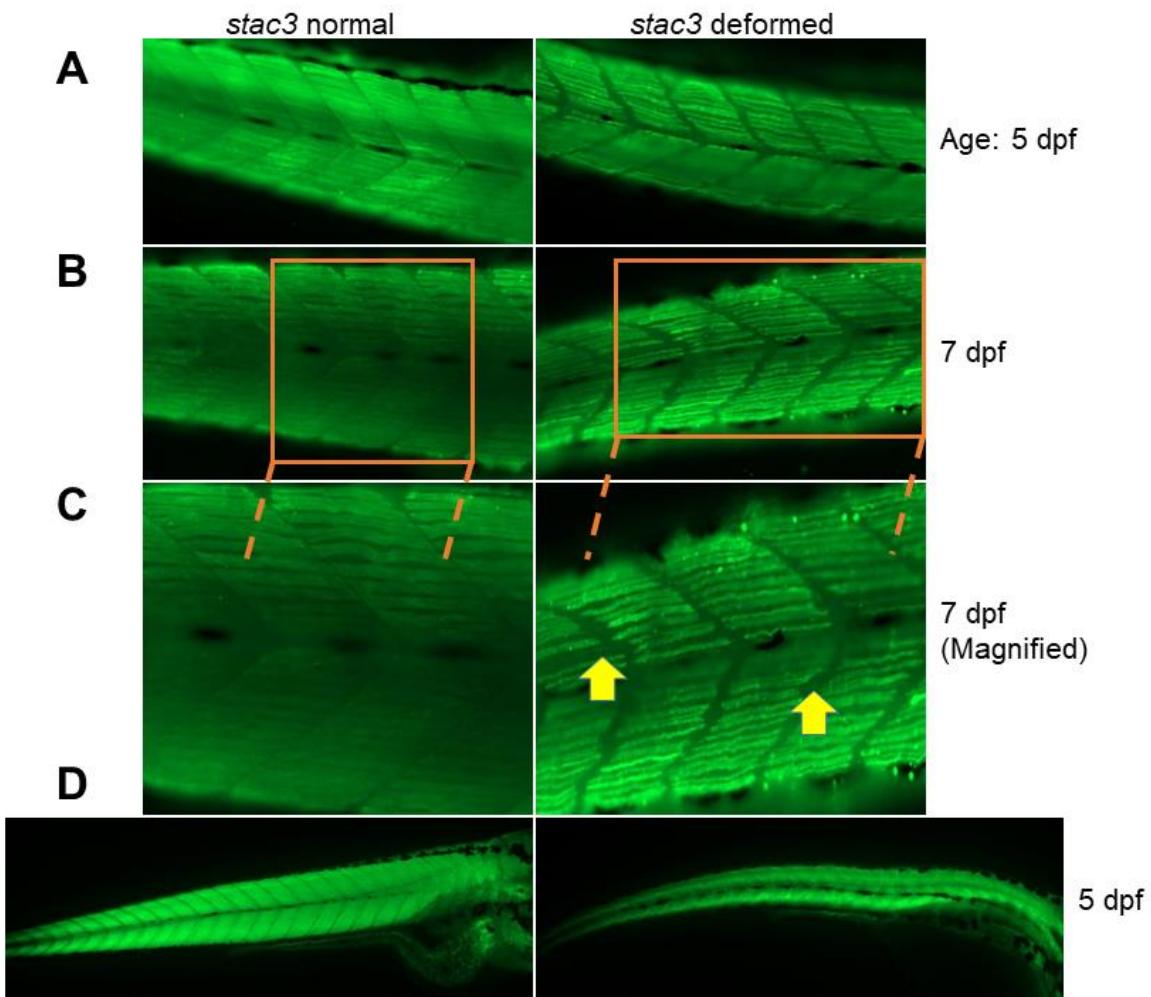


767

768 (A). Hatching of *stac3*^{-/-} larvae at 3 and 4 days post-fertilization (dpf). The blue circle marks larvae
769 attempting to come out of the chorion (delayed hatching: knockout), while violet color indicates
770 unhatched (knockout) larvae, hatched (*stac3*^{+/+} and *stac3*^{+/−}: wild type and heterozygous siblings)
771 larvae are outlined in red. (B). Percentage of delayed hatching of *stac3*^{-/-} larvae at different time
772 points, T-Test, *p=0.018 (4 dpf), *p=0.012 (5 dpf). (C). Whole-body Ca²⁺ levels in *stac3*^{-/-} larvae,
773 T-Test, *p=0.0351. (D). Ryanodine receptor 1a (*rry1a*) gene expression, T-Test, *p=0.0171. Data
774 are presented as mean ± standard deviation.

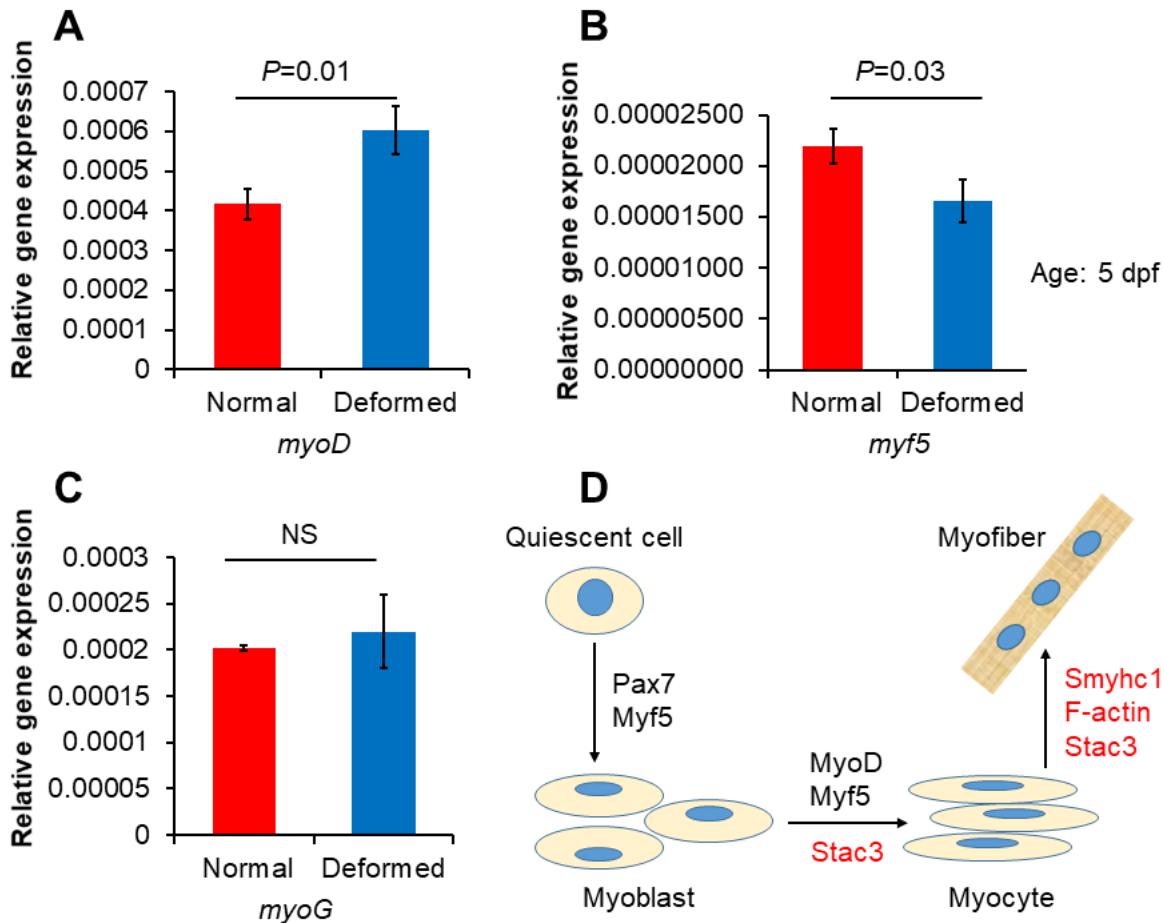
775

776 3. Slow muscle myosin staining of KO larvae



778 (A). Whole-mount immunostaining of slow muscle myosin isoforms with anti-Myhc1 antibody, in
779 the trunk region, *stac3* KO larvae showed thin, curly muscle fibers, and smaller gaps compared
780 to *stac3* WT and heterozygous fish at 5 dpf. (B). In the trunk region, *stac3* KO larvae (right)
781 exhibited protruding breaks between somite boundaries compared to muscle fibers patterning's
782 in *stac3^{+/+}* and *stac3⁺⁻* control siblings (left) at 7 dpf. (C). Bottom panels (B) are magnified;
783 protruding breaks between somite boundaries are marked with yellow arrow heads. (D). Wild type
784 and heterozygous *stac3* and *stac3* knockout larvae stained with phalloidin at 5 dpf. Reduced
785 amounts of filamentous (F-actin) fibers were noted in whole body of *stac3* knockout larvae (right)
786 compared to *stac3^{+/+}* and *stac3⁺⁻* control siblings (left).

787 4. Knockout of *stac3* alters myogenesis in zebrafish larvae



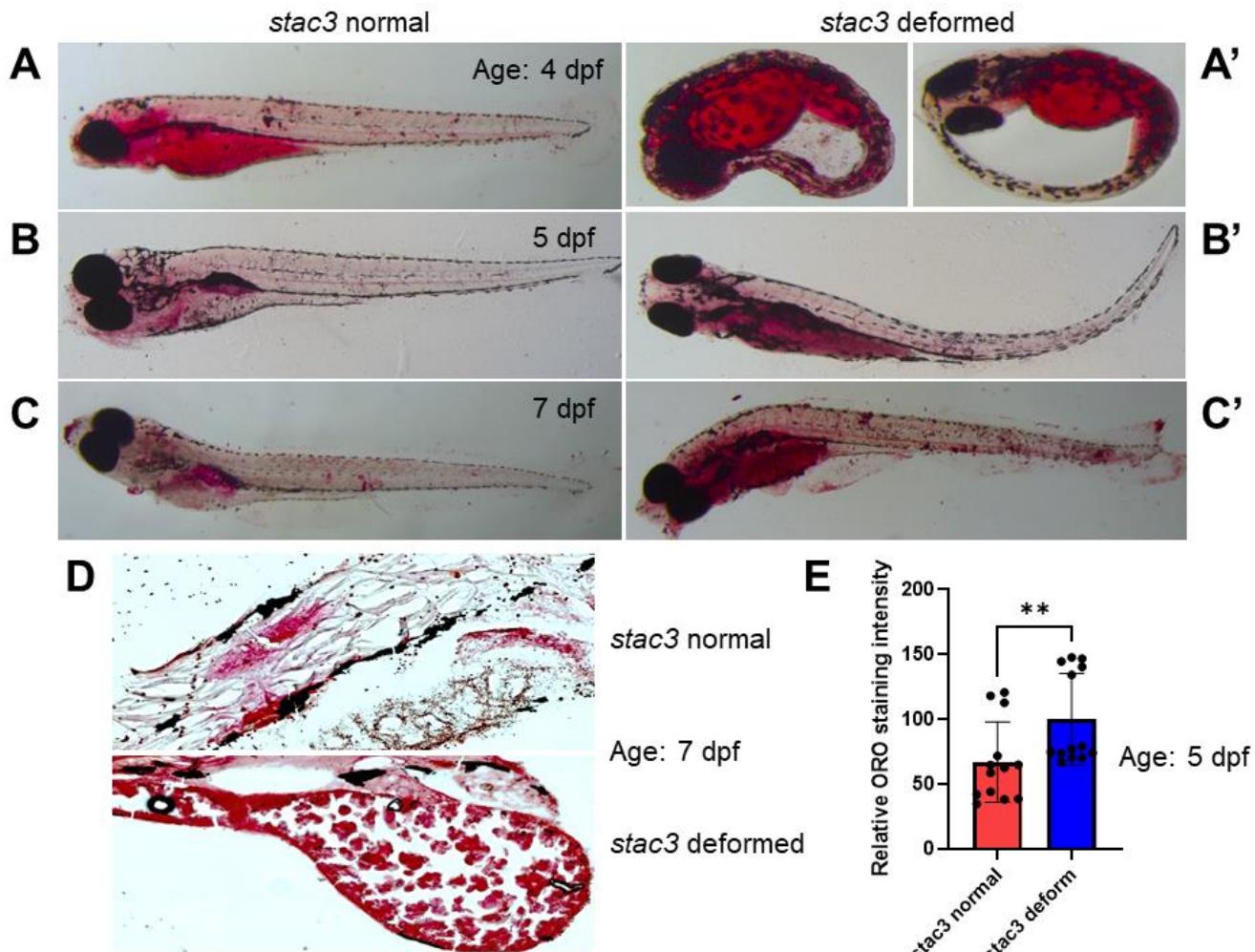
788

789 (A). RT-PCR analysis revealed that gene expression of *myoD* was significantly upregulated in
790 *stac3*^{-/-} larvae compared to *stac3* wild type and heterozygotes (B). *myf5* gene expression was
791 significantly downregulated in *stac3*^{-/-} larvae compared to wild type and heterozygous siblings.
792 (C). *stac3* knockout had no effect on mRNA levels of *myoG*. (D). A suggested mechanistic action
793 of *stac3* in early muscle formation (red color denote functional regulators). T-Test, * $p=0.01$,
794 * $p=0.03$, Non-significant (NS), n=10 animals for each group. Data are presented as mean \pm
795 standard deviation.

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797

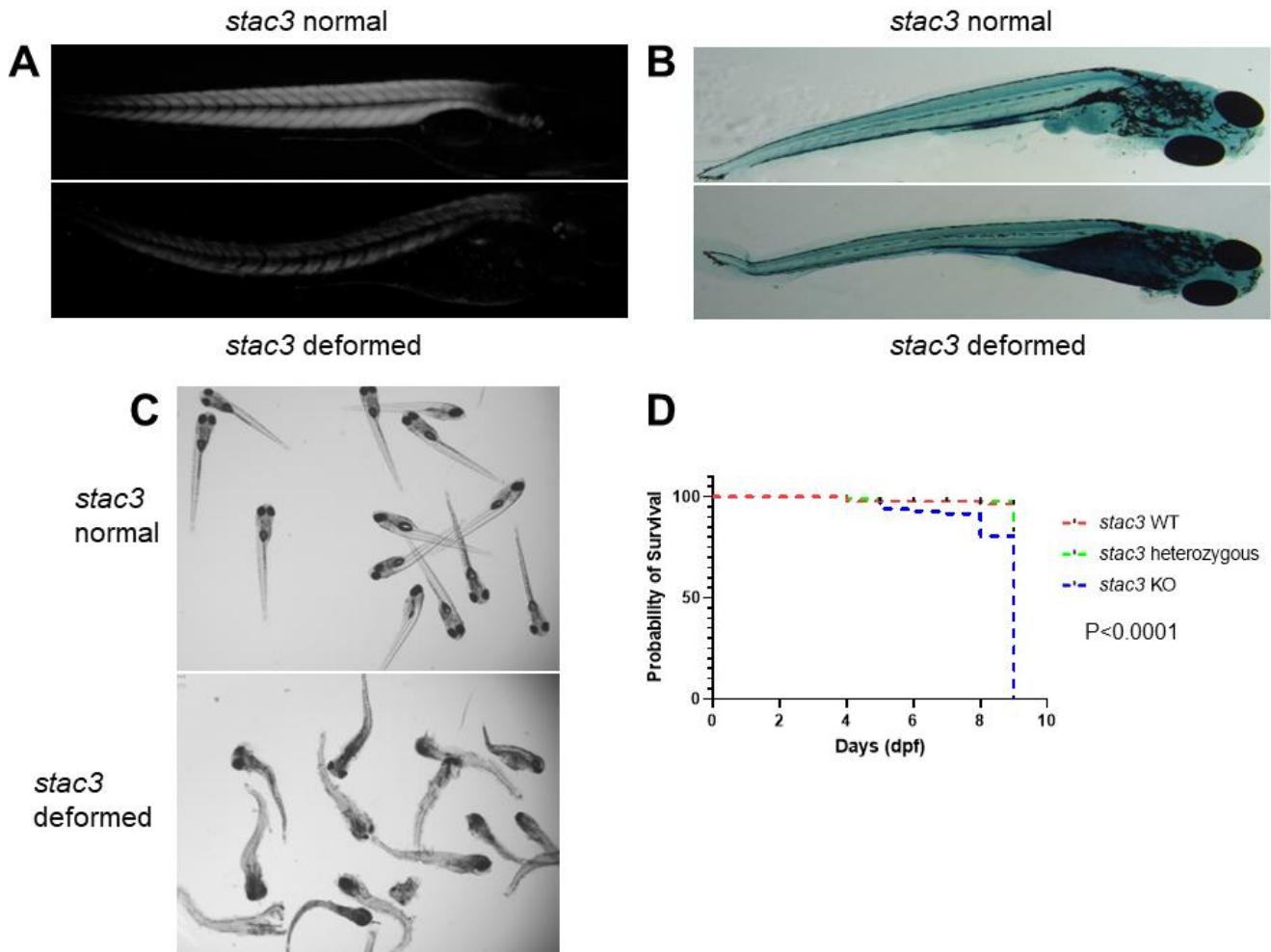
798 5. Lipid staining of *stac3* KO larvae using Oil Red O (ORO)



799

800 (A-A'). Hatched (*stac3* wild type and heterozygous) and unhatched (*stac3* knockout) larvae were
801 stained with ORO dye for neutral lipid visualization, both groups displayed similar levels of neutral
802 lipids at 4 dpf (scale bar 3 mm). At 5 dpf (B-B') and 7 dpf (C-C'): scale bar 3 mm), deformed (*stac3*
803 knockout: B' and C') larvae exhibited higher neutral lipids compared to *stac3* wild type and
804 heterozygous (B and C). (D). OCT based yolk sac sections of *stac3* normal and knockout larvae
805 were stained with ORO dye. *stac3* knockout larvae (bottom) displayed more lipids compared to
806 *stac3* normal (top) at 7 dpf. (E). Quantification red color indicated relative ORO staining intensity
807 of the larvae. At each stage n=15 animals utilized per group. Mann Whitney test, **p=0.0023.
808 Genotype of larvae (n=10) per group was confirmed as described in FigureS1A, B.

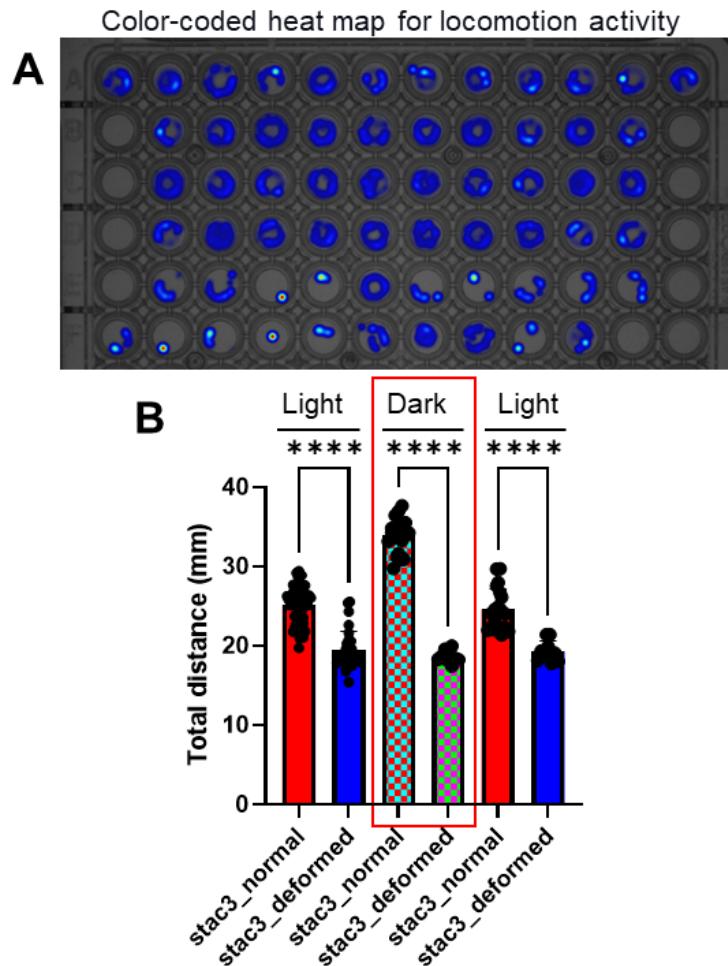
809 6. Death of *stac3* knockout might be due to metabolic dysfunction



810

811 (A). Birefringence assay demonstrated reduced chevron formation of muscle fibers in *stac3*
812 knockout (bottom) compared to wild type *stac3* larvae (top) at 5 dpf. (B). *stac3* knockout larvae
813 exhibited increased cellular senescence mostly in the yolk sac region (bottom) in comparison to
814 wild type *stac3* (top), as demonstrated by SA- β -gal staining at 6 dpf. (C). Dead larvae are
815 identified as *stac3* knockout (bottom) compared to *stac3* wild type and heterozygous siblings (top)
816 at 5 dpf. (D). Survival analysis of *stac3*^{-/-}, wild type and heterozygous siblings at 1-10 dpf. Post
817 birefringence analysis, larvae (n=5-10) per group were utilized for genotype confirmation. Log-
818 rank test applied for *stac3* WT, *stac3* heterozygous, and *stac3* KO zebrafish larva groups,
819 ****p<0.0001. The genotype of dead larvae (n=80) was confirmed as described in FigureS1A, B.

820 7. Swimming performance (locomotion) of *stac3* knockout in early life



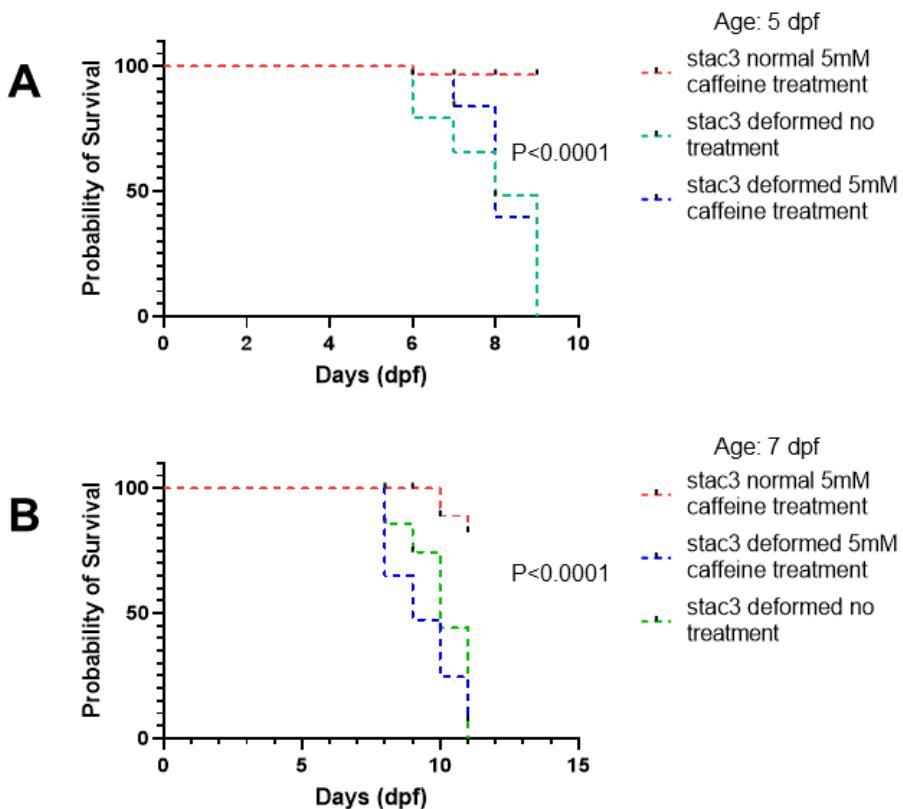
821

822 (A). Merged, color-coded heat map for the total distance travelled by each larvae at 5 dpf. The
823 blue color corresponds to a longer distance travelled, while pale yellow-red corresponds to no
824 movement activity. (B). The locomotion tracking protocol included 15 minutes light on, 5 minutes
825 light off, then 5 minutes light on. One-way ANOVA, ***p=0.0001, *stac3*^{+/+} and *stac3*^{-/-} (n=48) and
826 *stac3*^{-/-} (n=22) animals for each group. Genotype of larvae (n=10) per group was confirmed as
827 described in FigureS1A, B. Each dot represents the total distance travelled by the larvae on graph
828 Data are presented as mean \pm standard deviation.

829

830

831 8. Survival of the *stac3*^{-/-} larvae at 5 dpf and 7 dpf after caffeine treatment



832

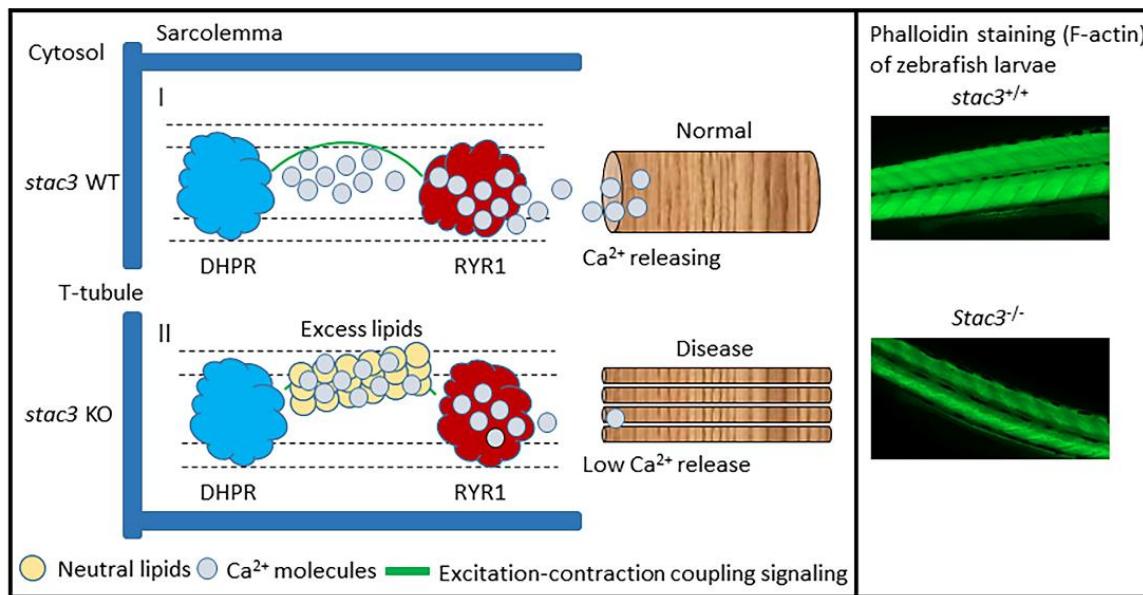
833 (A-B). Calculation of survival percentage of *stac3* KO (deformed) and *stac3* normal (*stac3*^{+/+} and
834 *stac3*⁺⁻ control siblings) zebrafish larvae with and without 5mM caffeine at 5 and 7 dpf. In both
835 age groups, survival analysis was performed in 2-3 replicates, and larvae growth and survival
836 were monitored for 96 hours. Log-rank test applied between treated and untreated deformed
837 *stac3* zebrafish groups, ***p<0.0001.

838

839

840

841 9. Working model of *stac3* gene in early life of zebrafish



842

843 In *stac3* knockout, increased lipids can directly reduce Ca²⁺ release at DHPR via RYR1 (*ryr1a*)
844 receptor which seemingly delays the functional organization of F-actin and slow muscle fibers of
845 zebrafish larva at early life.

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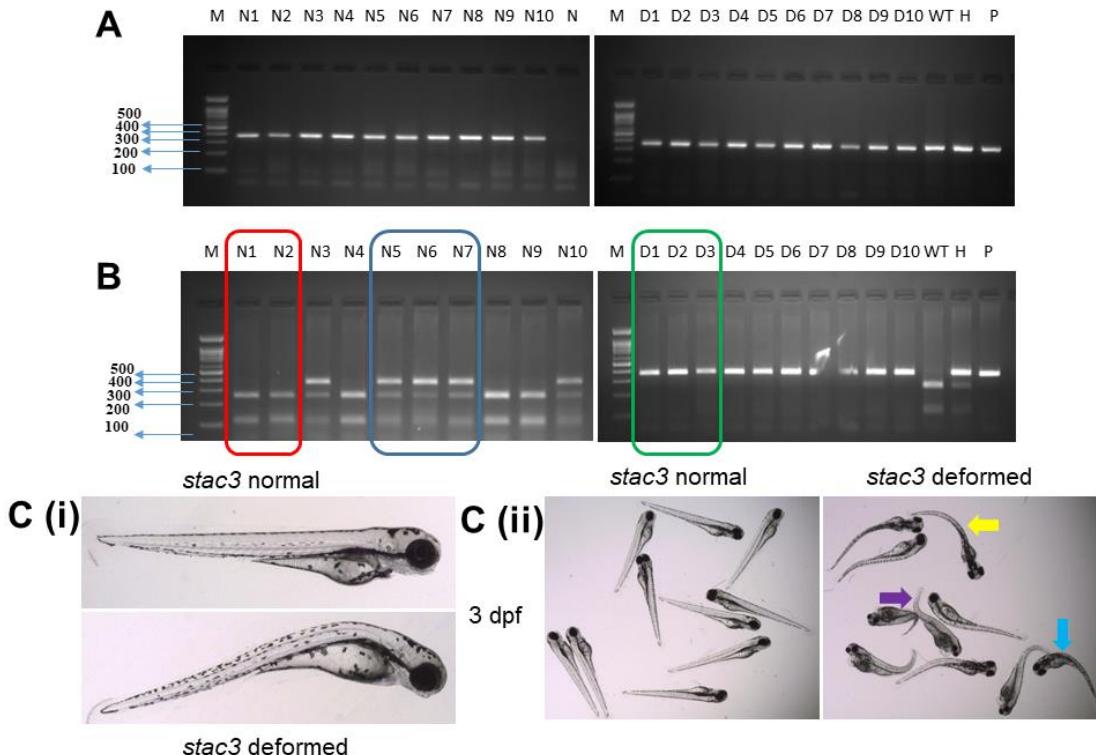
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Supplementary figures and legends

855 **S1. Knockout confirmation of the *stac3* gene in zebrafish**

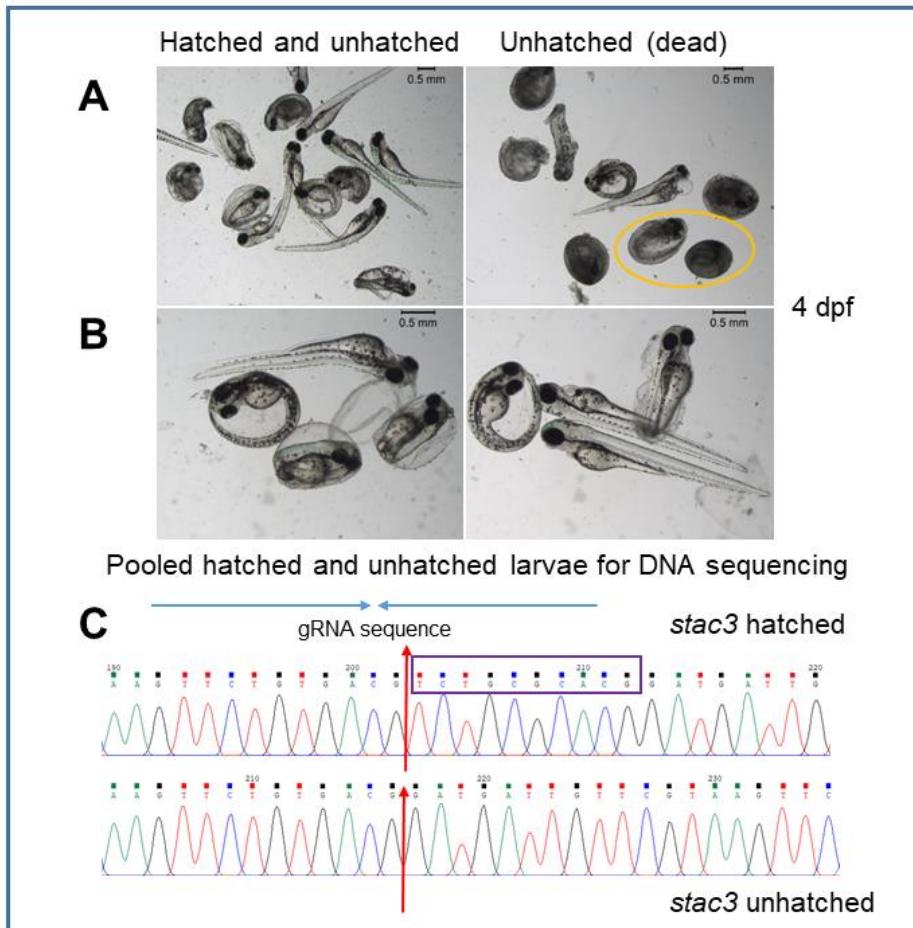


856

857 (A). DNA was extracted from wild type and heterozygous and knockout *stac3* larvae at the age of
858 3 dpf and amplified in a polymerase chain reaction (PCR) using *stac3* gene-specific primers (top).
859 (B). The PCR product was confirmed on a 2% agarose gel, which showed a single band at 363
860 base pairs (bp). The PCR product was digested using the *FSP1* enzyme. The wild type had two
861 digested DNA products (250 bp and 113 bp, red). The heterozygotes had three digested DNA
862 products (113 bp, 250 bp, and 363 bp, blue). The knockout undigested PCR product was a single
863 band at 363 bp (green) (bottom). M-marker, N1-N10 (normal: *stac3*^{+/+} and *stac3*⁺⁻ siblings): wild
864 type and heterozygous, D1-D10 (deformed: *stac3*^{-/-}): knockout, WT-wild type, H-heterozygote, P-
865 positive control. (C). At 3 days post-fertilization, *stac3*^{-/-} mutant larvae were distinguishable from

866 *stac3^{+/+}* and *stac3^{+/-}* siblings (i), and displayed multiple congenital musculoskeletal defects, which
867 included bending at the head (blue), trunk (yellow), and tail regions (violet) (ii). Scale bar: 1 mm.

868 **S2. Genotype confirmation of delayed hatching of larvae by sequencing at 4 dpf**

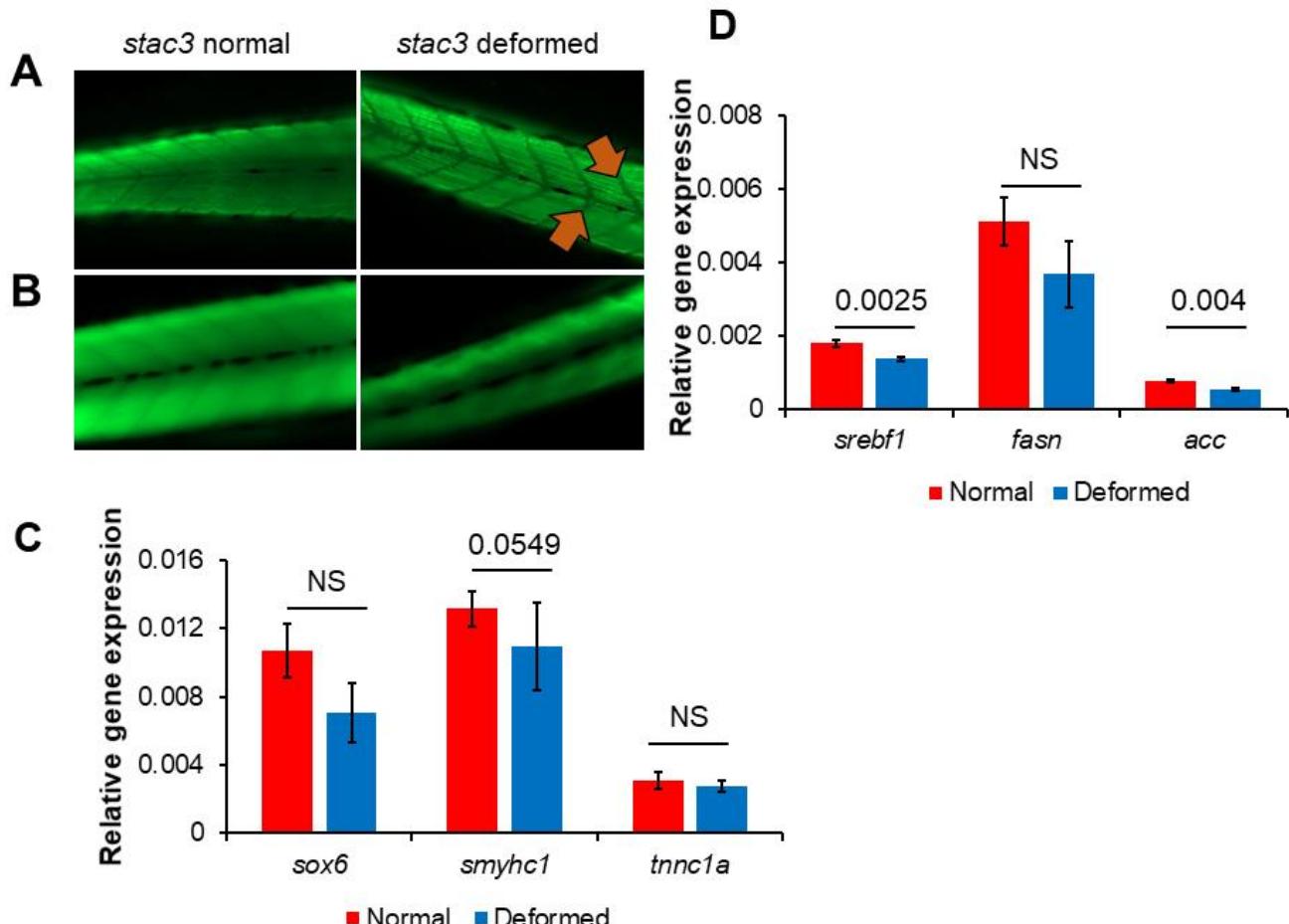


869

870 (A). Hatched and unhatched *stac3* larvae (left panel). Dead *stac3^{-/-}* larvae within chorion are
871 shown in the yellow circle in the right panel. (B). Representative images of hatched and unhatched
872 *stac3* larvae (n=10) pooled for sequencing (left and right panel). (C). All hatched and unhatched
873 larvae confirmed as wild type (top), heterozygous (not shown), and knockout larvae (bottom)
874 respectively. Knockout larvae acquired a 10 bp deletion mutation, the TCTGCGCACG sequence
875 is marked by a violet box. Scale bar: 0.5 mm.

876

877 **S3. F-actin and slow muscle myosin staining of knockout larvae**

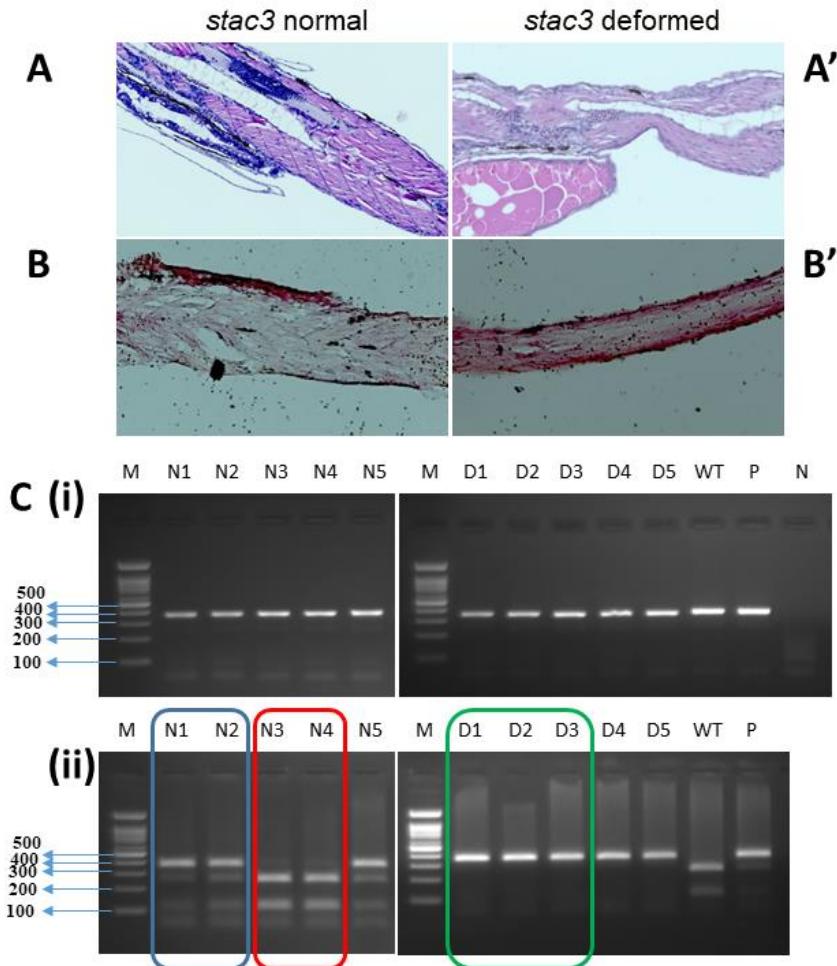


878

879 (A). Tail region of wild type and heterozygous *stac3* and *stac3* knockout larvae stained with F59
880 antibody. *stac3*^{-/-} larvae (right) showed disorganized slow muscle fibers compared to *stac3* wild
881 type and heterozygous siblings (left), while orange arrowheads indicate alterations in muscle
882 fibers. (B). Reduced amounts of filamentous (F-actin) fibers were noted in whole body of *stac3*
883 knockout larvae (right) compared to control siblings (left) stained with phalloidin and followed by
884 '20X' magnification at 5 dpf. (C). RT-PCR analysis revealed that gene expression of skeletal
885 muscle markers such as *sox6*, *smyhc1*, and *tnnc1a* was unaltered, (D). whereas *srebf1* and its
886 down-stream target acetyl co-enzyme-A were significantly down regulated, in *stac3*^{-/-} larvae

887 compared to *stac3* “normal” (wild type and heterozygotes). T-Test, **p=0.0025 and p=0.004. Non-
888 significant (NS), n=10 animals for each group. Data are presented as mean \pm standard deviation

889 **S4. Muscle histology of *stac3*^{-/-} larvae**

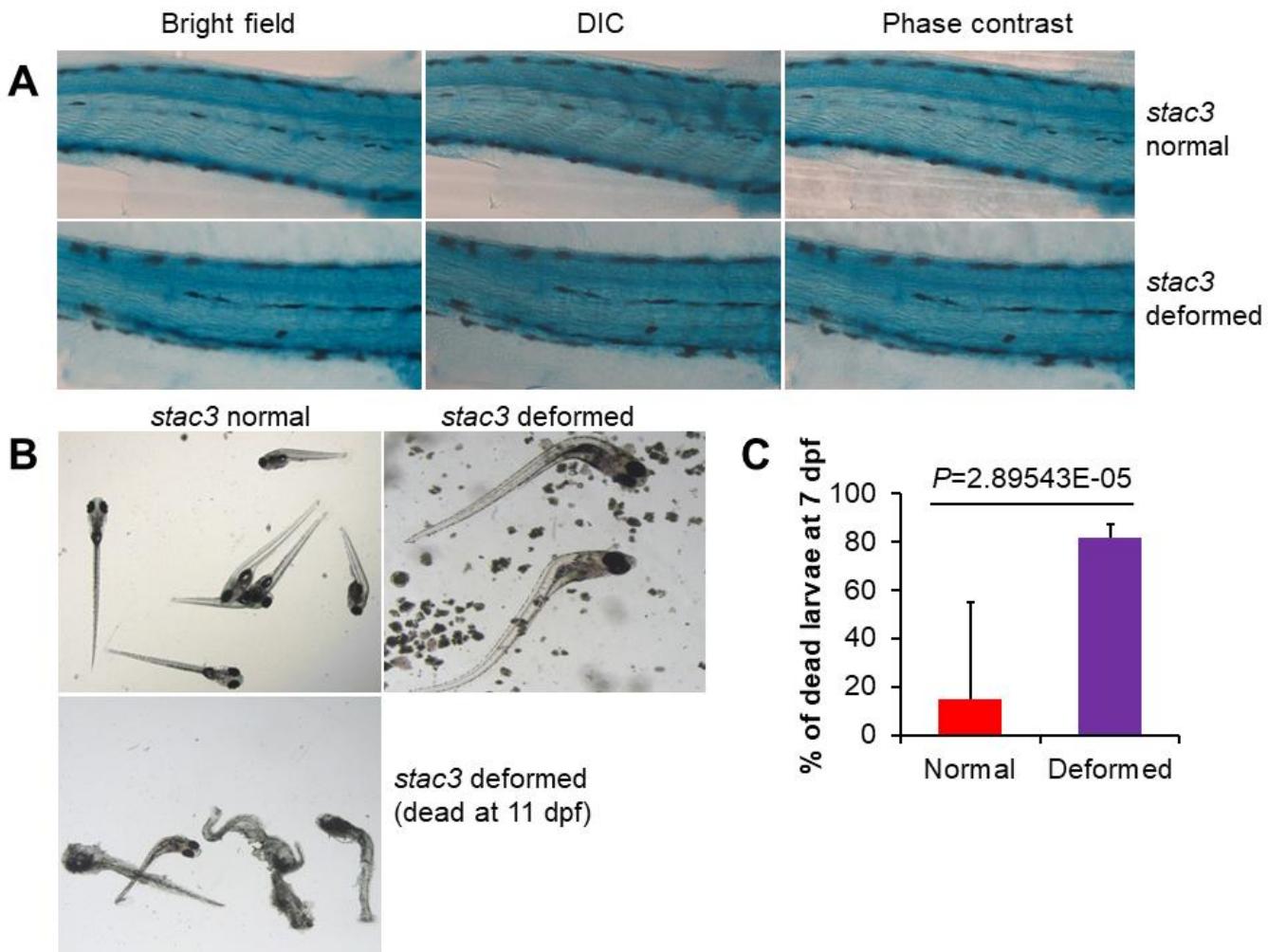


890

891 (A-A'). Paraffin based muscle sections of *stac3* wild type and heterozygous siblings and *stac3*
892 knockout larvae stained with hematoxylin showed disorganized fibers in the knockout fish (A').
893 (B-B'). OCT based muscle sections of *stac3*^{+/+} and *stac3*^{-/-} control siblings and knockout larvae
894 were stained with ORO dye. *stac3* knockout larvae displayed more red staining (higher lipids)
895 compared to *stac3* *stac3*^{+/+} and *stac3*^{-/-} control siblings at 5 dpf. (D). Genotype of larvae was
896 confirmed as described in FigureS1A, B.

897

898 **S5. Death of *stac3*^{-/-} larvae at the early age**



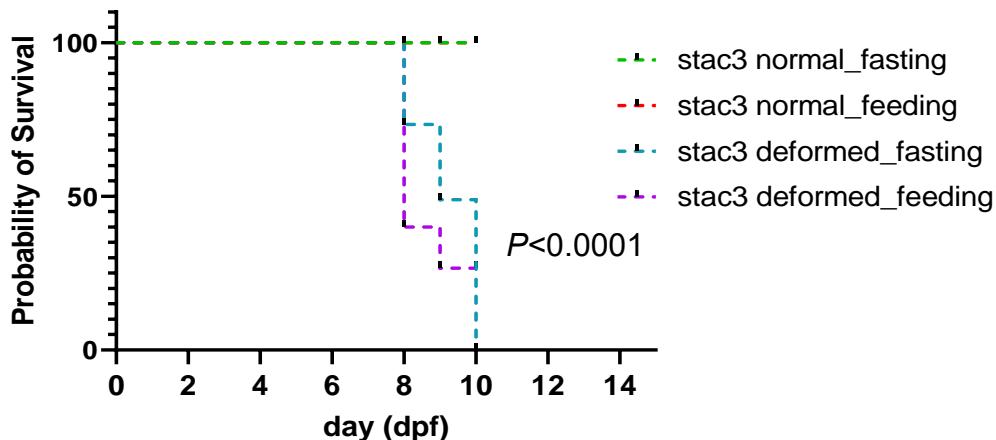
899

900 (A). Altered integrity of muscle fibers observed in *stac3* KO larvae (bottom panel) compared to
901 *stac3* wild type and heterozygous siblings (top panel), as measured by SA- β -gal staining at 6 dpf.
902 (B). *stac3* wild type and heterozygous siblings and KO larvae raised in an incubator confirmed
903 that *stac3*^{-/-} larvae expeditiously die by 11 dpf. *stac3* wild type and heterozygous sibling (left: top)
904 at 9 dpf, *stac3* knockout (middle) 10 dpf, and *stac3* knockout larvae (left: bottom) 11 dpf. (C).
905 Percentage (%) calculation of dead *stac3* knockout larvae at 7 dpf. Scale bar: 0.5-1 mm.

906

907

908 **S6. Fasted *stac3* KO larvae show a better short-term survival**



914 Feeding was introduced in some 7 dpf old *stac3* KO (deformed) and *stac3*^{+/+} and *stac3*⁺⁻ (normal)
915 control siblings. Larval growth and survival were monitored for 72 hours and the fasted and fed
916 animals were compared. Survival percentage was calculated between fasting vs. feed *stac3* KO,
917 *stac3*^{+/+} and *stac3*⁺⁻ groups using Log-rank test, ****p<0.0001.

918 **Supplementary table1. Primers used for RTqPCR**

Gene name	Forward primer	Reverse primer
<i>stac3</i>	ATCAATAACCCGGGTAGCA	GGCCATGATCACTCCGACTC
<i>ryr1a</i>	GAT GAA ACA GAG CAC ACT G	CCA CAT TTA TCC AAG CTG
<i>myod</i>	AACATTACAGTGGAGACTCTG	GTCATAGCTGTTCCGTCTTC
<i>myog</i>	GCTCCACATACTGGGTGTC	TATAGGCAGGGACACAGTGA
<i>myf5</i>	TACTACAGCCTGCCATGG	GACTGTTGCAGTCACCAGTC
<i>sox6</i>	TCCACAAACAAACCTCCCCTG	TTGCTGTCCGATTCCATGCG
<i>smyhc1</i>	GCTAACAGGCAGGCATCAGA	TGCATTGGGAATCCTTGACA
<i>tnnc1a</i>	AAAAATGAGTTCCGTGCAGC	CTTCAGGGTAGGGTTCTGG

<i>fasn</i>	TTCTGTAACGTTGCCGGGAG	TTCTTGAATCTGAACGCGGG
<i>srebf1</i>	ATGAATCTGTCTTGACGACACTTCT	TCAGGTGGATGTGACGGTG
<i>rpl32</i>	GAATCCAGAGGGCAGCATGT	GTCAAGATCAGGGCAAACTGG

919

Supplementary table2. Percentage (%) of dead *stac3* KO larvae at 7 dpf

Larva condition		Age: 7 dpf				
		exp1	exp2	exp3	Average	% in total population
<i>stac3^{+/+}</i> and <i>stac3⁺⁻</i> control siblings	324	379	300	334.33	87.14	14.76
<i>stac3^{-/-}</i> (KO)	45	47	56	49.33	12.86	81.76
<i>stac3</i> dead larvae (belong to KO group)	45	47	29	40.33	10.51	
Total	369	426	356	383.67	100.00	

920

Supplementary table3. Muscle pathology identification by blind analysis of zebrafish

921

larva at 6 dpf

S.NO	Birefringence image ID	Bright image ID	Muscle pathology	Remarks	Genotype
1	Mil_11593	Mil_11594	none	out of focus	<i>stac3⁺⁻</i>
2	Mil_11596	Mil_11597/9	none		<i>stac3⁺⁻</i>
3	Mil_11603	Mil_11604	none	out of focus	<i>stac3⁺⁻</i>
4	Mil_11605	Mil_11606	none	out of focus	<i>stac3^{+/+}</i>
5	Mil_11608	Mil_11609		very weak signal	<i>stac3^{-/-}</i>
6	Mil_11612	Mil_11613	patchy	very weak signal	<i>stac3^{-/-}</i>
7	Mil_11614	Mil_11615	patchy	very weak signal	<i>stac3^{-/-}</i>

8	Mil_11616	Mil_11617	"condensed"	very weak signal	<i>stac3</i> ^{-/-}
9	Mil_11618	Mil_11619	patchy	very weak signal	<i>stac3</i> ^{-/-}
10	Mil_11620	Mil_11621	patchy	very weak signal	<i>stac3</i> ^{-/-}
11	Mil_11622	Mil_11623	"condensed"	very weak signal	<i>stac3</i> ^{-/-}
12	Mil_11624	Mil_11625	patchy		<i>stac3</i> ^{-/-}
13	Mil_11629	Mil_11630	patchy	weak signal	<i>stac3</i> ^{-/-}
14	Mil_11631	Mil_11632	none		<i>stac3</i> ^{+/+}
15	Mil_11633	Mil_11634	none	out of focus	<i>stac3</i> ^{+/+}
16	Mil_11636	Mil_11637	none	out of focus	<i>stac3</i> ^{-/-}
17	Mil_11638	Mil_11639	none	out of focus	<i>stac3</i> ^{+/+}
18	Mil_11640	Mil_11641	none	out of focus	<i>stac3</i> ^{-/-}
19	Mil_11642	Mil_11643	none	out of focus	<i>stac3</i> ^{+/+}
20	Mil_11645	Mil_11646	none	out of focus	<i>stac3</i> ^{-/-}
21	Mil_11647	Mil_11648	none	out of focus	<i>stac3</i> ^{-/-}
22	Mil_11649	Mil_11650	none	out of focus	<i>not genotyped</i>
23	Mil_11651	Mil_11652	none		<i>not genotyped</i>
24	Mil_11655	Mil_11656	none	out of focus	<i>not genotyped</i>
25	Mil_11657	Mil_11658	none	out of focus	<i>not genotyped</i>

26	Mil_11659	Mil_11660	none		<i>not genotyped</i>
27	Mil_11661	Mil_11662	none	out of focus	<i>not genotyped</i>
28	Mil_11663	Mil_11664	patchy		<i>not genotyped</i>
29	Mil_11667	Mil_11668	patchy	very weak signal	<i>not genotyped</i>

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Supplementary materials and methods

924 With the guide RNA targeted exon-4 sequence of *stac3* gene, we identify suitable restriction
925 enzymes using NEB Cutter (<https://nc3.neb.com/NEBcutter/>) for genotyping zebrafish larvae,
926 while the guide RNA sequence has a restriction digestion site at its 3 prime for genotyping.

927 **stac3**

928 >NM_001003505.1 Danio rerio SH3 and cysteine rich domain 3 (*stac3*), mRNA
929 CAAACTCTGTACAAGTGTCAATCCTCAGTCAGAGCAGTCTGGTTATTACTGAGTTAAGATTATCTTC
930 TGTGCTAAAGCAAATATTAACCTATCTGTATCAGGGTCACACCTCGCTGACTGAAACTGGGATTATAGC
931 ACAGAAACAGATCATCTACAGGTGCGGATTCTGCTGACTGACTGCGTCTGAAGAGGCTGGACTGATGGC
932 TCAATATGACCAACTGGAGGATAAAGACTCGCTGGACATCCACGATAACCCTCCAGCGCCAGAGAATGTG
933 GTGAAAGAGGACGACAACACTGTGTATTTGTGTATGATGAAGAGGTGGAGGAAGAAGCTCCTCCGC
934 CACCCACCCCGGAGCCATAGTCCAGGTCAATGACAAACCACACAATTCAAGGACCACTACTGCAAGAA
935 ACCCAAGTTCTGTGACGTCTGCGCACGGATGATTGTTCTCAATAATAAGTTGCGCTGCCTGTAAAAAC
936 TGCAAGACCAACATCCACCCTCTGCCAGTCATACGTGCAAGTCCAGAGATGCTTCGGAAAATACCTC
937 CTGGGTTCAGACGGCGTACAGCTCTCTCTATGACCAGGAGATCAATAACCCGGGTAGCAGAACCG
938 CACAGATCCGGTGGTCGACACGCTGAGAGTCGGAGTGATCATGCCAATAAGAGAGGAAGAAGAGGCTCA
939 GAGGACAAGAAGAACATGATGATGATGGAGACAAGAAAGACAAACCGCAACAGATGACAAGAACAGCA
940 AGGGTGCTGAGGGAAAGCAAGATGGAGACAAGAAAGACAAACCGCAACAGATGACAAGAACAGCA

941 GCAGCAGACCTCAGTCAGTCGATTATTATGGCTCTGTATCGCTTAAAGCCATCGAGAAAGATGAT
942 CTGGACTTCATCCAGGAGATCGTATAACTGTTGGATGACTCTAATGAGGAGTGGTGGAGGGCAAGA
943 TTGGTGAGAAGACGGGTATTACCCATGACCTACATCATCCGGGTTCGAGCTGGCGAGCGGGTTATAA
944 AGTGACCCGATCTTGAGGAAACCGAGAGATGGGCCAGATCACCTGAAGAAAGACCAGATCGTGGT
945 AAGAAAGGAGAGGAGGTGAACGGATATCTGAAGGTCAGCACACTGGCGTAAACTGGCTTCTCCCTGCG
946 ATCTGCTGCATGAGCTCTAATAATCAGCAGACCAGCAGAAGGATGAAGAGAGACGGAGCATGCACACAGC
947 GGCTGCGTTACACTCCACTTTACACACACACTCACAAACATCCCTCACTTGTGACCGTACGCAAATCC
948 CTGCCGCTATTATGAAGTACATACCTGTTCATCAAGTGAACGAGTGAAGATTAGATGTTGACCGTAGCT
949 TTTGTCAGATTTCAAGTCTGCTCATATGTCTAATATATGTCCTAGAATGCGATGTGATTGTGACGTC
950 TCAACAGTGTAGTGCTCAAGTCAAGGGTTAGGGCACTCCATTAAACCCATTCAAGATTCTCCGCCA
951 GTAGTGGACACATATGTAACCTCATTAATGCTGTACATCCAAGGAAACGAGATGAAGGAAAGTTAGTGAG
952 TCAAGAACATAAAATATTGTACTGGAACGCAGCCCCAGTCTCATTCAACACACAATACACTCTTCCAAA
953 TAAATGTGCCATGAAGAGCAAAAAAAAAAAAAAAA
954 **gRNA**

955 **AGTTCTGTGACGTC****TGCGCA**

956 **stac3 exon 4**

957 ttgcattactaacatctaactaatggactattattgagtagtgtagtgttcaacgttagttctgctggggcttcataatgagacacctgcagtgtta
958 cggtagcacacagccactcggtcatcctctcagggttatttctcatcctcag**GTGTATTTGTGTATGATGAAGAGG**
959 **TGGAGGAAGAAGAAGCTCCTCCGCCACCCACCCCGGAGCCCATAGTCCAGGTCAA****CGAC**
960 **AAACCACACAAATTCAAGGACCACTACTGCAAGAAACCA****AGTTCTGTGACGTCTGCGCAC**
961 **GGATGATTGTT**gtaagttcacattgatagcaagttacaataacagcacacatattgtctcaacccttgccatattttgcatt
962 gttaaaggtaactgttctgccaaatgaatgatcagatacacacataagttgcagaagaactctacgatattgaaacgtatgcgcgtt
963 gtgggtctctacttcag

964 **Fspl restriction enzyme**

965 **5'...T G C[▼] G C A... 3'**

966 **3'...A C G_▲ C G T... 5'**

967 Pcr product 363bp

968 After cut 247bp 113bp

969 Genotyping of *stac3*^{-/-} vs. *stac3*^{+/+} and *stac3*⁺⁻ control siblings was confirmed as described in
970 FigureS1A, B. Blue: coding region, green color marks the gRNA region in exon4 of the *stac3* gene
971 and protospacer adjacent motif sequence is in yellow, while red denotes restriction digestion site.

972