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The ion channel Trpc6a regulates the cardiomyocyte regenerative response to mechanical stretch.

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20 **Abstract**

21 Myocardial damage caused for example by cardiac ischemia leads to ventricular volume
22 overload resulting in increased stretch of the remaining myocardium. In adult mammals, these
23 changes trigger an adaptive cardiomyocyte hypertrophic response which, if the damage is
24 extensive, will ultimately lead to pathological hypertrophy and heart failure. Conversely, in
25 response to extensive myocardial damage, cardiomyocytes in the adult zebrafish heart and
26 neonatal mice proliferate and completely regenerate the damaged myocardium. We therefore
27 hypothesized that in adult zebrafish, changes in mechanical loading due to myocardial damage
28 may act as a trigger to induce cardiac regeneration. Based, on this notion we sought to identify
29 mechanosensors which could be involved in detecting changes in mechanical loading and
30 triggering regeneration. Here we show using a combination of knockout animals, RNAseq and
31 *in vitro* assays that the mechanosensitive ion channel Trpc6a is required by cardiomyocytes for
32 successful cardiac regeneration in adult zebrafish. Furthermore, using a cyclic cell stretch assay,
33 we have determined that Trpc6a induces the expression of components of the AP1 transcription
34 complex in response to mechanical stretch. Our data highlights how changes in mechanical
35 forces due to myocardial damage can be detected by mechanosensors which in turn can trigger
36 cardiac regeneration.

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46 **Introduction**

47 Following a myocardial infarction, the loss of cardiac tissue results in significant
48 changes in the mechanical loads exerted on the heart. As dynamic/elastic myocardium is
49 replaced by relatively stiff non-contractile scar tissue, the resulting elevated preload and
50 changes in tissue composition increases the amount of stretch exerted on the remaining
51 myocardium, triggering an increased force of contraction and concomitant cardiomyocyte
52 hypertrophy (Frank-Starling law)¹. While this adaptive mechanism initially compensates for
53 the increased mechanical load, prolonged stress will become maladaptive and, without medical
54 intervention, will ultimately result in pathological hypertrophy and heart failure. In contrast to
55 adult mammals, neonatal mice and adult zebrafish can fully regenerate their hearts after cardiac
56 injury^{2,3}. Rather than undergoing compensatory hypertrophy, neonatal mouse and adult
57 zebrafish cardiomyocytes proliferate in response to the loss of myocardium. This suggests that
58 the changes in mechanical loading which occur after cardiac injury in neonates/zebrafish may
59 act as a trigger to induce cardiomyocyte proliferation and ultimately regeneration. Although
60 decades of research have been devoted to understanding the effects of mechanical stretch on
61 non-proliferative adult cardiomyocytes there is very little information regarding the effects on
62 cardiomyocytes which are capable of proliferation. However, *in vitro* evidence studying the
63 effects of mechanical loading on embryonic mouse cardiomyocytes indicates an increase in the
64 proliferation index following 24 hours of cyclic stretch⁴. Likewise, transcriptomic analysis of
65 neonatal rat ventricular cardiomyocytes subjected to cyclic stretch *in vitro* showed a significant
66 upregulation of genes associated with cell proliferation⁵. To understand whether increased
67 mechanical stretch can induce cardiomyocyte proliferation *in vivo*, in models which are capable
68 of cardiac regeneration, it is important to assess whether changes in mechanical force occur
69 following cardiac injury. Analysis of adult zebrafish indicates that following cardiac injury, the
70 initial response is similar to that observed in adult humans resulting in a stiffer myocardium
71 due to the extensive fibrosis at the site of injury⁶. Furthermore, the resulting volume overload

72 initially induces an elongation of cardiomyocyte sarcomere length which reverts following
73 cardiac regeneration. This indicates that following cardiac injury in adult zebrafish, the heart is
74 subjected to significant changes in mechanical loading. Whether these forces can induce a
75 proliferative response is at present unclear⁶. However, other situations that can also increase
76 cardiomyocyte stretch do support this hypothesis. In humans vigorous exercise results in an
77 elevated preload resulting in increased cardiomyocyte stretch. These conditions trigger an
78 adaptive hypertrophic response resulting in an essentially larger more powerful heart¹. On the
79 other hand, in adult zebrafish, exercise can induce cardiomyocytes to proliferate rather than
80 undergo hypertrophy⁷. This indicates that increased mechanical load can act as a stimulus to
81 induce cardiomyocyte proliferation. But care must be taken when extrapolating exercise
82 induced cardiomyocyte hypertrophy vs damage induced hypertrophy as it appears that different
83 mechanisms are at play depending on the conditions⁸. Therefore, understanding how increased
84 stretch can influence cardiomyocyte proliferation, particularly *in vivo*, may provide invaluable
85 information on how this phenomenon could be harnessed to induce a regenerative response.
86 Increased myocardial stretch can be sensed by a wide variety of mechanosensory mechanisms
87 present in cardiomyocytes such as cell surface receptors, sarcomeric components, intercalated
88 discs and stretch activated ion channels¹. The transient receptor potential (Trp) channels are a
89 family of non-selective cation channels which can be regulated by a variety of stimuli including
90 mechanical stretch. Of these, *TRPC3* and *TRPC6* are highly expressed in the heart and have
91 been directly linked to pathological cardiac hypertrophy in response to chronic overload. In
92 particular, stretch elevates intracellular Ca^{2+} which activates the CALCINEURIN/NUCLEAR
93 FACTOR OF ACTIVATED T CELLS (NFAT) pathway triggering pathological hypertrophy
94 and remodelling^{9,10}. Both *TRPC3* and *TRPC6* have been shown to be responsible for the stretch
95 induced increase in Ca^{2+} ⁹. Indeed, targeting both of these ion channels can inhibit pathological
96 cardiomyocyte hypertrophy¹¹. Because of the role *TRPC6* plays in regulating the

97 cardiomyocyte hypertrophic response to increased stretch in mammals we surmised that Trpc6
98 may also regulate cardiomyocyte proliferation in adult zebrafish regenerating hearts in response
99 to the chronic mechanical overload associated with cardiac injury.

100 In this study we examined how the loss of *trpc6a* could affect cardiac regeneration in
101 zebrafish. Similar to reports in mammals, we found that *trpc6a* knockout (KO) did not result in
102 any observable cardiac developmental defects. However, adult *trpc6a* KO zebrafish failed to
103 regenerate their hearts following apical resection due to a reduction in cardiomyocyte
104 proliferation. Transcriptomic analysis indicated that following cardiac injury, *trpc6a* KO
105 zebrafish did not upregulate the expression of genes required for this process. In particular, this
106 included orthologous components of the ACTIVATOR PROTEIN 1 (AP1) transcription factor
107 complex which is critical for cardiac regeneration in adult zebrafish¹². Furthermore, we
108 demonstrate that the stretch induced expression of AP1 components is markedly reduced in
109 *trpc6a* KO zebrafish. Taken together these findings indicate that Trpc6a positively regulates
110 the expression of pro-regenerative genes in response stretch and that this process is required for
111 successful cardiac regeneration.

112

113 **Results**

114 **Loss of *trpc6a* does not affect cardiac development**

115 To understand the role Trpc6a plays during cardiac development and regeneration, we utilised
116 a KO zebrafish line which harbours a single base pair substitution G637T resulting in a
117 premature stop codon in exon2 of *trpc6a* (Fig.1.A). To confirm that this mutation results in a
118 loss of Trpc6a protein, we performed immunohistochemistry (IHC) using a Trpc6 antibody. In
119 this manner we could detect Trpc6 in the myocardium of adult *trpc6a*^{+/+} zebrafish hearts
120 (Fig.1.B). In comparison, Trpc6 was absent in adult *trpc6a*^{-/-} zebrafish hearts indicating that no
121 functional Trpc6a protein was present in the KO line (Fig.1.C). In parallel, we also assessed

122 sarcomere structure in *trpc6a*^{-/-} using a Tropomyosin (*trpm*) antibody. We could not detect any
123 observable differences in tropomyosin labelling between *trpc6a*^{-/-} and their *trpc6a*^{+/+} siblings
124 (Fig.1.D,E). We next determined whether loss of Trpc6a affects cardiac development in
125 zebrafish larvae as this could potentially impact processes occurring in adulthood, such as
126 cardiac regeneration. Because of the role TRPC6 plays in hypertrophy in mammals we first
127 measured the ventricular wall of 3 days post fertilisation (dpf) larvae (n=10/group) (Fig.1.F,G).
128 In this manner we could not detect any significant differences between *trpc6a*^{-/-} and *trpc6a*^{+/+}
129 larvae (Fig.1.H). Mutations in *TRPC6* have also been associated with cardiac arrhythmias
130 which could, if present, disrupt heart regeneration at later stages. Therefore, we analysed a
131 variety of cardiac physiological parameters in both *trpc6a*^{-/-} and *trpc6a*^{+/+} larvae.
132 Measurements of ventricular and atrial heart rates indicated there was no significant difference
133 between *trpc6a*^{-/-} and *trpc6a*^{+/+} larvae (n=10/group) (Fig.1.I). Lastly, we measured the blood
134 flow rate and calculated the cardiac output in both *trpc6a*^{-/-} and *trpc6a*^{+/+} larvae. Our data
135 indicates that there are no significant differences in these parameters between *trpc6a*^{-/-} and
136 *trpc6a*^{+/+} larvae indicating that loss of Trpc6a does not appear to affect overall cardiac
137 performance (n=10/group) (Fig.1.J,K). These data indicate that heart development and cardiac
138 performance are not significantly affected in *trpc6a*^{-/-} zebrafish larvae.

139

140 **Trpc6a is required for cardiac regeneration**

141 Because of the role TRPC6 plays in sensing changes in mechanical load after cardiac injury in
142 mammals, we assessed whether the loss of Trpc6a affected cardiac regeneration in adult
143 zebrafish. To achieve this, we performed apical resection of adult *trpc6a*^{-/-} and *trpc6a*^{+/+}
144 zebrafish. At 30 days post amputation (dpa), histological staining using acid fuchsin orange G
145 (AFOG) indicated that the loss of Trpc6a inhibited cardiac regeneration resulting in the
146 presence of a significant fibrin/collagen scar (n=5/group) (Fig.2.A-C). Previous research

147 indicates that TRPC6 can also play a role in angiogenesis¹³. During cardiac regeneration in
148 adult zebrafish, revascularization of the wound region is a critical early event which could be
149 affected by the loss of *Trpc6a*. To address this possibility, we analysed wound revascularization
150 at 7dpa. In this manner we could detect numerous vessels forming a vascular plexus within the
151 wound region of both *trpc6a*^{-/-} and *trpc6a*^{+/+} zebrafish hearts indicating that this process appears
152 largely unaffected and is unlikely the cause of the defective regeneration we observed in *trpc6a*⁻
153 ⁻ hearts (n=5/group)(Fig.2.D-G). We next sought to determine whether cardiomyocyte
154 proliferation had been affected in *trpc6a*^{-/-} zebrafish. To meet this end, we performed EdU
155 labelling of resected *trpc6a*^{-/-} and *trpc6a*^{+/+} zebrafish hearts at 14dpa. Our analysis indicates
156 that there is a significant reduction in the number of EdU labelled cardiomyocytes in the *trpc6a*⁻
157 ⁻ hearts compared to their *trpc6a*^{+/+} siblings (n=3/group)(Fig.2.H-J). Taken together this data
158 indicates that the loss of *trpc6a* disrupts cardiac regeneration due to a significant reduction in
159 cardiomyocyte proliferation.

160

161 **Loss of *Trpc6a* results in misregulated of gene expression during regeneration**

162 To determine what effect the loss of *Trpc6a* had on the cardiac transcriptome during heart
163 regeneration, we performed bulk RNA sequencing of sham operated and 7dpa resected *trpc6a*⁻
164 ⁻ and *trpc6a*^{+/+} hearts (n=5/group). Analysis of sham vs 7dpa samples indicates that 480 genes
165 are significantly upregulated in 7dpa *trpc6a*^{+/+} hearts compared with 293 in 7dpa *trpc6a*^{-/-}
166 (Fig.3.A-C). Of these, 135 genes are upregulated in both *trpc6a*^{-/-} and *trpc6a*^{+/+} 7 dpa hearts
167 while 345 genes are specifically upregulated in *trpc6a*^{+/+} hearts and 158 genes are specific to
168 *trpc6a*^{-/-} hearts (Fig.3.C). Analysis of significantly downregulated genes indicates that 8 are
169 common to both *trpc6a*^{-/-} and *trpc6a*^{+/+} 7 dpa hearts while 48 are specifically downregulated in
170 *trpc6a*^{+/+} hearts compared with 68 downregulated genes specific to *trpc6a*^{-/-} hearts (Fig.3.A-
171 C). Lastly, 1 gene was significantly upregulated in *trpc6a*^{-/-} hearts and downregulated in

172 *trpc6a*^{+/+} hearts while 2 genes were significantly upregulated in *trpc6a*^{+/+} hearts and
173 downregulated in *trpc6a*^{-/-} hearts (Fig.3.C). We next focused on the 345 genes which were
174 specifically upregulated in *trpc6a*^{+/+} hearts as these likely included genes which are induced
175 following Trpc6a activation. In this manner we identified a number of transcription factors
176 which were significantly upregulated in the *trpc6a*^{+/+} hearts but not in *trpc6a*^{-/-} hearts (Fig.3.D).
177 Of particular interest were a number of components of the AP1 transcription factor complex
178 (*fosl1a*, *june* and *fosab*), a critical regulator of cardiac regeneration in adult zebrafish¹².

179

180 **Trpc6a regulates the stretch induced expression of AP1 transcription factor components.**

181 Previous data indicates that pathologically stretching cardiomyocytes *in vivo* activates TRPC6,
182 which in turn induces downstream gene expression¹¹. Based on this, we assessed whether
183 Trpc6a regulated the expression of the AP1 transcription factor components *fosl1a* and *fosab*
184 in response to mechanical stretch. To achieve this, we dissociated and isolated cardiomyocytes
185 from either *trpc6*^{-/-} or *trpc6a*^{+/+} hearts and subjected them to 24 hours of cyclic stretch
186 (n=5/group) (Fig.4.A). Following the completion of this protocol, we harvested the
187 cardiomyocytes and performed RT qPCR for both *fosl1a* and *fosab* (Fig.4.A). In this manner,
188 we observed an approximately 5-fold increase in expression of both *fosl1a* and *fosab* in
189 stretched *trpc6a*^{+/+} cardiomyocytes when compared to *trpc6*^{-/-} cardiomyocytes (Fig.4.B,C).
190 These data indicate that loss of Trpc6a in cardiomyocytes results in a failure to upregulate the
191 expression of the AP1 transcription factor components *fosl1a* and *fosab* in response to
192 mechanical stretch.

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194

195

196 **Discussion**

197 The TRP ion channel TRPC6 is responsible for detecting increased mechanical stretch in
198 cardiomyocytes and activating the CALCINEURIN/NFAT pathway¹⁰. Under
199 pathophysiological conditions of chronic elevated cardiomyocyte stretch, for example volume
200 overload caused by a cardiac ischemia, this will lead to pathological hypertrophy and ultimately
201 heart failure¹⁰. Whether TRPC6 could also play a role in detecting increased cardiomyocyte
202 stretch and triggering cardiac regeneration in animal models capable of this feat is currently
203 unknown. The results of this study indicate that *Trpc6a* is an essential component of the cardiac
204 regenerative response in adult zebrafish. Our data demonstrates that loss of *Trpc6a* results in a
205 failure to regenerate the heart following cardiac resection. Early regenerative processes such as
206 revascularization appear largely unaffected, however cardiomyocyte proliferation is
207 significantly impeded in the absence of *Trp6a* signaling leading to the persistence of extensive
208 scarring. Furthermore, comparative transcriptomic analysis of *trpc6a*^{-/-} and *trpc6a*^{+/+} resected
209 hearts indicates that loss of *Trp6a* substantially impacts gene expression. In particular
210 components of the AP1 transcription factor complex, which are required for successful cardiac
211 regeneration, are not upregulated in the absence *Trp6a*. Lastly, our data indicates that *Trpc6a*
212 regulates the expression of AP1 transcription factor complex components in response to
213 mechanical stretch. Together these results indicate that, in adult zebrafish, increased/chronic
214 cardiomyocyte stretch associated with cardiac injury is sensed by *Trpc6a* which subsequently
215 activates downstream signaling pathways resulting in the expression of genes, such as AP1
216 transcription factor complex components, which are involved in driving cardiac regeneration.

217 The Frank-Starling law was described over a century ago and explains how elevated
218 ventricular preloading, which stretches cardiomyocytes, results in an increased force of
219 contraction in order to maintain circulatory homeostasis¹⁴. Stretching cardiomyocytes increases
220 the calcium sensitivity of their sarcomeres resulting in enhanced contractility. In situations

221 where ventricular preloading is maintained, there is a further progressive increase in the force
222 of contractility termed the slow force response (SFR) which is driven by elevated, TRPC6
223 dependent⁹, Ca²⁺ transients¹⁵. Chronic ventricular loading, for example after myocardial
224 ischemia, results in cardiac remodeling and pathological cardiomyocyte hypertrophy leading,
225 ultimately, to heart failure. The molecular mechanisms which drive pathological cardiac
226 hypertrophy (as opposed to physiological hypertrophy induced by exercise) are largely driven
227 by CALCINEURIN and its downstream effector NFAT⁸. Chronic increases in mechanical load
228 result in elevated intracellular Ca²⁺ which in turn activates CALCINEURIN. CALCINEURIN
229 subsequently dephosphorylates NFAT which translocates into the nucleus and regulates the
230 expression of genes which drive pathological hypertrophy. The ion channel TRPC6 is
231 responsible for detecting increases in cardiomyocyte stretch and generating the sustained Ca²⁺
232 transients which drive this pathological process¹⁰. It is apparent then that TRPC6 plays a central
233 role in the cardiac mechanosensitive response to volume overload which results in pathological
234 hypertrophy. Conversely, our data indicates that, in adult zebrafish, it appears that the response
235 to volume overload regulated by *Trpc6a* results in cardiomyocyte proliferation and ultimately
236 cardiac regeneration. In mice, global KO of *Trpc6* results in increased mortality after
237 myocardial infarction, however this is primarily due to the role *Trpc6* plays in cardiac fibroblast
238 activation, a process which is essential for early scar formation in order to avoid cardiac
239 rupture¹⁶. Blood pressure in adult zebrafish is around 50 times lower than mice (2.5mmHg vs
240 100mmHg)¹⁷ and as such the formation of a clot is sufficient to avoid excessive blood loss
241 following damage to the myocardium. Because of the role TRPC6 plays in different cell types
242 in mammals, it will be interesting to determine the effect that conditional, cardiomyocyte
243 specific, deletion of *Trpc6* has following myocardial infarction. Conversely, constitutive,
244 cardiomyocyte specific, over-expression of *Trpc6* in mice activates the CALCINEURIN/NFAT
245 pathway resulting in pathological hypertrophy and lethality¹⁰. However, it would also be of

246 interest to assess what effect *Trpc6* overexpression has at earlier stages of development when
247 cardiomyocytes are still capable of proliferating. Downstream of TRPC6, cardiomyocyte
248 specific overexpression of an active-CALCINEURIN isoform in adult mice is sufficient to
249 trigger pathological hypertrophy and heart failure¹⁸. Furthermore, similar experiments
250 performed in neonatal mice indicates that active-CALCINEURIN induces a switch from
251 proliferation to hypertrophic growth in cardiomyocytes which are normally hyperplastic at this
252 stage of development¹⁹. While these data seem at odds with our finding that *Trpc6a* plays a
253 beneficial role during cardiac regeneration, our transcriptomic data indicates that other *Trpc6a*
254 mediated mechanisms are also involved. Although much focus has been placed on the TRPC6-
255 CALCINEURIN-NFAT axis, TRPC6 also activates other signalling mechanisms such as AP1
256 mediated gene transcription²⁰. We found that, during cardiac regeneration, there is a significant
257 increase in the expression the AP1 transcription factor components *fosab* and *fosl1a* which does
258 no occur when *Trpc6a* is absent. This is in-line with previous *in vitro* data indicating that
259 activation of *Trpc6* results in increased *c-fos* expression²¹. More recently, the AP1 transcription
260 factor complex has been shown to be a critical regulator of cardiac regeneration in adult
261 zebrafish¹². In particular, cardiomyocyte specific expression of a dominant-negative *Fos*
262 isoform significantly inhibits cardiac regeneration. This loss of AP1 function results in
263 defective cardiomyocyte sarcomere disassembly and proliferation and also affects their ability
264 to extend protrusions into the site of injury¹². Interestingly, although the AP1 components *JunB*
265 and *Fosl1* are upregulated in adult zebrafish hearts after injury, the same is not true for adult
266 mice after myocardial infarction^{12,22}. Furthermore, overexpression of *JunB* and *Fosl1* in
267 neonatal rat cardiomyocytes is sufficient to induce protrusive behaviour in these cells similar
268 to that observed in zebrafish cardiomyocytes¹². These data indicate a difference in AP1
269 signalling between adult zebrafish and mammals which may partly explain the differences we
270 observed following KO of *trpc6a*. Lastly, we have demonstrated that *Trpc6a* regulates the

271 expression of the AP1 transcription factor components *fosab* and *foslla* in response to
272 mechanical stretch, similar to reports in mammalian cardiomyocytes^{5,23}. Although care must be
273 taken when extrapolating *in vitro* data, it is likely that this is also the situation which occurs
274 during cardiac regeneration in adult zebrafish. The increase in myocardial stretch caused by
275 volume overload following cardiac injury could activate *Trpc6a* and induce the expression of
276 AP1 components required for cardiac regeneration. In summary we have identified *Trpc6a* as a
277 critical regulator of cardiac regeneration which can induce the expression of AP1 components
278 in response to mechanical stretch. Future studies will be required to establish exactly why
279 *trpc6a* induces a regenerative response in adult zebrafish compared to the pathological response
280 in mammals.

281

282 **Methods**

283 **Zebrafish line and husbandry**

284 *Trpc6a* KO G637T (sa23930) zebrafish transgenic line was purchased from ZIRC and
285 maintained under standardized conditions²⁴. Experiments were conducted in accordance with
286 local approval and the European Communities council directive 2010/63/EU.

287

288

289 **Larval heart rate and blood flow analysis**

290 3dpf larvae were anaesthetised and mounted in low melt agarose. 30 seconds videos of either
291 cardiac contractions or blood flow were recorded using a Point Grey GRAS-03K2C-C high
292 speed camera. Heart rate and blood flow were analysed using ViewPoint MicroZebraLab
293 software and ImageJ software. T-test statistical analysis was performed using GraphPad Prism.

294

295

296

297 **Resection**

298 Cardiac resection were performed on 6 to 10-month-old zebrafish as previously described², in
299 accordance with local approval (APAFIS#2021021117336492 v5).

300

301 **Immunohistochemistry and histological staining**

302 Immunohistochemistry and histological stainings were performed on 10 μ m heart sections as
303 previously described²⁵. The antibodies used in this manuscript are listed below:

304 anti-Trpc6 (OST00081W, Osenses)

305 anti-Trpm (T2780, Sigma)

306 EdU labelling was performed according to the manufacturer's instructions (Click-iT EdU Kit
307 C10337, Molecular Probes). Acid Fuchsin-Orange G (AFOG) staining was performed as
308 previously described²⁶ and the size of the scar area was calculated using ImageJ software. T-
309 test statistical analyses was performed using GraphPad Prism. Alkaline phosphate staining was
310 performed on whole-mount heart as previously described²⁵.

311

312 **Imaging**

313 A Zeiss Discovery V20 fluorescence stereomicroscope fitted with a Tucsen FL20 microscope
314 camera was used for histological imaging and either a Zeiss Axio Imager equipped with an
315 Apotome 3 module or a Leica TCS SP-8 confocal microscope were used for imaging
316 immunohistochemistry labelled sections.

317

318 **EdU labelling**

319 To label proliferating cells, amputated fish were anesthetized in Tricaine and injected with 50 μ L
320 of 240 μ g/mL of EdU solution daily. At 14dpa, fish were euthanized (excess of tricaine), the

321 hearts were collected, and processed for immunohistochemistry. Following imaging, EdU+
322 cardiomyocytes were counted using IMARIS software. T-test statistical analysis was performed
323 using GraphPad Prism.

324

325 **RNA sequencing**

326 Adult fish were anesthetized in Tricaine. For each group, 5 hearts were pooled and RNA was
327 extracted using Trizol/choloform. Genewiz performed the RNAsequencing and bioinformatic
328 analysis of the data using DESeq2. The Wald test was used to generate p-values and log2 fold
329 changes (log2FC). Genes with a p-value < 0.05 were considered differentially expressed.
330 Heatmaps were generated with iDEP 1.0 using the top 100 most variable genes and the distance
331 between groups was calculated using the Pearson's correlation coefficient.

332 **Cardiomyocyte isolation and cyclic stretch**

333 For each group, 5 hearts were collected and pooled. Cardiomyocytes were isolated as previously
334 described²⁷. Cardiomyocytes were plated on BioFlex® culture plates coated with Collagen I
335 (Flexcell®) and concentrated at the center of the wells using a 12-hour static tension protocol
336 applied with the Flexcell Tension System (FX-6000T, Flexcell®) at 28°C and with 5% CO₂.
337 Following this, the culture medium was changed and a cyclic stretch protocol was applied for
338 24h (Sine, 16% elongation, 0.5Hz).

339 **Real-time quantitative PCR**

340 RNA was extracted from isolated cardiomyocytes using Trizol/chloroform. cDNA was
341 obtained after reverse transcription using a First strand cDNA synthesis RT-PCR kit(Roche)
342 and quantitative PCR was performed using SYBR Green (Roche) and a LightCycler 480 system
343 (Roche). The primer sequences used are as follow:

344 *tubulin alpha* Forward: 5' CGGCCAAGCAACACTACTAGA 3'

345 *tubulin alpha* Reverse: 5' AGTTCCTCCAGCAGGCATTG 3'

346 *fosl1a* Forward: 5' AAGGGAACGCAACAAAATGG 3'

347 *fosl1a* Reverse: 5' AGCTTCTCCTTTTCCTTCTGG 3'

348 *fosab* Forward: 5' GTGCTTTTCGACTTTTGACAGG 3'

349 *fosab* Reverse: 5' GTCTGGTTGAGCGGGTAATAC 3'

350

351

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360

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425

426

427 **Figure Legends**

428 **Figure 1. Loss of *trpc6a* does not affect cardiac development**

429 **A.** Description of the point mutation carried by the sa23930 zebrafish transgenic line. The
430 G637T mutation causes a premature stop codon in the Exon 2 of the *trpc6a*. **B-C.** IHC images
431 from adult heart sections showing the presence of Trpc6 in the myocardium of *trpc6a*^{+/+} but
432 absent from the myocardium of *trpc6a*^{-/-} zebrafish. Trpc6: green, DAPI: blue. **D-E.** IHC images
433 from adult heart sections showing the organization of tropomyosin (Trpm) in the myocardium
434 of *trpc6a*^{+/+} and in *trpc6a*^{-/-} zebrafish. Trpm: green, DAPI: blue. **F-G.** Representative
435 morphology of the ventricular wall of 3dpf larvae from control (*trpc6a*^{+/+}, **F**) and *trpc6a* KO
436 (*trpc6a*^{-/-}, **G**) groups. **H.** Ventricular wall thickness measurements of 3dpf larvae during
437 diastole. t-test was used for statistical analysis. **I.** Atrial and ventricular contraction rate (in
438 bpm) of *trpc6a*^{+/+} and *trpc6a*^{-/-} 3dpf larvae. 1-way ANOVA was used for statistical analysis. **J.**
439 Blood flow velocity (in nL/s) measured in the caudal vein at 3dpf. t-test was used for statistical

440 analysis. **K.** Cardiac output (in nL/beat) of *trpc6a*^{+/+} and *trpc6a*^{-/-} 3dpf larvae. t-test was used
441 for statistical analysis. **F-K.** Data obtained on n=10 larvae per group.

442

443 **Figure 2. Trpc6a is required for cardiac regeneration.**

444 **A-C.** AFOG staining images and quantification of the scar area at 30dpa. Representative image
445 of AFOG staining obtained for *trpc6a*^{+/+} (**A**) and *trpc6a*^{-/-} (**B**). Scale bars: 200µm. The dashed
446 line outlines the scar region. The quantification of the scar area (n=5/group) (**C**). t-test was used
447 for statistical analysis. **: p value < 0.01. **D-G.** Representative images of alkaline phosphatase
448 staining showing the vasculature on 7dpa whole mount hearts. Low (**D-E**) and high (**F-G**)
449 magnification of the vascular plexus present in the wound region of *trpc6a*^{+/+} (**D, F**) and *trpc6a*^{-/-}
450 (**E, G**) zebrafish heart. Scale bars: 200µm. **H-J.** Cardiomyocyte proliferation measured at
451 14dpa. Representative IHC images showing Mef2c (green), EdU (red) and DAPI (blue) for
452 *trpc6a*^{+/+} (**H**) and *trpc6a*^{-/-} (**I**). The white box depicts a higher magnification image in the upper
453 right corner. Scale bars: 100µm. **J.** Quantification of EdU+ cardiomyocytes (n=3/group). t-test
454 was used for statistical analysis. ***: p value < 0.001.

455

456 **Figure 3. Loss of Trpc6a results in misregulated gene expression during regeneration.**

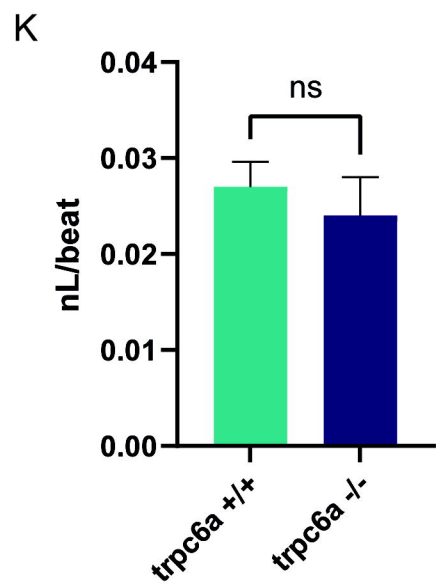
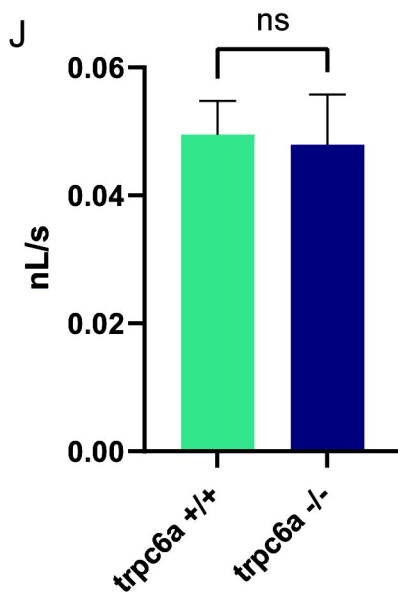
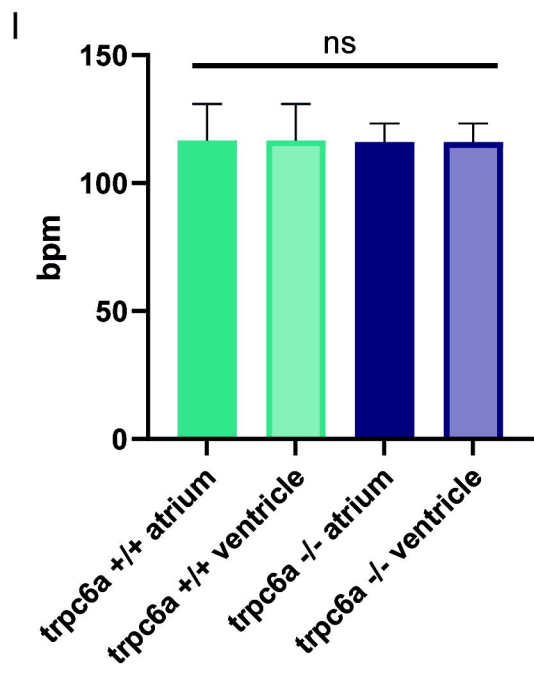
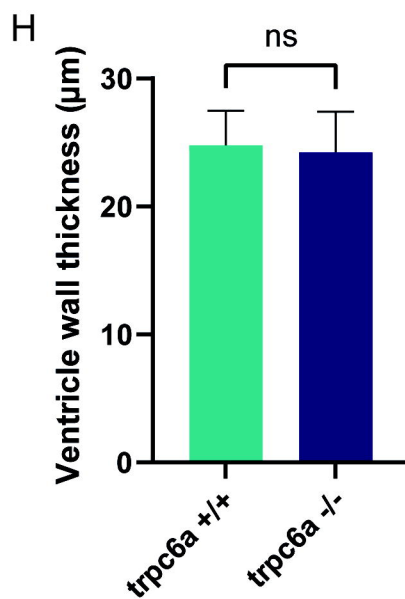
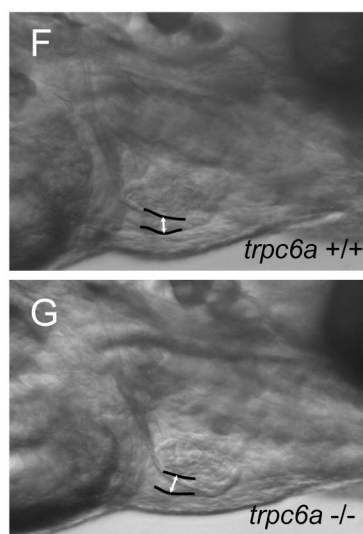
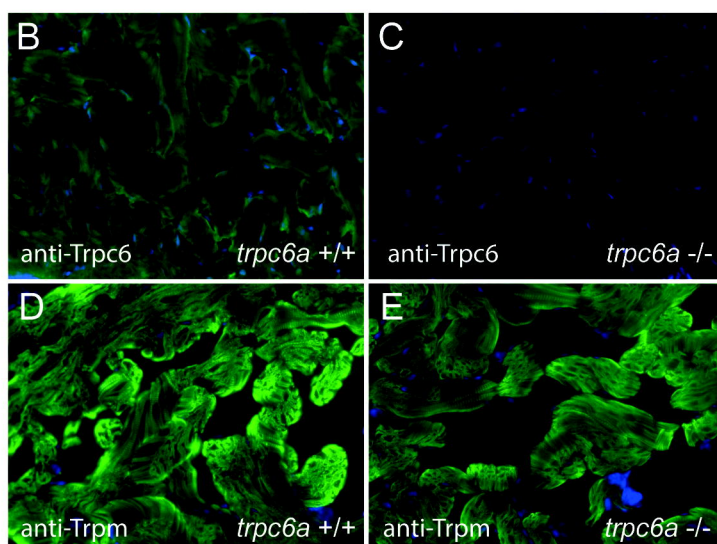
457 **A-B.** Heatmaps showing the 100 most differentially regulated genes between uninjured and
458 7dpa hearts of *trpc6a*^{+/+} (**A**) and *trpc6a*^{-/-} (**B**). Color key represents the relative distance between
459 groups and was calculated using Pearson's correlation coefficient. **C.** Venn diagram
460 representing the number of genes significantly up or down regulated after injury in *trpc6a*^{+/+}
461 and *trpc6a*^{-/-} hearts. **D.** Table of transcription factors which are significantly upregulated in
462 *trpc6a*^{+/+} hearts but not in *trpc6a*^{-/-} hearts following injury (compared to their respective sham
463 controls).

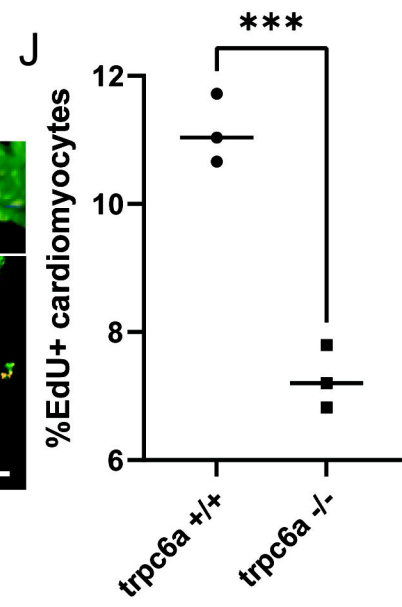
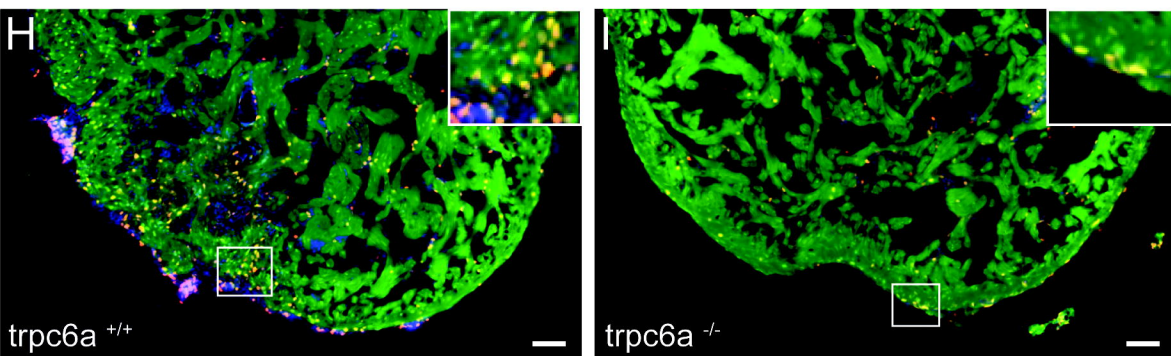
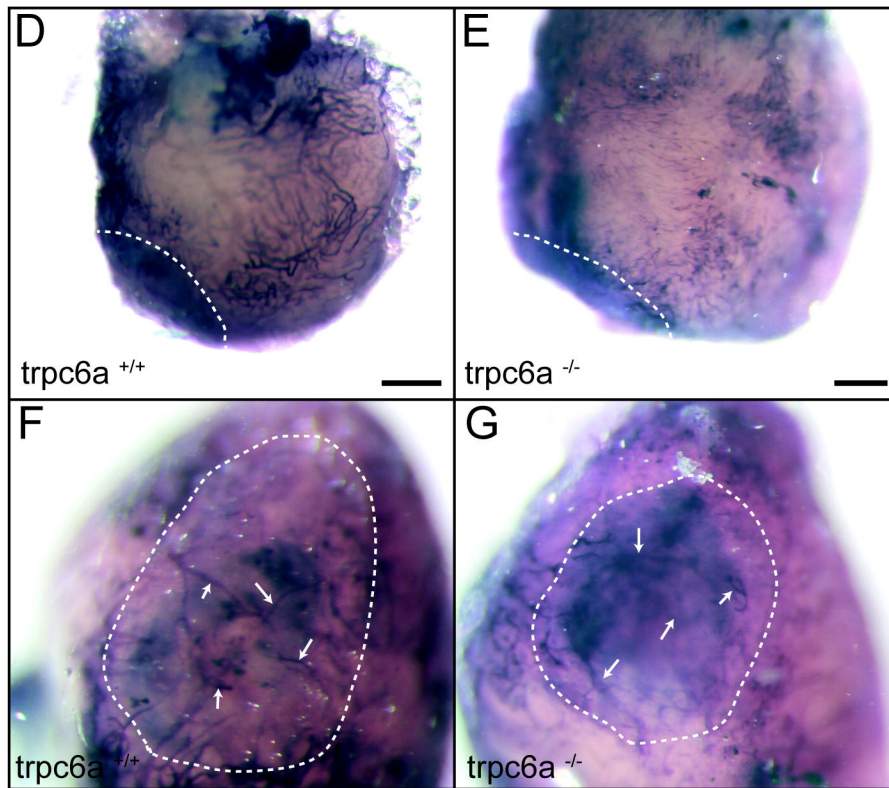
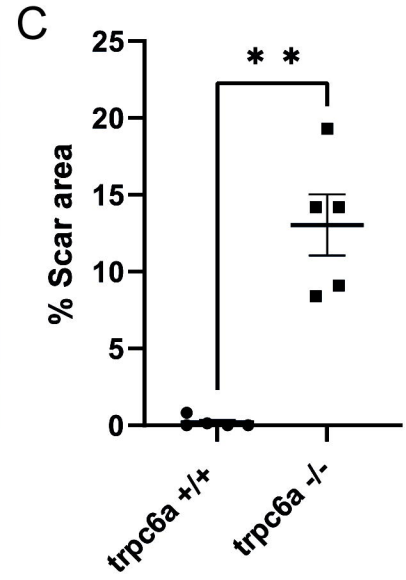
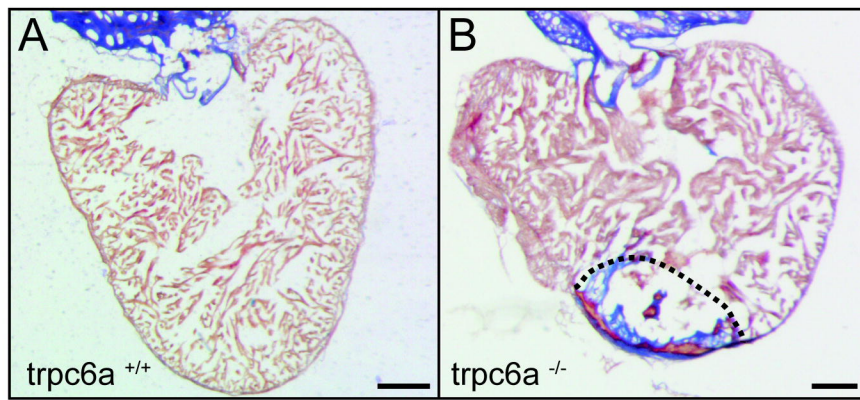
464 **Figure 4. Trpc6a regulates the stretch induced expression of AP1 transcription factor**
465 **components.**

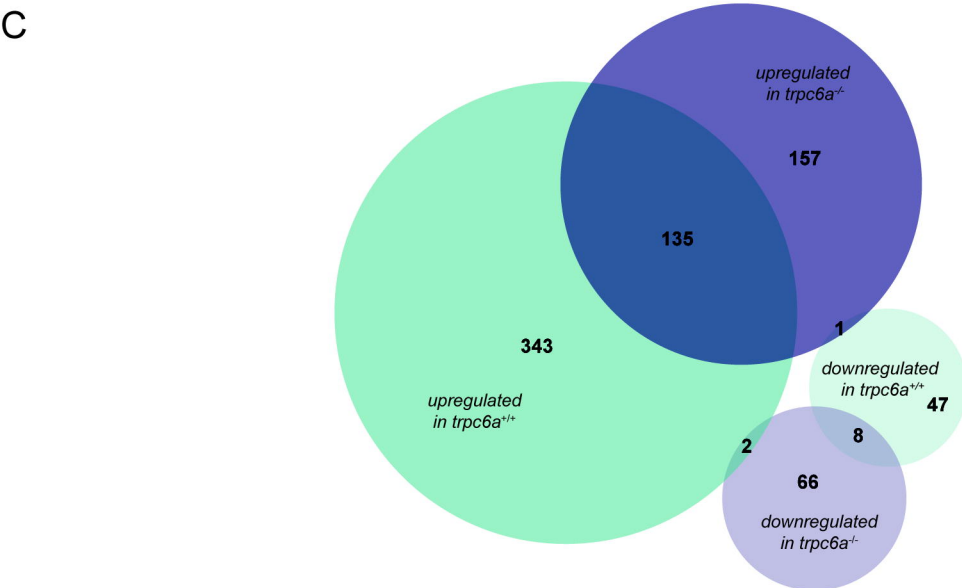
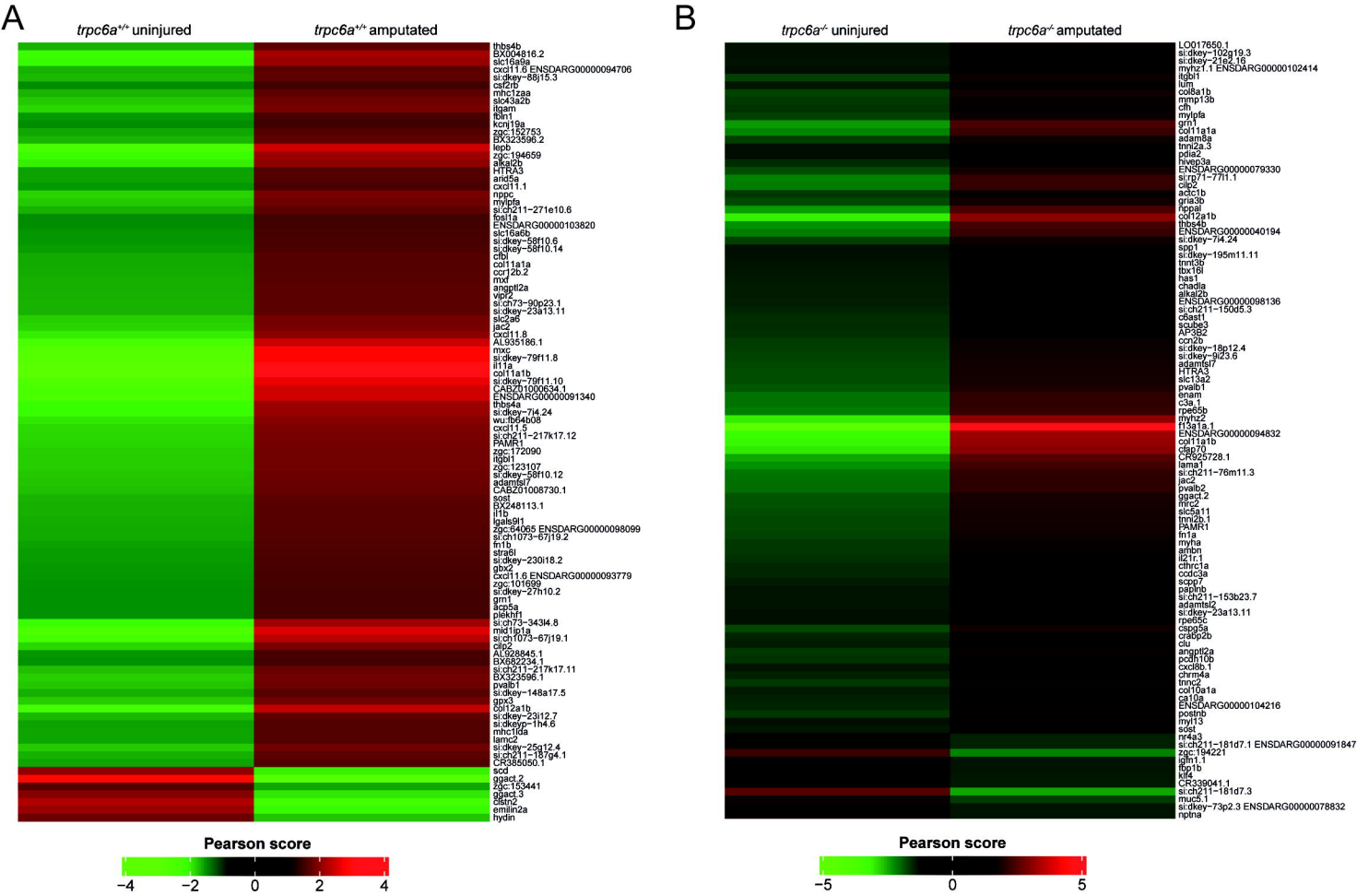
466 **A.** Schematic representation of the experimental design. Cardiomyocytes were isolated from
467 extracted hearts and plated onto Collagen I-coated plates. A static tension protocol was applied
468 overnight to promote cell adhesion and to concentrate cardiomyocytes in the center of the plate.
469 The following day, cell medium was changed and a cyclic stretch protocol was applied for 24h
470 before RNA extraction and RT-qPCR. **B.** Relative expression of *fosab* in *trpc6a*^{+/+} and *trpc6a*^{-/-}
471 ^{-/-} cardiomyocytes subjected to cyclic stretch. **C.** Relative expression of *fosl1a* in *trpc6a*^{+/+} and
472 *trpc6a*^{-/-} cardiomyocytes subjected to cyclic stretch.

A

trpc6a KO (sa23930) G637T

Exon 2-CCTCTCACTGTCACTAGTACGAGATCGTG
stop

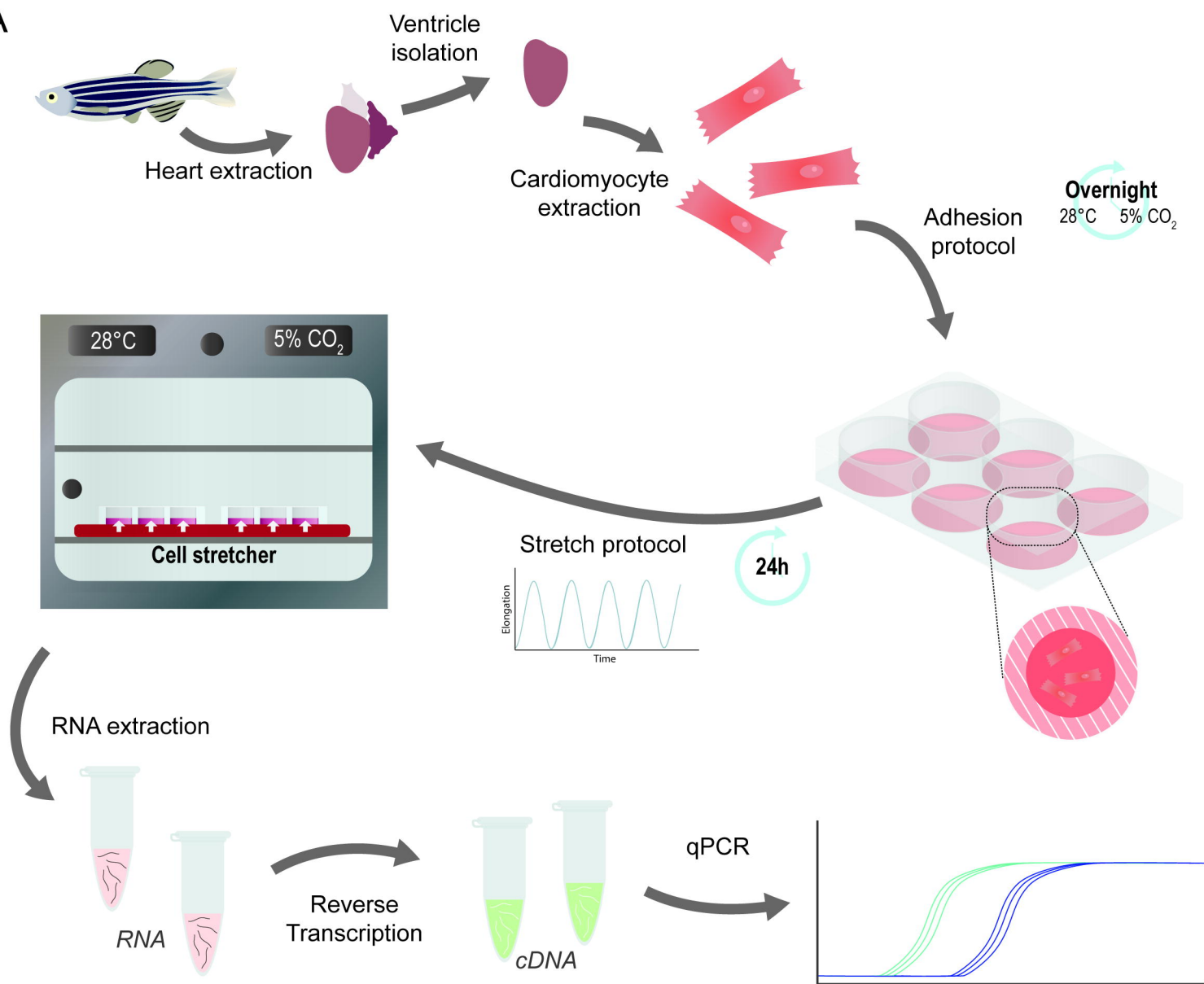




D

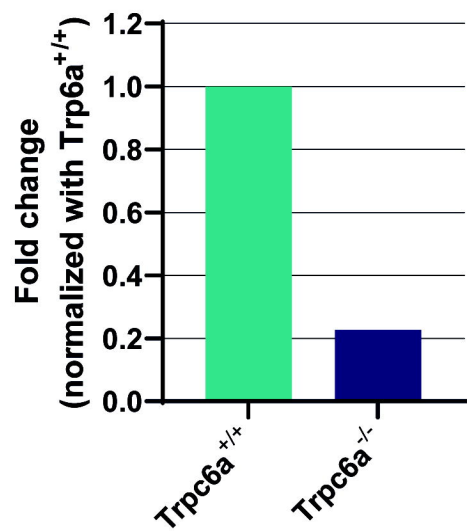
ID	Gene name	<i>trpc6a^{+/+}</i>		<i>trpc6a^{-/-}</i>	
		log ₂ FC	p value	log ₂ FC	p value
ENSDARG00000036074	CCAAT enhancer binding protein alpha (<i>cebpa</i>)	1,492	0,046	0,362	0,457
ENSDARG00000015355	FOS like 1, AP-1 transcription factor subunit a (<i>fosl1a</i>)	3,156	0,003	0,284	0,766
ENSDARG00000104090	JunE proto-oncogene, AP-1 transcription factor subunit (<i>june</i>)	1,614	0,042	-0,495	0,414
ENSDARG00000103980	v-ets avian erythroblastosis virus E26 oncogene homolog 2 (<i>ets2</i>)	1,693	0,036	0,500	0,375
ENSDARG00000031683	v-fos FBJ murine osteosarcoma viral oncogene homolog Ab (<i>fosab</i>)	2,496	0,011	0,025	0,975
ENSDARG00000070542	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog Bb (<i>mafbb</i>)	2,975	0,012	1,311	0,289

A



B

fosab expression



C

fosl1a expression

