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1 **Which actin genes are necessary for zebrafish heart development and function?**

2

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13

14 Keywords zebrafish, CRISPR, cardiac actin, genome modification

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15 **Abstract:**

16

17 Heart failure is the number one cause of mortality in the world, contributed to by cardiovascular
18 disease. Many diseases of the heart muscle are caused by mutations in genes encoding
19 contractile proteins, including cardiac actin mutations. Zebrafish are an advantageous system for
20 modeling cardiac disease since embryos can develop without a functional heart. However,
21 genome duplication in the teleost lineage creates a unique obstacle by increasing the number of
22 genes involved in heart development. Four actin genes are expressed in the zebrafish heart:
23 *acta1b; actc1c;* and duplicates of *actc1a* on chromosome 19 and 20. Here, we characterize the
24 actin genes involved in early zebrafish heart development using *in situ* hybridization and CRISPR
25 targeting to determine which gene is best to model changes seen in human patients with heart
26 disease. The *actc1a* and *acta1b* genes are predominant during embryonic heart development,
27 resulting in severe cardiac phenotypes when targeted with CRISPRs. Targeting these two cardiac
28 genes with CRISPRs simultaneously results in a more severe phenotype than their individual
29 counterparts, with the results suggesting compensation for lost actin genes by other actin
30 paralogues. Given the duplication of the *actc1a* gene, we recommend *acta1b* as the best gene
31 for targeted cardiac actin research.

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32 **Introduction**

33

34 Heart failure is the number one cause of death worldwide, with cardiovascular disease being a
35 major contributor [1]. Cardiomyopathies are called "diseases of the sarcomere" because mutations
36 in genes encoding the proteins of the sarcomere contractile machinery are a main cause of
37 cardiomyopathies, including myosin, troponin, tropomyosin, cardiac myosin binding protein C,
38 and cardiac actin (ACTC) [2,3]. Recent efforts have targeted some of these proteins for drug
39 development to treat cardiomyopathies [4].

40

41 Testing drugs with a whole animal system is vital for developing treatments for diseases. We
42 seek to understand how changes in the cardiac actin gene (*ACTC*) in people lead to different
43 cardiomyopathies. We have studied several ACTC variants at the molecular level [5–9]; however,
44 our goal is to integrate our molecular knowledge of ACTC biochemical changes with physiological
45 dysfunction in a whole animal by gene editing the cardiac actin gene in the model organism.

46

47 The zebrafish is an excellent model for cardiac research [10,11]: their embryos are transparent with
48 heartbeats detectable at 24 hours post-fertilization (hpf), and they do not require a fully
49 functional heart for viability for the first 5 days post-fertilization (dpf) due to diffusion of oxygen
50 through the tissues; hence, embryonic lethal heart mutations seen in mammals can be studied
51 more readily in zebrafish. In addition, zebrafish are small and easy to maintain, with quick growth,
52 large numbers of progeny, and the genome of the Tübingen (Tü) strain has been completely
53 sequenced.

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54

55 However, unlike the single α-cardiac actin gene (*ACTC*) in humans, the zebrafish genome contains
56 four actin genes that are expressed in the heart (*zfactc* genes): *acta1b; actc1c;* and duplicates of
57 *actc1a* on chromosome 19 and 20. Cardiac-related effects of mutations in *actc1a* and *acta1b*
58 have been studied previously [12–14], while we identified and performed preliminary
59 characterization of *actc1c* [15].

60

61 Shih et al. (2015) performed transcriptome analysis of *actc1a* and *acta1b* in embryonic (96 hpf)
62 and adult (6 month-old) zebrafish hearts, showing higher *acta1b* expression in early development
63 while *actc1a* is expressed at higher levels during adulthood, suggesting developmental regulation
64 of *zfactc* genes [16]. Functional and spatiotemporal characterization of these *zfactc* genes is needed
65 to assign a cardiac designation with high confidence.

66

67 While early expression of *actc1a* and *acta1b* has been characterized with *in situ* hybridization
68 (ISH), this previous work focused primarily on the somites of the tail. The *actc1a* gene was the
69 subject of expression characterization in the somites and included the heart at the 1-4 somites
70 to 7 dpf stages [16–18], with *acta1b* ISH analysis focused on the somites [18–20]. Our preliminary
71 characterization of *actc1c* showed expression in the heart and the somites at 36 hpf [15]. Owing to
72 teleost gene duplication, duplicate *actc1a* genes located on chromosomes 19 and 20 are identical
73 in sequence up to about 600 bp before the start codon, making designing *in situ* hybridization
74 probes or CRISPR sgRNA that distinguish between the duplicate genes extremely challenging.

75

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76 To determine the *zfactc* genes necessary for zebrafish heart development and function and which
77 is the best to edit and model human cardiac diseases resulting from *ACTC* mutations**,** we studied
78 the spatiotemporal expression of these genes in the heart, employing *in situ* hybridization in
79 embryos from 24 hpf to 96 hpf and observing the functional consequences of CRISPRs targeting
80 the genes. All three *zfactc* genes are expressed in the heart at initial stages of heart development;
81 however, the *actc1a* and *acta1b* genes are the predominant paralogues expressed during
82 embryonic heart development and result in severe cardiac phenotypes when targeted with
83 CRISPRs. The *actc1c* gene seems to be a minor player, with its expression occurring primarily in
84 the first 2 dpf. At the same time, CRISPR work targeting *actc1c* results in a cardiac phenotype,
85 suggesting that this gene plays a role in cardiac development. Targeting two *zfactc* genes with
86 CRISPRs simultaneously results in a more severe phenotype than their individual counterparts,
87 with the results suggesting compensation for lost *zfactc* genes by other actin paralogues. Given
88 the gene duplication of the *actc1a* gene, we suggest that the *acta1b* gene is the best candidate
89 for cardiac actin research.

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90 **Materials and Methods**

91

92 *Ethics Statement*

93

94 All protocols were carried out according the guidelines stipulated by the Canadian Council for
95 Animal Care and the University of Guelph's Office of Research Animal Care Committee (Animal
96 Use Protocol license: 4309)

97

98 *Zebrafish Maintenance*

99

100 Adult zebrafish (Tübingen strain) were maintained according to guidelines by the Canadian
101 Council on Animal Care and kept on a 12/12-hour light and dark cycle at 28°C. Adults were fed
102 brine shrimp (Hikari Bio-Pure Brine Shrimp) and fish flakes (Omega One) daily in a cycled-water
103 aquatic facility. Embryos were collected from crossing wild-type adult zebrafish and grown at
104 28°C in zebrafish embryo medium [21] for up to 6 days prior to fixation.

105

106 *In situ hybridization*

107

108 Zebrafish embryos were staged and fixed in 4% paraformaldehyde/PBS overnight at 4°C. Cardiac
109 actin probes were cloned into TOPO-plasmids (Thermofisher) for probe synthesis (Table 1).
110 Antisense RNA probes were synthesized from the TOPO construct using SP6 RNA polymerase
111 (Thermofisher).

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112

113 *In situ* hybridizations were carried out as previously described [22] using an Intavis *In Situ* Pro Liquid
114 Handling Robot (Intavis, Koeln) with the exception of the proteinase K digestion and staining
115 reaction steps, which were performed by hand.

116

117 *CRISPR sgRNA Preparation and Microinjections*

118

119 CRISPR single guide RNA (sgRNA) was designed for *actc1a, actc1c* and *acta1b* using CHOPCHOP
120 (https://chopchop.cbu.uib.no; danRer11/GRCz11)[23], selecting the sgRNA that returned the
121 fewest off-target sites (Table 2). Given the extreme identity between the two *actc1a* genes
122 physically located on chromosomes 19 and 20, one sgRNA was designed that targets both genes.

123

124 sgRNA was synthesized using SP6 RNA Polymerase (Thermofisher). Cas9 (1 mg/ml; CP01-200,
125 PNA Bio Inc) and fresh sgRNA (1 ug) was injected into 1-cell zebrafish embryos that were allowed
126 to recover in zebrafish embryo medium at 28°C. Zebrafish embryos were monitored daily for the
127 appearance of phenotypes and imaged using an iPhone 6 camera (8 megapixel, 1080p HD video
128 at 60 fps; Apple Inc.)

129

130 *High Resolution Melt Curve Analysis Screening and Sequencing*

131

132 For screening using High Resolution Melt Curves (HRM), genomic DNA was extracted from
133 zebrafish embryos by individually lysing tissue in 10 ul of 0.5 M NaOH at 95°C for 45 mins,

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134 followed by neutralization with 0.2 mM Tris-HCl (pH 8). DNA was diluted 1/20 for best
135 amplification results during HRM. The HRM amplicons were designed to be no larger than 200
136 bp and centered on the CRISPR-Cas9 cut site (Table 3). High Resolution Melt Curves were
137 produced using the saturating dye, EvaGreen (Type-it HRM PCR Kit, Qiagen) and the
138 manufacturers recommended protocol for use. HRM was performed using a StepOne Plus
139 Thermocycler (Thermofisher) and results interpreted using High Resolution Melt Software v3.0.1
140 (Thermofisher).

141

142 Embryos that demonstrated melt curves with melting temperature at 50% of the maximum
143 temperature ($Melt_{50}$) values that differed at least 0.5°C from their un-injected control siblings
144 were sent for sequencing. Positive HRM samples were isolated using a PCR purification kit
145 (Qiagen) and submitted to the University of Guelph, Agriculture and Food Laboratory for Sanger
146 Sequencing. Sequences were analyzed using TIDE [24] and Gear-Indigo (https://www.gear-
147 genomics.com) to identify nucleotide changes between un-injected CRISPR control and CRISPR-
148 injected samples.

149

150 *Heart Rate Acquisition and Analysis*

151

152 The heart rates of all CRISPR-injected embryos were video-recorded daily from 1-6 dpf using an
153 iPhone 6 camera (8 megapixel, 1080p HD video at 60 fps; Apple Inc.). Captured videos were
154 analyzed with DanioScope software (Noldus) to determine heart rates. Genomic DNA was
155 extracted from these embryos and screened for mutations using HRM as above and hits were

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156 sent for Sanger Sequencing. Embryos with mutated sequences had their heart rates graphed in

157 comparison to wild-type controls.

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158 **Results**

159

160 ***actc1a and acta1b are the predominant actin paralogues expressed during striated muscle***
161 ***development –*** To identify which actin paralogues are necessary for heart development, we
162 analyzed the expression of *actc1a, acta1b* and *actc1c* with *in situ* hybridization (ISH) during early
163 stages of embryogenesis (Fig 1). At 24 hpf, all 3 actin genes are expressed in the linear heart tube
164 (Fig 1, A&B; white arrowheads), although *actc1c* appears to be limited to ventricle
165 cardiomyocytes (Fig 1C) [25]. *actc1a* is expressed in the zebrafish heart at all stages examined with
166 the strongest expression observed in the ventricle (Fig 1, A,D,E,J,K,P&O). The continued
167 expression of *actc1a* throughout embryogenesis suggests *actc1a* is required for cardiac muscle
168 development.

169

170 Similar to *actc1a*, *acta1b* is also expressed in the heart for significant stages of heart
171 development. *acta1b* is expressed strongly in the ventricle and weakly in the atrium from 24-48
172 hpf (Fig 1, B,F,G,L&M). By 72 hpf, *acta1b* is no longer expressed in the heart and is restricted to
173 the skeletal muscle of the head and trunk (Fig 1, R&S). Unlike *actc1a*, *acta1b* is only expressed
174 during the stages of heart morphogenesis and cardiac sarcomere formation, suggesting *acta1b*
175 may be required for the assembly but not the maintenance of the zebrafish heart muscle.

176

177 *actc1c* expression is not observed in the heart at stages beyond 24 hpf (Fig 1, C,H&I) and ceases
178 expression in skeletal muscle by 48 hpf (Fig 1, N,O,T&U). This lack of cardiac expression after 24
179 hpf suggests that *actc1c* is not required throughout heart development but does not rule out the

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180 possibility that *actc1c* may be required for the initial formation of cardiac sarcomeres in the
181 ventricle.
182
183 ***actc1a, acta1b and actc1c are all required for normal heart development and function –*** We
184 demonstrated that *actc1a*, *acta1b* and *actc1c* are expressed at the earliest stages of heart
185 development at 24 hpf but have different temporal and spatial expression patterns from 36-72
186 hpf (Fig 1). Since all three paralogues were expressed in the developing linear heart tube, we
187 tested the necessity for each actin gene in heart development by disrupting each paralogue using
188 the CRISPR/Cas9 system.
189
190 All CRISPR/Cas9-injected embryos displayed similar cardiac phenotypes ranging from normal to
191 significantly reduced heart rates, and unlooped and minor to severely degenerated hearts.
192 Embryos injected with either *acta1b*- or *actc1c*-targeting CRISPR sgRNA displayed pericardial
193 edema when compared to mock-injected and embryos injected with *actc1a*-targeting CRISPR
194 sgRNA embryos at 72 hpf (Fig 2, A-D; black arrowheads). At 96 hpf, *actc1a*-CRISPR-injected
195 embryos demonstrated pericardial edema like *acta1b* or *actc1c*-injected embryos (Fig 2, C-F).
196
197 Although the severity of cardiac phenotypes varied across embryos injected with the same
198 CRISPR sgRNA, we suspect genetic mosaicism produced by CRISPR/Cas9 cutting and non-
199 homologous end joining repair accounts for this variation.
200

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201 ***Simultaneous CRISPR/Cas9 against actc1a and acta1b results in more severe cardiac***
202 ***phenotypes –*** We have shown that *actc1a* is expressed at all early stages of heart development
203 when compared to either *acta1b* or *actc1c*, yet a *actc1a*-CRISPR-mutant phenotype appears later
204 than the cardiac phenotypes of *acta1b* or *actc1c* (Fig 2). Based on these data, we hypothesize
205 that *acta1b* and possibly *actc1c*, compensates for the absence of *actc1a* protein in initial stages
206 of development during *acta1b* expression up to 72 hpf. To test this hypothesis, we injected
207 embryos with both *actc1a*- and *acta1b*- targeting CRISPR sgRNA and compared their heart rates
208 to wild-type and embryos injected with either *actc1a*- or *acta1b*-targeting CRISPR sgRNA alone.
209 We would expect double *actc1a-acta1b* mutants to display heart rates similar to *acta1b*-CRISPR
210 mutant heart rates at 72 hpf since expression of both predominant cardiac actin genes is absent
211 and *acta1b* cannot compensate for *actc1a*.
212
213 At 48 and 72 hpf, the double *actc1a-acta1b*-targeting CRISPR sgRNA-injected embryos did not
214 display significantly different heart rates when compared to wild-type, *actc1a*- or *acta1b*-
215 targeting CRISPR sgRNA-injected embryos (Fig 3). However, by 96 hpf, the average heart rates of
216 *actc1a-acta1b*-targeting CRISPR sgRNA-injected embryos were significantly lower than the heart
217 rates observed with wild-type embryos. The difference between wild-type and *zfactc*-targeting
218 CRISPR sgRNA-injected embryo heart rates continues to increase as embryogenesis progresses,
219 suggesting that the hearts of embryos injected with *zfactc*-targeting CRISPR sgRNA do not
220 continue proper development.

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221 **Discussion**

222

223 Zebrafish are an excellent system for modeling human cardiac disease and dissecting the
224 mechanisms behind disease progression and treatment. However, a genome duplication event
225 unique to the teleost lineage can complicate specific gene targeting studies due to genetic
226 compensation. Zebrafish cardiac actin genes are no exception to this complication with 4
227 identified genes (*actc1a* (chromosome 19 and 20), *acta1b* and *actc1c*) contributing to heart
228 development. Based on *in situ* expression (Fig 1), and previous data [16], *actc1a* has continuous
229 transcription in the heart throughout development; however, *actc1a* is present as two identical
230 genes on chromosomes 19 and 20. Sequencing of these regions reveals extreme sequence
231 identity between the two occurrences of *actc1a,* so modifying and analyzing one *actc1a* isoform
232 is very challenging without modifying one isoform first to differentiate the two.

233

234 *acta1b* is expressed in both chambers during the initial stages of heart development that include
235 formation of the cardiac sarcomeres and heart looping. Additionally, there is only one *acta1b*
236 gene, making targeting and analysis of human cardiac mutations more efficient and feasible than
237 *actc1a* (19/20). *actc1c* demonstrated a very brief expression profile in the zebrafish heart,
238 suggesting it is not as necessary for heart development as the other *zfactc* genes.

239

240 When we combine the expression profiles with the phenotypes of CRISPR-targeted *zfactc* genes,
241 *acta1b* is required for normal heart development. When *acta1b* was targeted by CRISPR, cardiac
242 phenotypes manifested early and became progressively worse over time (Fig 2 and 3). Targeting

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243 *actc1a* (19/20) with CRISPR resulted in phenotypes that appeared later than *acta1b* but followed
244 a similar phenotype progression. The phenotypic delay with *actc1a* targeting suggests that
245 *acta1b* and/or *actc1c* compensate for the lack of functional *actc1a*. We also considered that one
246 wild-type isoform of *actc1c* (19 or 20) could still be expressed in these CRISPR mutants. However,
247 due to the identical sequences of the two *actc1a* genes, we would hypothesize that we achieved
248 a minimum threshold for completely modifying both *actc1a* genes within CRISPR-injected
249 embryos. Simultaneously targeting *acta1b* and *actc1a* with CRISPR/Cas9 resulted in significantly
250 worse heart phenotypes than targeting either single gene (Fig 3), supporting a compensation
251 model by cardiac actin paralogues.
252
253 Taken together, our data suggests *acta1b* is the best zebrafish cardiac actin gene for modeling
254 human heart diseases resulting from mutations in the cardiac actin gene . *acta1b* mutants
255 demonstrated minimal compensation by other *zfactc* gene early in embryogenesis; ideal
256 conditions for characterizing the phenotype and disease mechanism of a specific human actin
257 mutation.
258
259 This work provides a foundation to model human actin mutations in zebrafish. Future work will
260 focus on introducing human cardiac actin mutations into *acta1b* and characterizing the disease
261 as well as exploring methods of treatment. Additionally, determining the changes in cardiac actin
262 paralogue expression in response to single actin knockouts would further dissect the actin gene
263 compensation hypothesis.
264

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334 **Tables**

335

336 **Table 1:** Primers used to amplify *in situ* hybridization probes.

Gene	Gene ID	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
actc1a	408256	CCATCGTCCACAGAAAGTGC	GGACACATCAGAACTTTTATTAC
acta1b	407658	CAAGCAGGAACAGGACGA	ATTTATTAACACATATGC
actc1c	337770	ATGTAATGATGCCCAGGATAC	AAAAAATGTGCACTTTGG

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338

339 **Table 2**: CRISPR single guide RNA designed to target the *zfactc* genes.

Target Gene	CRISPR Oligo Sequence (5' to 3')
actc1a	ACCATTGTCGCACACGAGTGCGG
acta1b	GTGACGGGGGCGACCAACGATGG
actc1c	GCGAGGACGACCAACAATGGAGG

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341

342 **Table 3:** Primers employed for high resolution melting analysis of *zfactc* amplicons.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
actc1a	GAACCATCCTAAAAAACAGC	AACAATAGAGGGAAAGACAG
acta1b	CAAGATGTGTGACGACGA	AGAGGAAAATGCAGCAGA
actc1c	CTTGTATGTGCTGTGTTTGA	TGGATTTGCCGGAGATGA

343

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344 **Figure Legends**

345 **Figure 1. Expression of three actin paralogues throughout early embryo development.** At 24 hpf,
346 *in situ* hybridization reveals somite and heart tube-restricted expression of *actc1a* (A), *acta1b* (B)
347 and *actc1c* (C). By 36 hpf, *actc1a* becomes restricted further to the ventricle (D, E) while *acta1b* is
348 mainly expressed in the ventricle and faintly in the atrium (F, G). *Actc1c* demonstrates a ubiquitous
349 expression, with the exception of the heart, by 36 hpf (H, I). At 48 hpf, *actc1a* (J, K) and *acta1b* (L,
350 M) share nearly identical expression in the heart and somites with the atrium displaying low
351 expression for both paralogues. By 48 hpf, *actc1c* expression is only observed in the pectoral fin
352 buds (N, O). *Actc1a* expression becomes restricted to the heart and head muscles at 72 hpf (P, Q).
353 At 72 hpf, *acta1b* is only expressed in the head muscles and somites with no observable expression
354 in the heart (R, S). By 72 hpf, *actc1c* is not expressed in the striated muscle of the developing
355 zebrafish embryo (T, U). (white arrowheads = heart expression; v = ventricle, a = atrium; white
356 dotted lines outline ventricle; blue dotted lines outline atrium).
357

358 **Figure 2. Phenotypes of cardiac actin CRISPR-injected embryos.** When compared to mock-
359 injected control embryos (A), embryos injected with *actc1a*-targeting CRISPR sgRNA (B) displayed
360 only a slight pericardial edema while embryos injected with *acta1b*-targeting (C) or *actc1c*-
361 targeting CRISPR sgRNA (D) demonstrated pericardial edema (black arrowheads) and incompletely
362 looped hearts. By 96 hpf, embryos injected with *actc1a*-targeting CRISPR sgRNA exhibited
363 pericardial (black arrowhead) and yolk sac edema when compared to mock-injected control
364 embryos (E, F).
365

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366 **Figure 3. Heart Rates of embryos injected with cardiac actin-targeting CRISPR sgRNA throughout**
367 **embryogenesis.** The heart rates of wild-type and genotype-confirmed embryos injected with
368 cardiac actin-targeting CRISPR sgRNA were recorded daily from 2-5 days. Individual (circles) and
369 average (lines) heart rates are displayed for each genotype at every age. With the exception of
370 72 hpf, embryos injected with cardiac actin-targeting CRISPR sgRNA demonstrate a lower average
371 heart rate when compared to control wild-type embryos. By 96 hpf, the heart rate of the double
372 *actc1a-acta1b*-targeting CRISPR sgRNA-injected embryos have significantly lower heart rates than
373 embryos injected with either *actc1a*- or *acta1b*-targeting CRISPR sgRNA. (genotype confirmed:
374 wild-type, n=2; *actc1a/acta1b*, n=3; *actc1a*, n=6, *acta1b*, n=6).

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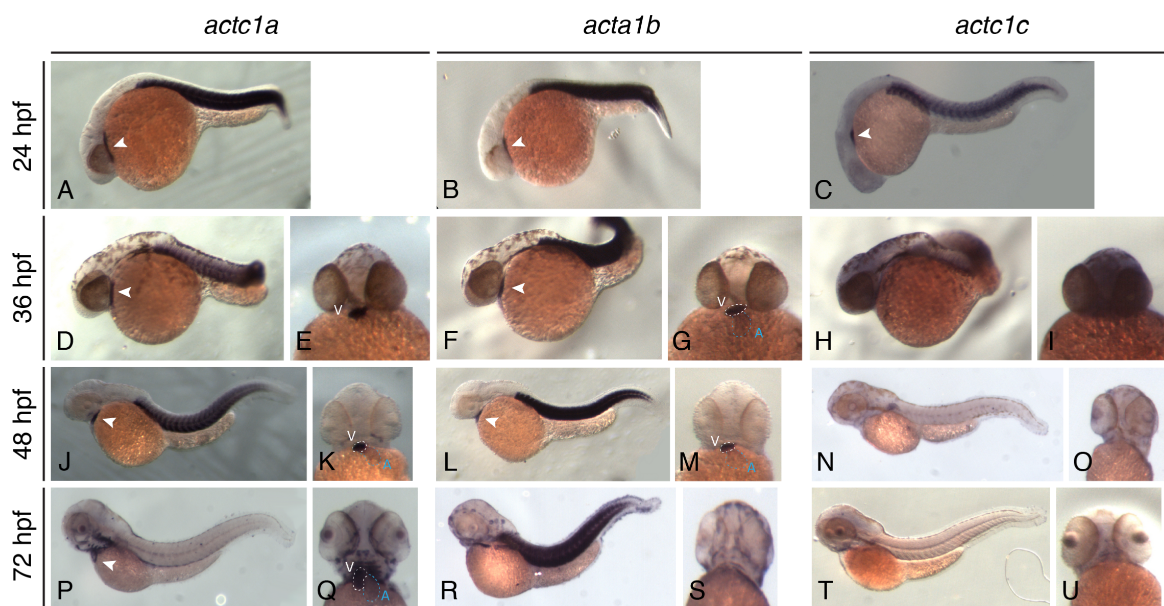
375 **Figure 1**

376

377



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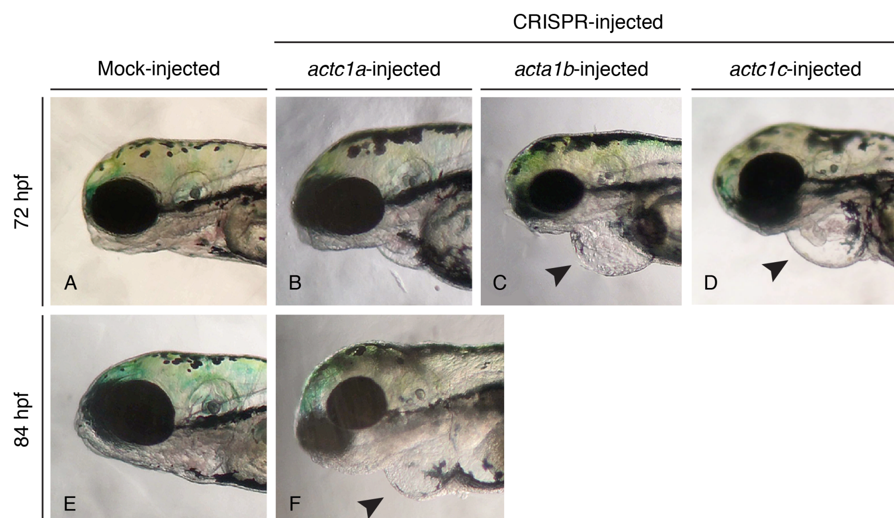
379 **Figure 2**

380

381



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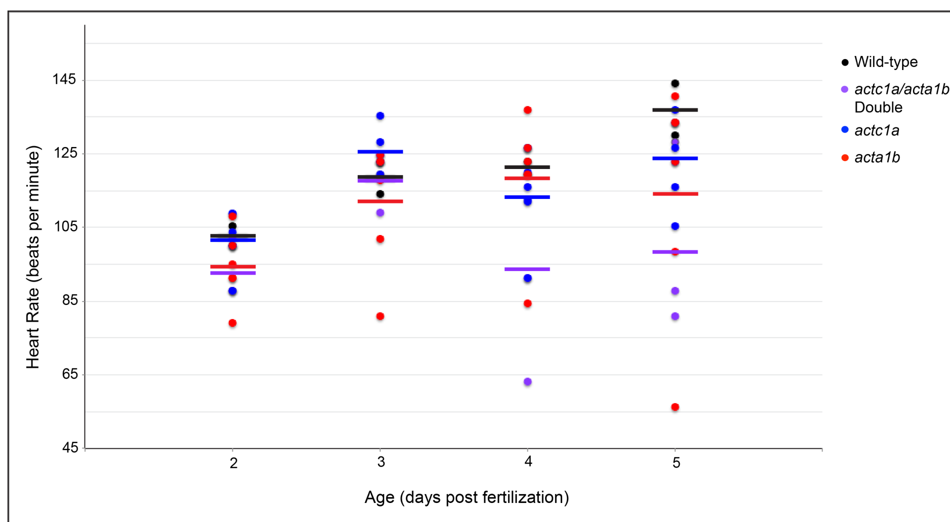
383 **Figure 3**

384

385



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