Hematopoietic and neural crest defects in zebrafish *shoc2* mutants: a novel vertebrate model for Noonan-like syndrome

HyeIn Jang[1], Erin Oakley[1], Marie Forbes-Osborne[2], Melissa Kesler[3], Rebecca Norcross[1], Ann C Morris[2], Emilia Galperin[1*]

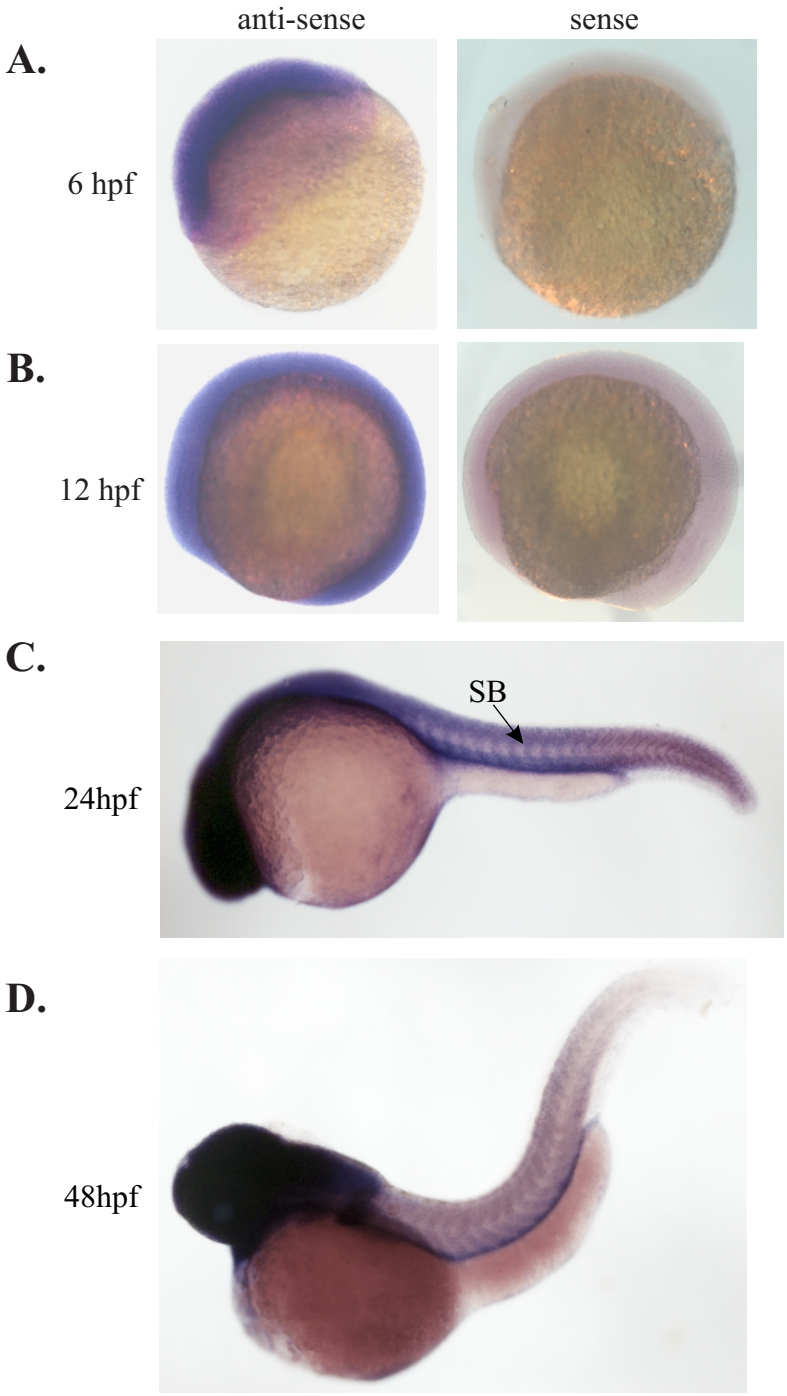
[1] Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, Kentucky 40536, United States of America

[2] Department of Biology, University of Kentucky, Lexington, Kentucky 40506, United States of America

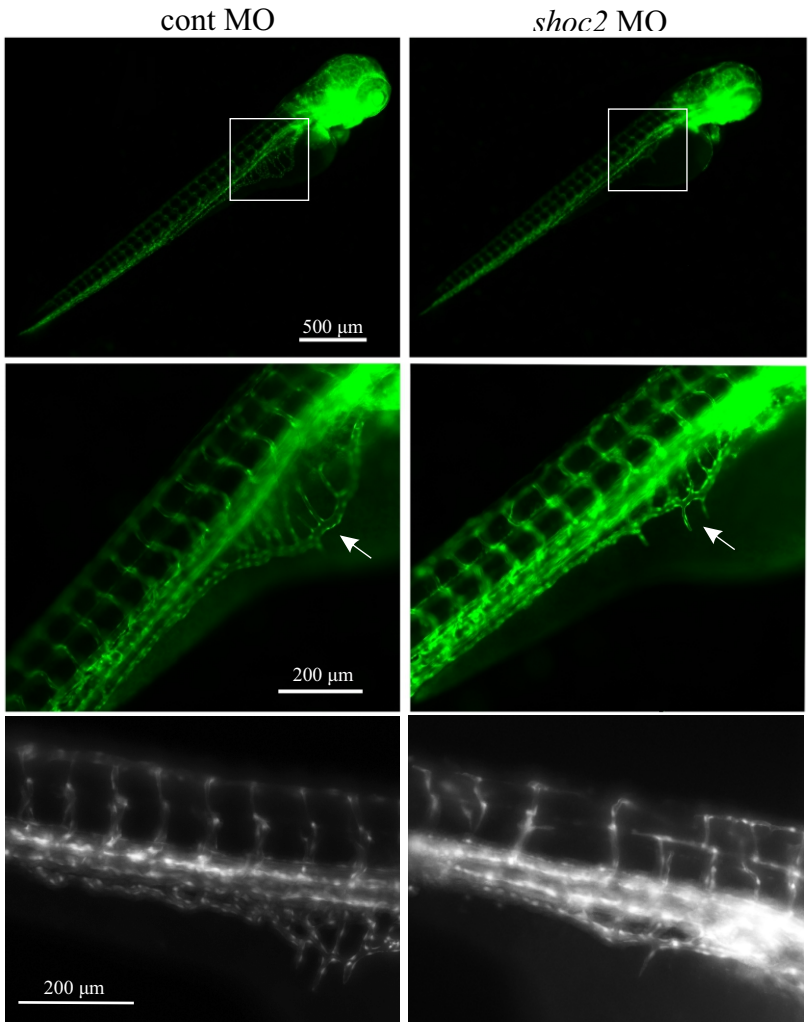
[3] Division of Pathology and Laboratory Medicine, Lexington, Kentucky 40536, United States of America

* Corresponding author
Emilia Galperin
E-mail: emilia.galperin@uky.edu
Telephone: +1 859-323-1796
Fax: +1 859-257-2283

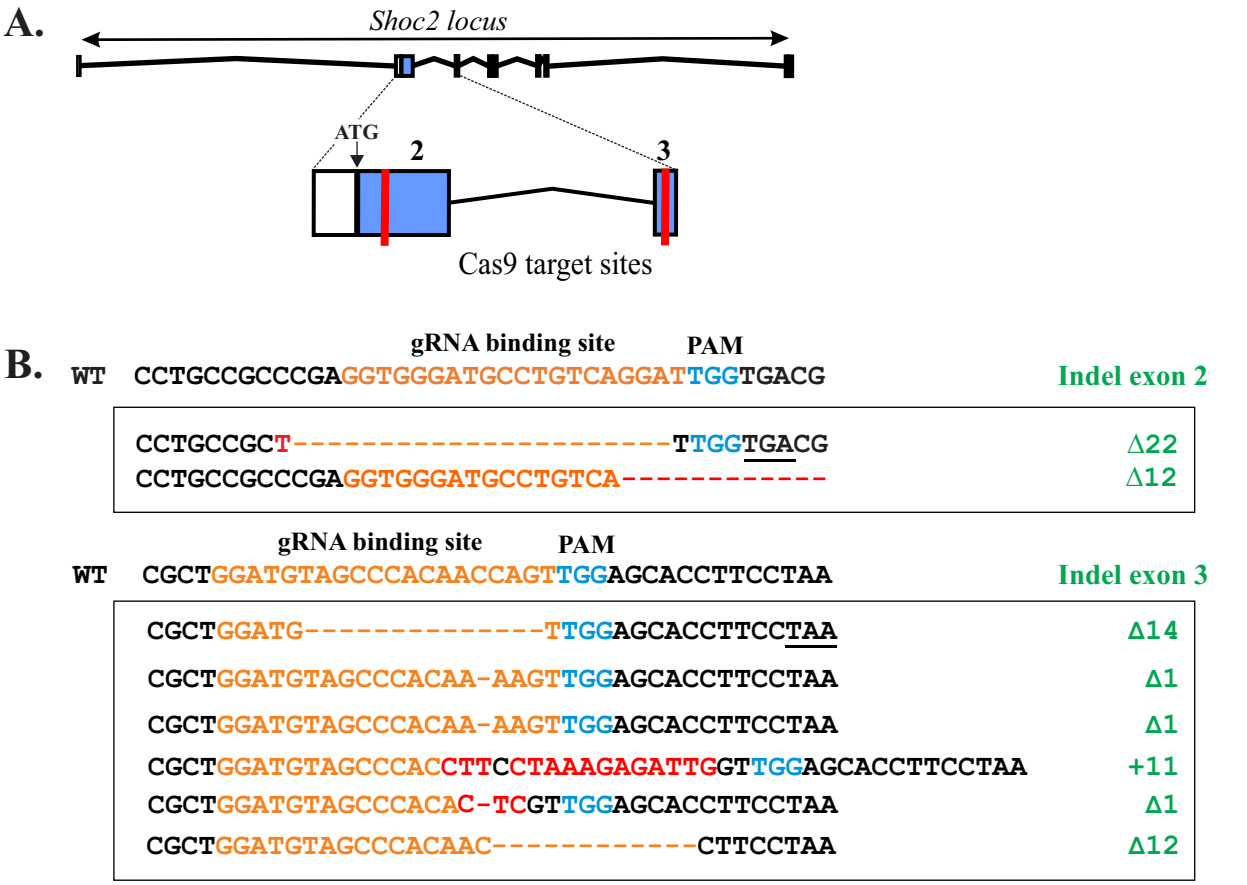
S1 Fig



S2 Fig

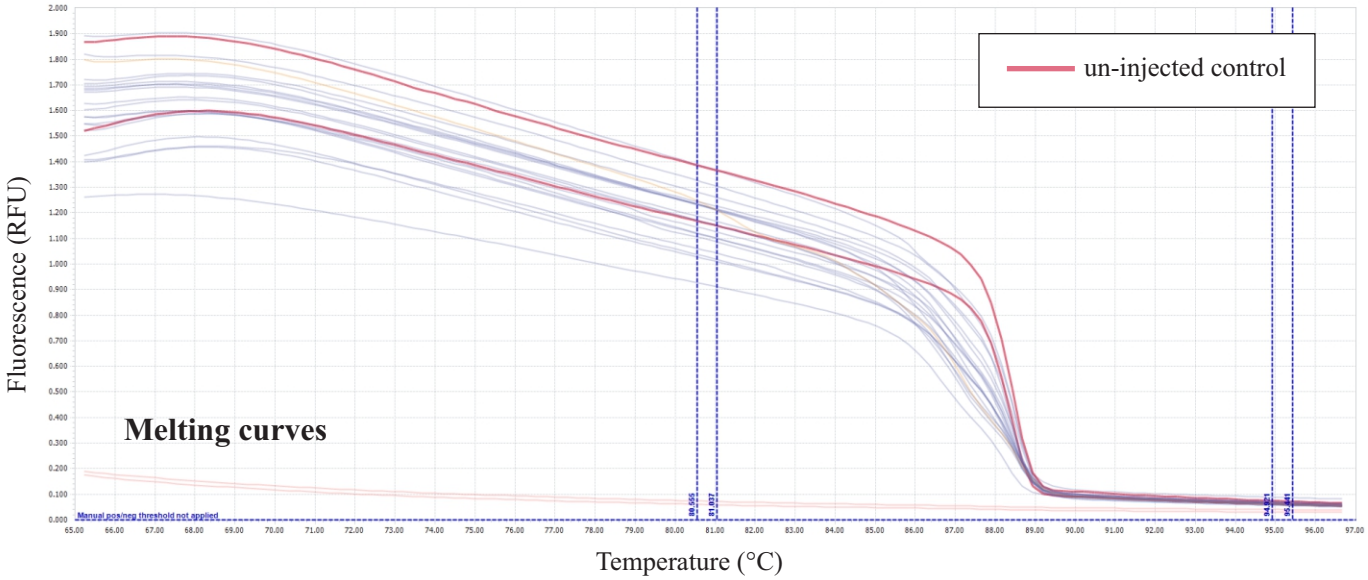


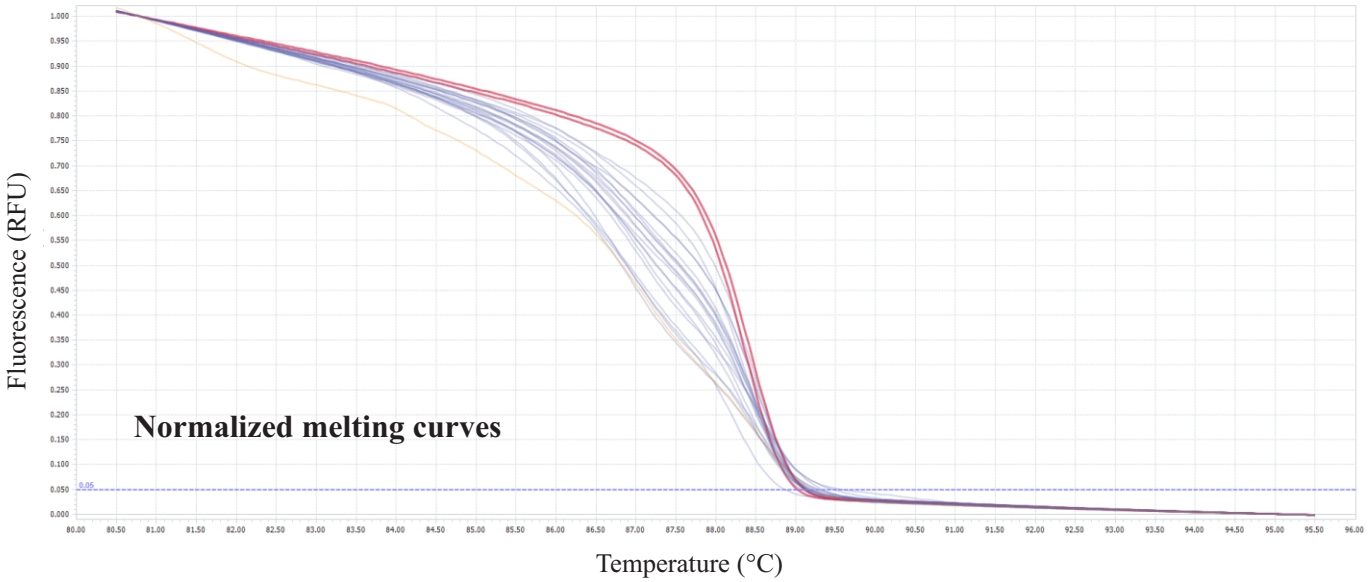
S3 Fig

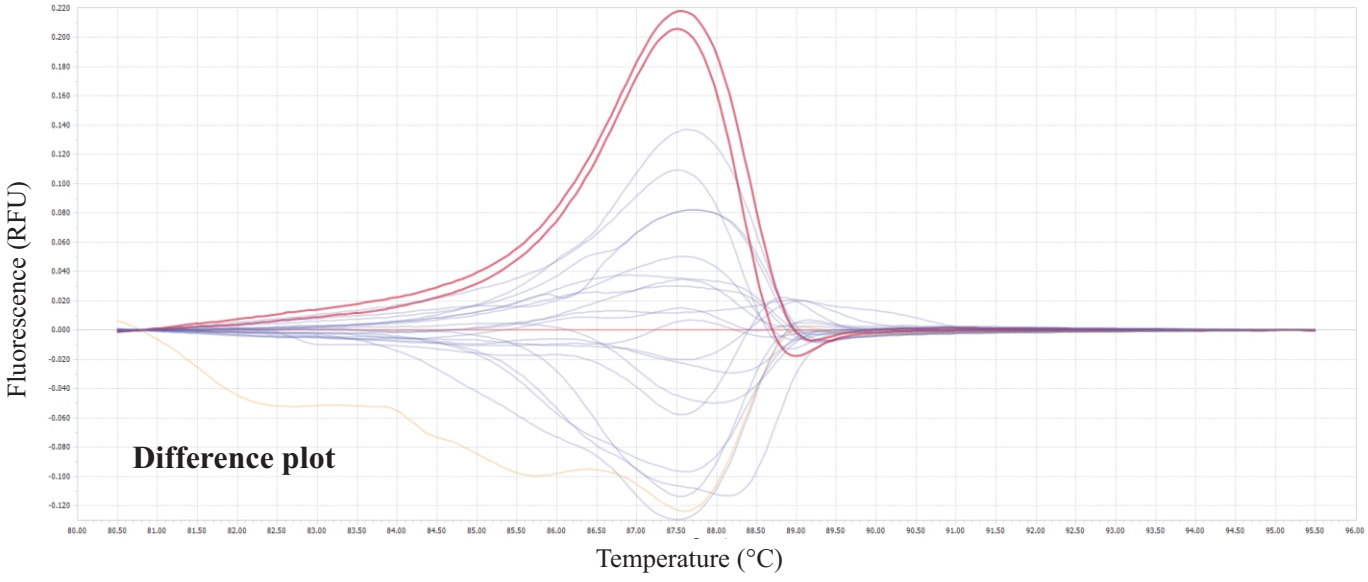

A.
Shoc2 locus
ATG
2
3
Cas9 target sites
B.
gRNA binding site
PAM
WT CCTGCCGCCCGAGGTGGGATGCCTGTCAGGATTGGTGACG
Indel exon 2
CCTGCCGCT----------TTGGTGACG Δ22
CCTGCCGCCCGAGGTGGGATGCCTGTCA---------- Δ12
gRNA binding site
PAM
WT CGCTGGATGTAGCCCACAACCAGTTGGAGCACCTTCCTAA
Indel exon 3
CGCTGGATG----------TTGGAGCACCTTCCTAA Δ14
CGCTGGATGTAGCCCACAA-AAGTTGGAGCACCTTCCTAA Δ1
CGCTGGATGTAGCCCACAA-AAGTTGGAGCACCTTCCTAA Δ1
CGCTGGATGTAGCCCACCTTCCTAAAGAGATTGGTTGGAGCACCTTCCTAA +11
CGCTGGATGTAGCCCACAC-TCGTTGGAGCACCTTCCTAA Δ1
CGCTGGATGTAGCCCACAAC----------CTTCCTAA Δ12
C.
M +/+ +/- -/- +/+ +/- -/-
200 bp
100 bp
Δ22
Δ14
D.
M WT Δ22 WT Δ14 M
500 bp
400 bp
300 bp
100 bp
E.
WT +/-
75 kDa
IB: Shoc2
37 kDa
IB: β-actin

S4A Fig

A. Exon 2 target site HRMA

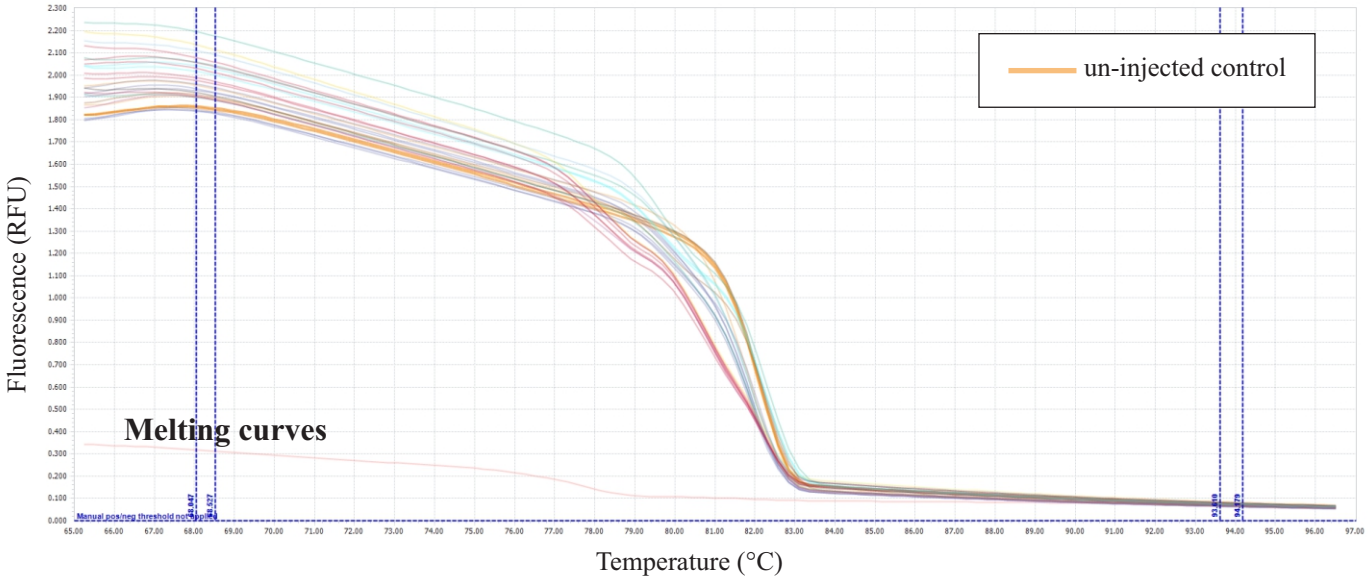


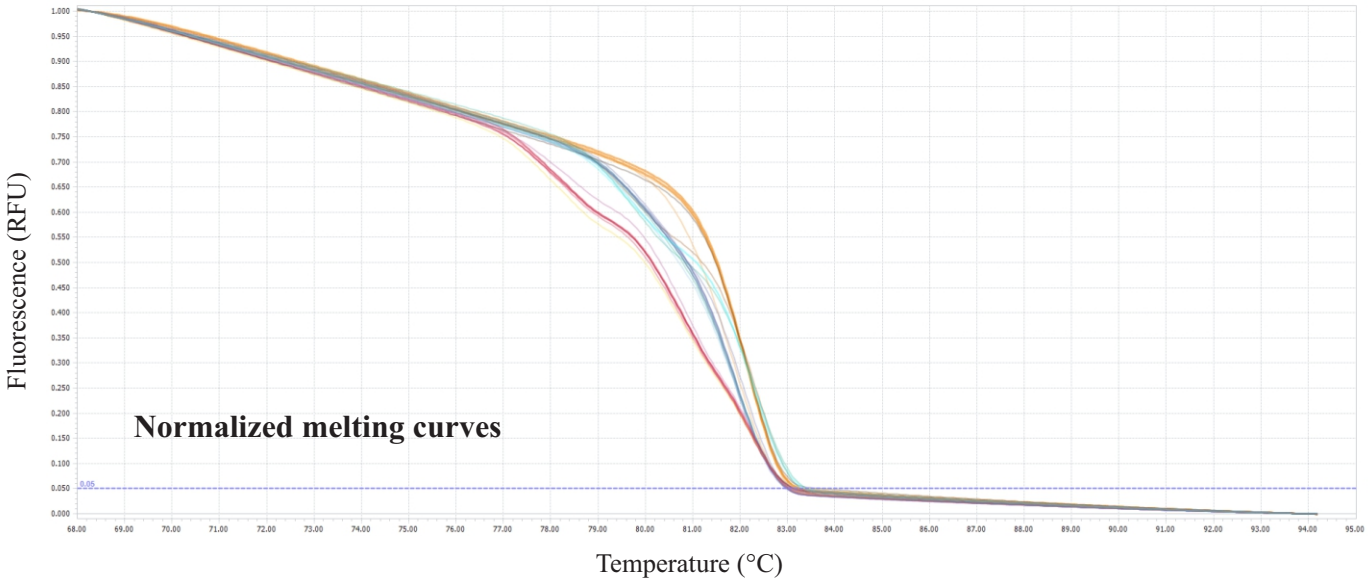


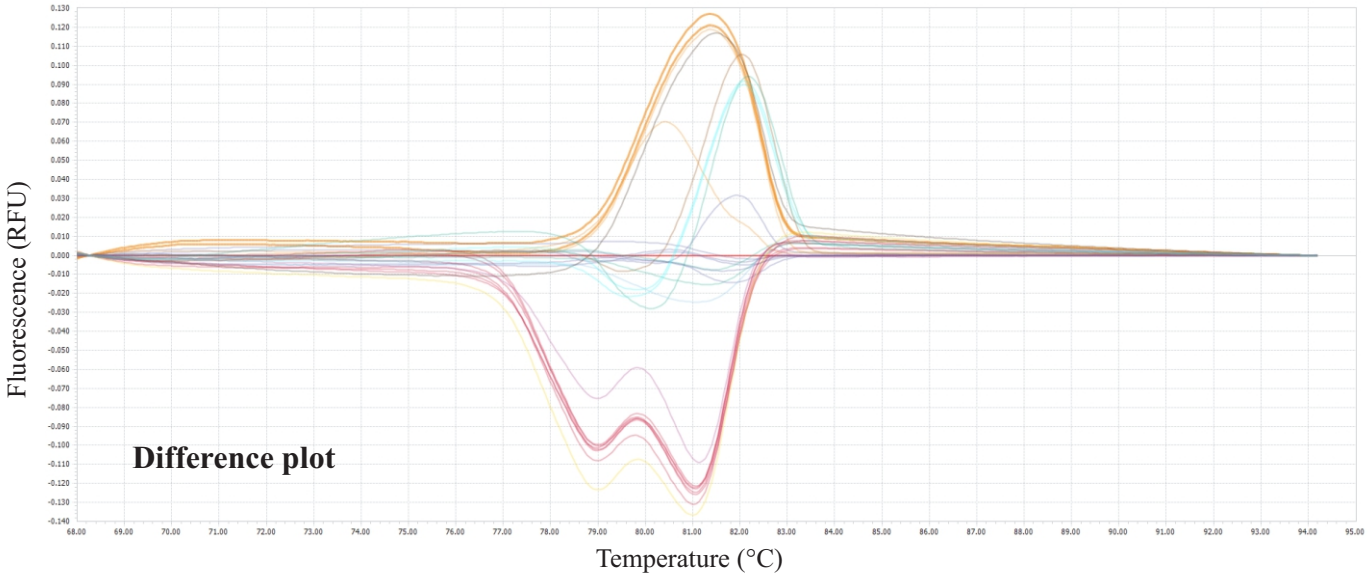


S4B Fig

B. Exon 3 target site HRMA

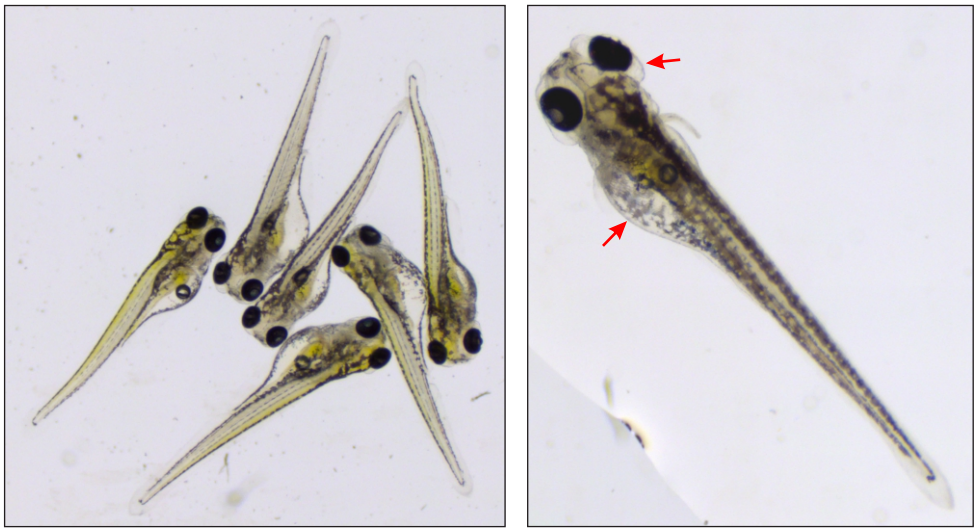




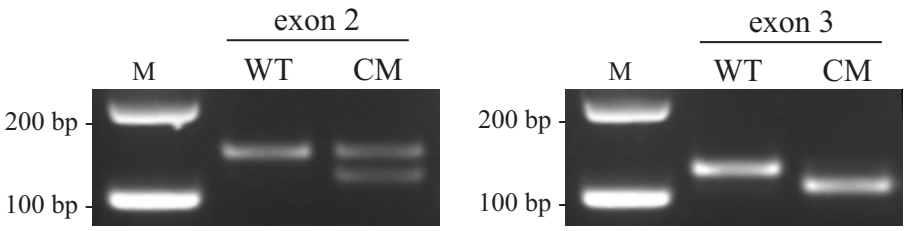


S5 Fig

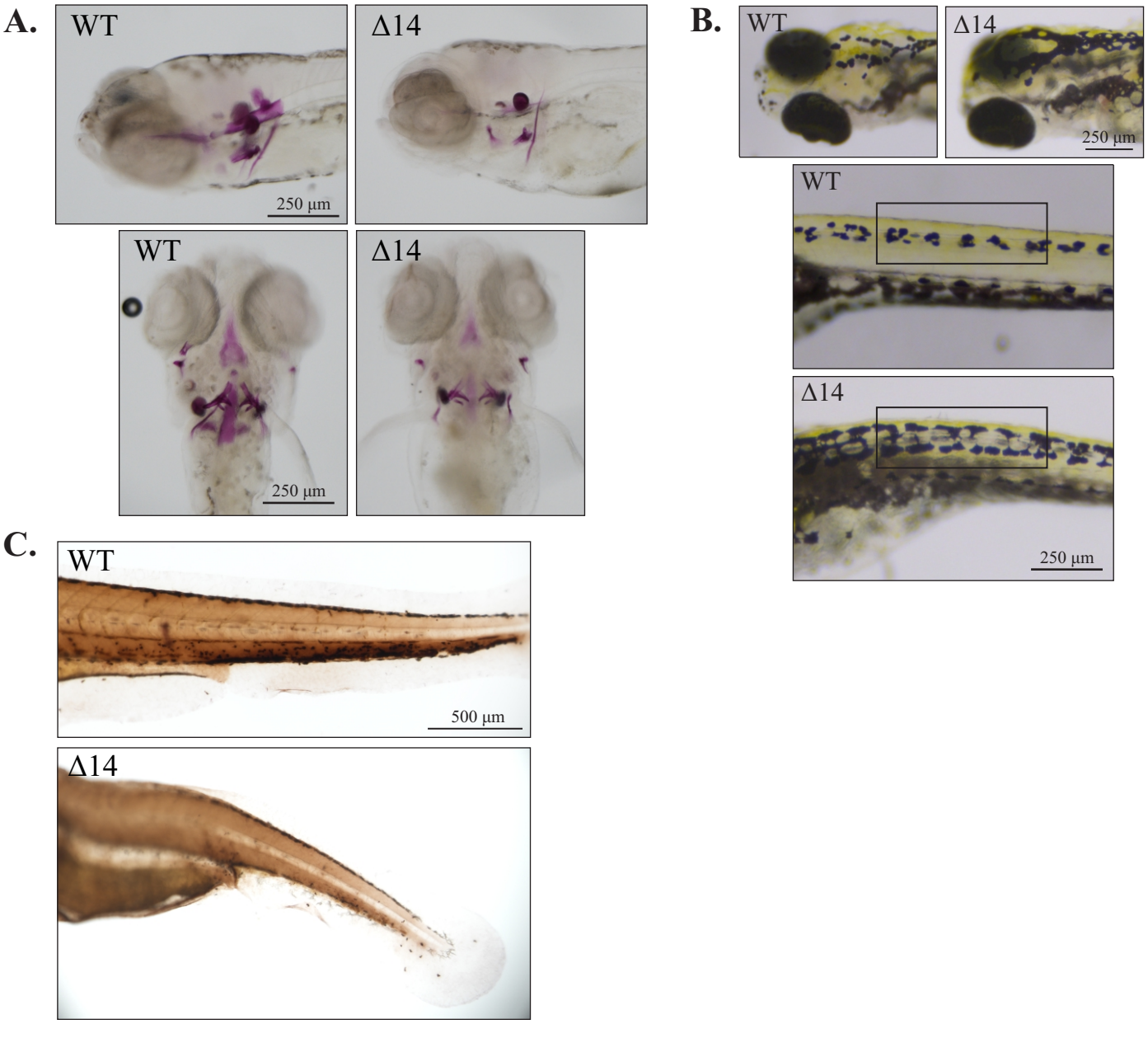
A.



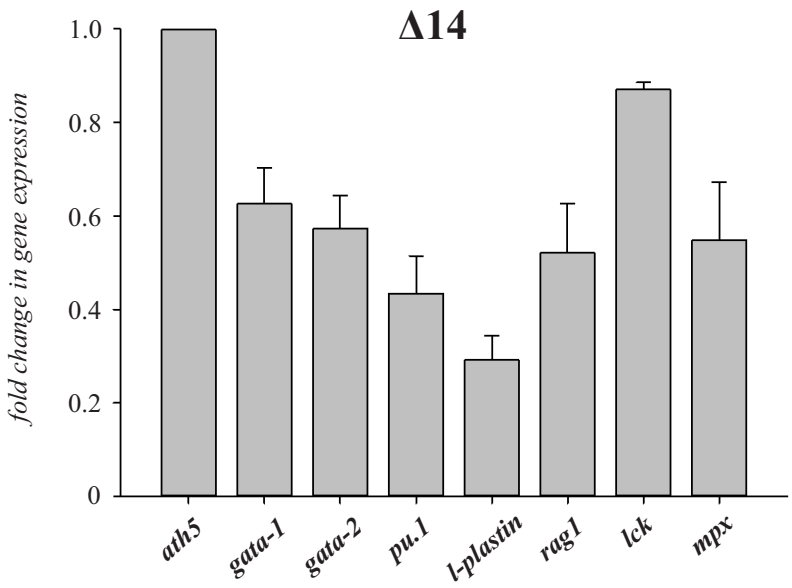
B.



S6 Fig

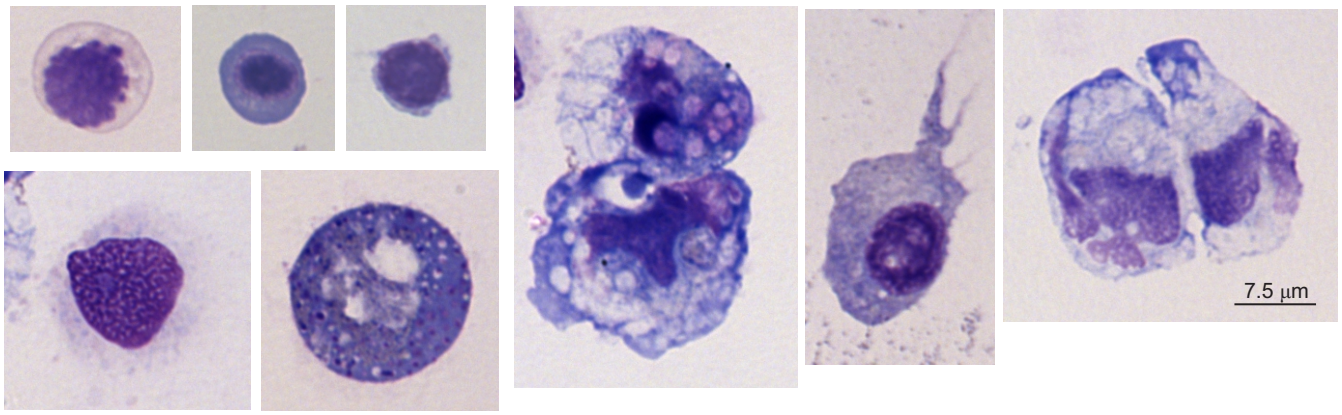

A.
WT
Δ14
250 μm
WT
Δ14
250 μm
B.
WT
Δ14
250 μm
WT
Δ14
250 μm
C.
WT
500 μm
Δ14

S7 Fig

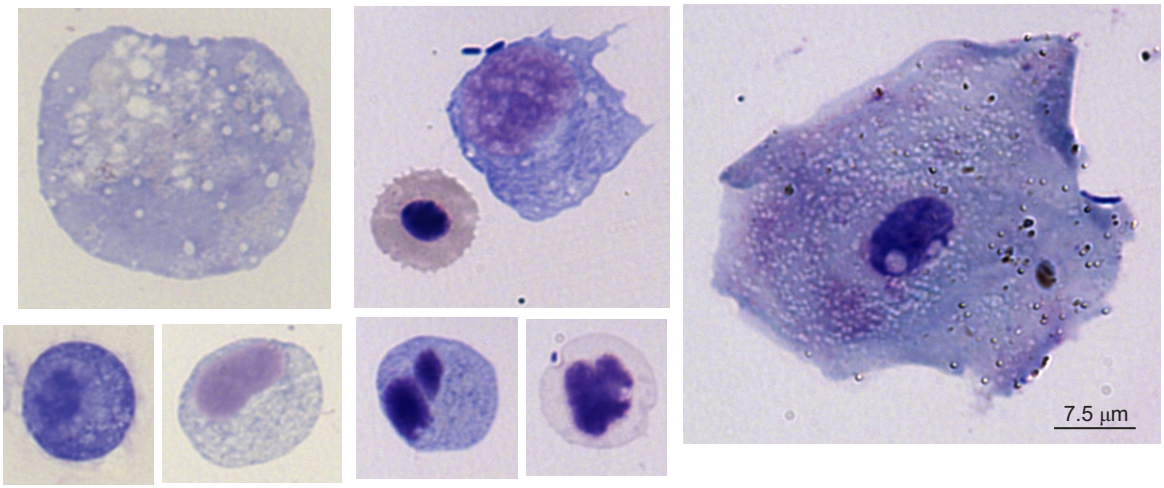

Δ14
fold change in gene expression
1.0
0.8
0.6
0.4
0.2
0
ath5
gata-1
gata-2
pu.1
l-plastin
rag1
lck
mpx

S8 Fig

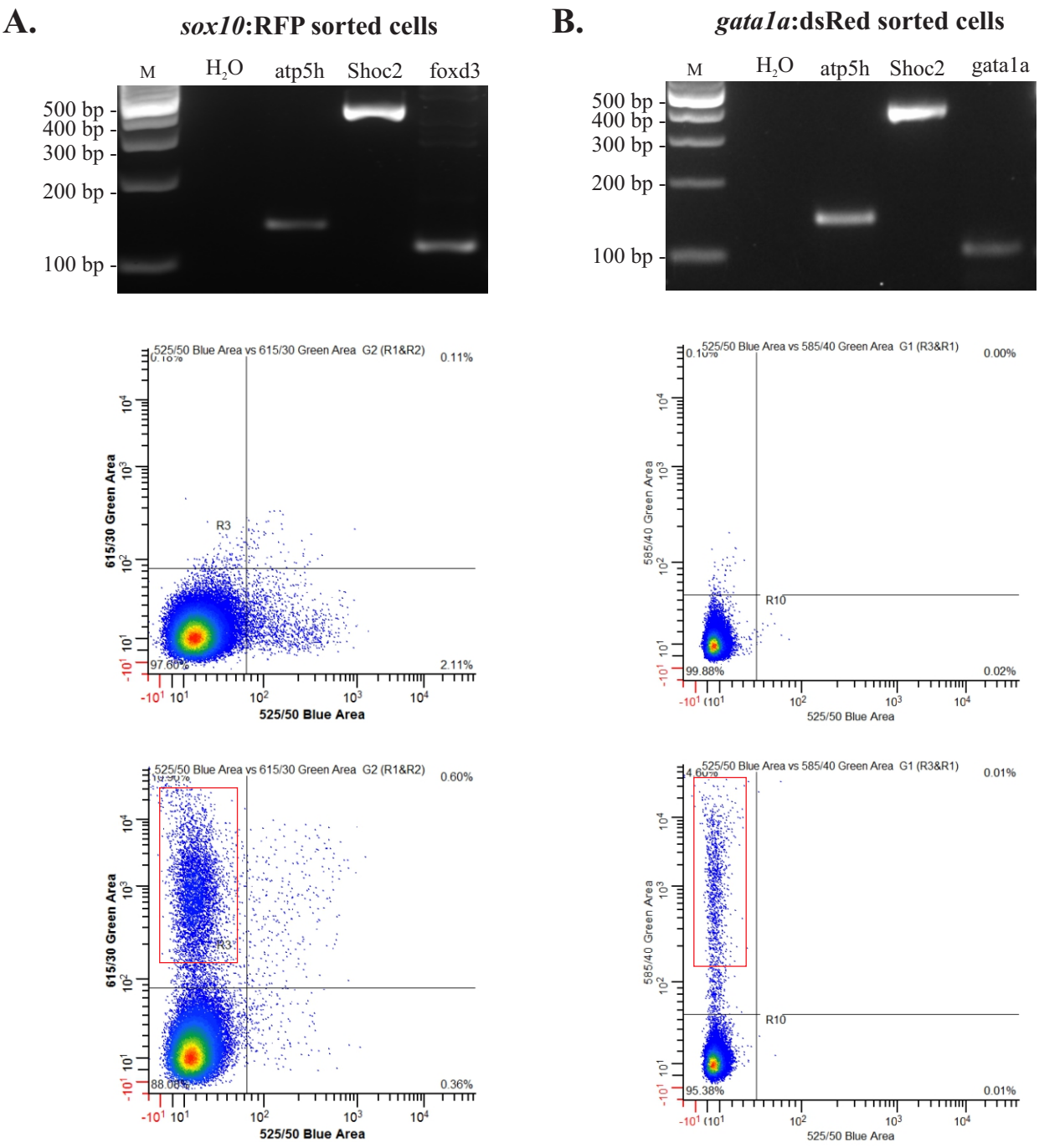
A.



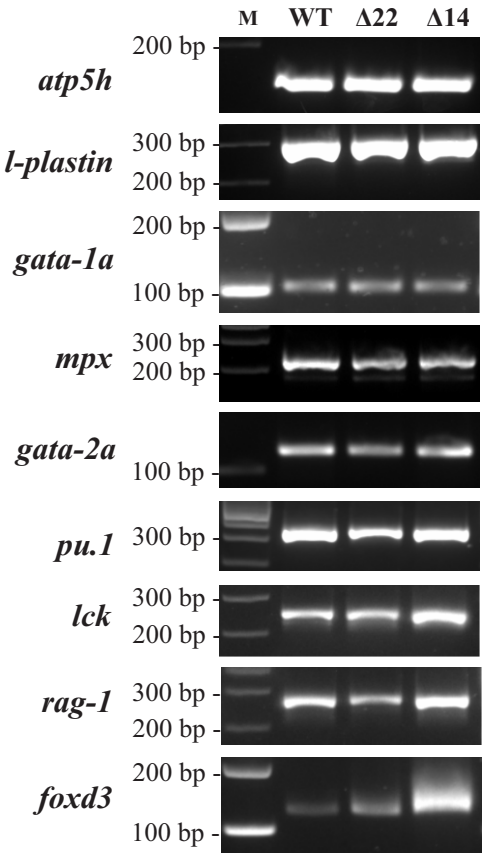
B.



S9 Fig


A.
sox10:RFP sorted cells
M
H2O
atp5h
Shoc2
foxd3
500 bp
400 bp
300 bp
200 bp
100 bp
525/50 Blue Area vs 615/30 Green Area G2 (R1&R2)
0.11%
R3
97.60%
2.11%
615/30 Green Area
525/50 Blue Area
525/50 Blue Area vs 615/30 Green Area G2 (R1&R2)
0.60%
R3
88.08%
0.36%
615/30 Green Area
525/50 Blue Area
B.
gata1a:dsRed sorted cells
M
H2O
atp5h
Shoc2
gata1a
500 bp
400 bp
300 bp
200 bp
100 bp
525/50 Blue Area vs 585/40 Green Area G1 (R3&R1)
0.00%
R10
99.88%
0.02%
585/40 Green Area
525/50 Blue Area
525/50 Blue Area vs 585/40 Green Area G1 (R3&R1)
0.01%
R10
95.38%
0.01%
585/40 Green Area
525/50 Blue Area

Supplemental Table 1



	Forward primer	Reverse primer
atp5h	5'-TGCCATCTCAGCAAAACTTG-3'	5'-CACAGGCTCAGGAACAGTCA-3'
l-plastin	5'-GTCGATGTGGATGGGAACGG-3'	5'-CCCTCCTCGGAGTATGAGTGC-3'
gata-1a	5'-AAGATGGGACAGGCCACTA-3'	5'-TGCTGACAATCAGCCTCTTTT-3'
mpx	5'-TTCTTTTTGTGGTGGGCTGC-3'	5'-CTCCCGTGTCTTTCGAGCTG-3'
gata-2a	5'-AACAATCGGGCTGTGGACAA-3'	5'-AGCACTCCAATCCTGCGTTT-3'
pu.1	5'-CAGAGCTACAAAGCGTGCAG-3'	5'-GCAGAAGGTCAAGCAGGAAC-3'
lck	5'-ACAATTTGGCAGCACATGTCTTT-3'	5'-GGCAGTTACACTGGTCACAC-3'
rag-1	5'-CCAGGTGAAGACATTTGCCG-3'	5'-ATTACGCAGAGTGTGCAGGG-3'
foxd3	5'-TTGACGCTCAGTGGAATC-3'	5'-TCTTGACGAAGCAGTCGT-3'

SUPPORTING FIGURE LEGENDS

S1 Fig. Expression pattern of *shoc2* in zebrafish embryonic development.

RNA *in situ* hybridization with antisense probes for Shoc2 in WT embryos at 6 **(A)**, 12 **(B)**, 24 **(C)** and 48 hpf **(D)**. An extensive anterior expression in the head region and the somite boundaries (black arrow) was observed at later stages **(C and D)**. Images are in lateral views, anterior to the left.

S2 Fig. Disruption of zebrafish *shoc2* results in vasculature defects.

fli1a:EGFP embryos were injected with control or *shoc2* MO. Control embryo showed well-organized inter-segmental vessels, while embryos injected with *shoc2* MO showed aberrant trunk blood vessels and disorganized sub-intestinal vein, indicated by arrow. All embryos shown in lateral view with anterior to the right and dorsal to the top. Images are taken at 3 dpf.

S3 Fig. **Heritable mutations of the Shoc2 gene.**

(A) Zebrafish *Shoc2* genomic locus on chromosome 22 with the DNA cleavage positions (in exon 2 and exon 3 indicated by the red bars).

(B) Details of the F1 offspring genotypes. The F1 genotypes contain deletions (dashes) and insertions (red letters) at the *shoc2* gRNA and #2 sites (orange letters) near proto-spacer adjacent motif sequences (blue letters). The numbers of deleted or inserted base pairs and resultant frameshift mutations are indicated, the premature stop codons are underlined.

(C) PCR analysis of genomic DNA allows for sensitive detection of WT and *shoc2*$^{\Delta 22}$ and *shoc2*$^{\Delta 14}$ mutant alleles in individual larvae (F3 generation). Acrylamide gel electrophoresis shows PCR amplicons of WT, heterozygous and homozygous *shoc2*$^{\Delta 22}$ and *shoc2*$^{\Delta 14}$ carriers. **(D)** RT-PCR analysis allows for sensitive detection of WT and *shoc2*$^{\Delta 22}$ and *shoc2*$^{\Delta 14}$ mutant RNA in individual larvae. Acrylamide gel electrophoresis shows PCR amplicons of WT, heterozygous and homozygous *shoc2*$^{\Delta 22}$ and *shoc2*$^{\Delta 14}$ carriers.
(E) Western blot analysis detects unaltered expression of Shoc2 protein in adult WT and *shoc2*$^{\Delta 22+/-}$ zebrafish. M- DNA Marker.

S4 Fig. Detection of *shoc2* mutant alleles in individual *shoc2* sgRNA/Cas9 injected embryos. The HRMA curve discriminates WT and mutant alleles. The genomic DNA was prepared from individual 1 day-post-injected embryos. The LightCycler® 480 High Resolution Melting Master and LightCycler® 96 system were used. The curves of embryos injected with *shoc2* sgRNA/Cas9 targeting exon 2 **(A)** and exon 3 **(B)** were shifted and irregular, indicating the presence of insertions/deletions.

S5 Fig. ***shoc2*$^{\Delta 22}$-*shoc2*$^{\Delta 14}$ compound mutants develop edemic phenotype.**
shoc2$^{\Delta 22+/-}$ and *shoc2*$^{\Delta 14+/-}$ adult fish were crossed to generate *shoc2*$^{\Delta 22}$-*shoc2*$^{\Delta 14}$ compound mutants. **(A)** Images of 6 dpf compound crispants show edemic phenotype similar to the observed in *shoc2*$^{\Delta 22}$ and *shoc2*$^{\Delta 14}$ crispants. Red arrows indicate edema around the eye, heart/yolk region of the larva. **(B)** PCR analysis of genomic DNA confirms the deletions in both alleles: exon2 with 22 bp deletion and exon3 with 14 bp deletion. M- DNA Marker. CM- compound mutant.

S6 Fig. Developmental impairments of *shoc2*$^{\Delta 14}$ crispant larvae.

(A) Alizarin Red S stain for the craniofacial ossification of 6 dpf WT and *shoc2*$^{\Delta 14}$ larvae detected defects in ossification of parasphenoid, ceratohyal, branchiostegal rays, opercle, ceratohyal and notochord. For schematic representation, see Fig 5B. Three biological replicates were performed for all experiments (n= 30/group).

(B) Dorsal (head) and lateral (trunk) views of 6 dpf larvae showing melanophores in WT and *shoc2*$^{\Delta 14}$ crispants. *shoc2*$^{\Delta 14}$ mutants presented with closed gaps in pigmentation pattern of head and lateral stipe (brackets) melanophores when compared with WT controls.

(C) Histochemical staining for mpx enzyme activity in 6 dpf WT and *shoc2*$^{\Delta 14}$ crispants. Larvae are shown in lateral view with the anterior to the left. The images are representative of ≥ 20 larvae in each group. The staining was repeated with similar results for three independent experiments.

S7 Fig. Loss of *shoc2* causes a reduction in the expression of blood cell markers.

The relative changes in the expression of hematopoietic markers were evaluated in *shoc2*$^{\Delta 14}$ crispants. Total RNA was extracted from 6 dpf WT and mutant larvae and levels of mRNA expression were quantified by qPCR. The data are presented as the fold change of the mRNA levels in WT larvae versus the mRNA levels in mutant larvae. *ath5* is a control mRNA. The results represent an average of three biological replicas. Error bars indicate means with SEM. $^{**}p < 0.05$ (Student's t-test).

S8 Fig. Blood cell types found in 6 dpf larvae.

Peripheral blood cells from **(A)** WT and **(B)** *shoc2*$^{\Delta 22}$ larvae at 6 dpf stained with May-Grünwald Giemsa. Blood cells isolated from six WT or six *shoc2*$^{\Delta 22}$ larvae were cytospun onto a coated slide and stained. Various blood cell subtypes were detected in both WT and *shoc2*$^{\Delta 22}$ larvae.

S9 Fig. ***shoc2*** **expressed in neural crest and hematopoietic cells.**

sox10:RFP and *gata1a:dsRed* positive cells were isolated from the corresponding transgene adult fish using flow cytometry. FACS profile of *sox10:RFP* and *gata1:dsRed* are shown in **(A)** and **(B)**, respectively. Red boxes in **(A)** and **(B)** indicate the collected cell populations. Total RNA was isolated and RT-PCR was performed. RT-PCR detected *shoc2* in both *sox10*- and *gata1a*-positive flow-sorted cells. *foxd3* **(A)** and *gata1a* **(B)** were included to confirm the specificity of flow sorting. M- DNA Marker.

S1 Movie: ***shoc2***$^{\Delta 22}$ **crispants display increased heart rate.**

WT **(A)** larvae had average 120 beats/minute while *shoc2*$^{\Delta 22}$ crispant **(B)** had 148 beats/min. Time-lapse images were acquired at 100 frames per sec during 1 min at room temperature using Nikon Eclipse Ti-U (Nikon Instruments).

S1 Table. RT-PCR of hematopoietic and non-hematopoietic genes.

RT-PCR of cells and WT, *shoc2*$^{\Delta 22}$ and *shoc2*$^{\Delta 14}$ larvae. Genes are indicated to the left, *atp5h* is used as a reference gene. Primer sequences are to the right of each gene.