SUPPLEMENTAL METHODS

Construction of expression vectors

Expression constructs were created by inserting cDNA sequences into the ubiquitin (*ubi*) regulated expression vector pDestTol2pA2_Ubi:EGFP,[1] followed by site-directed mutagenesis[2] using primers specific for the desired mutations (Table S1-2). IMAGE clone 7403818 (Open Biosystems) was amplified with *f10* primers tagged with BspHI sites using Phusion DNA polymerase (New England Biolabs), and cloned into the NcoI site of pDestTol2pA2-ubi:EGFP to produce pubi:zf10-EGFP. pubi:zf10-p2A-EGFP was generated by restriction free cloning[3] using p2A fragments flanked by fragments of *zf10* and *egfp*. Mutagenic primers (Table S1) were used to perform site-directed mutagenesis in pubi:zf10-EGFP or zf10-p2A-EGFP. The resulting expression vectors were verified by restriction digest and sequencing (primers in Table S1).

mRNA preparation and reverse transcription

Zebrafish embryos and larvae were homogenized in lysis buffer (Life Technologies or Qiagen) containing 1% 2–mercaptoethanol using syringes with 21 gauge needles. Total RNA was isolated with PureLink® RNA Mini Kit (Life Technologies) or RNA Mini Kit (Qiagen) according to manufacturers' instructions. Total RNA was treated with DNase I (Invitrogen) followed by first strand cDNA synthesis with oligo$(dT)_{12\text{-}18}$ primers (Superscript II, Invitrogen) per manufacturer's instructions. cDNA was used for RT-PCR and qPCR.

Quantitative PCR of coagulation factor mRNAs

Three dpf offspring derived from the intercross of *f10* heterozygotes were genotyped by PCR as described in Methods. Three pools of 25 individuals per genotype were used for RNA isolation and cDNA synthesis. Quantitative PCR (qPCR) was performed on each pool in triplicate using a Bio-Rad iCycler with *18S* as an internal control. The resulting mRNA levels of target genes were normalized to *18S* followed by statistical analysis as described.[4]

Measurement of larval size

Images were obtained using an Olympus stereoscope and cellSens software, calibrated with a stage micrometer slide. Anesthetized zebrafish larvae in 0.7% low melting point agarose were loaded into 1.5 - 1.8 mm outer diameter range glass capillaries (Pyrex) as described[5,6] and viewed at 27.4x magnification. Measurements of the rostrum to tail tip (length), eye-to-eye (width), and height at the yolk sac were obtained and averaged. Volumes were calculated by multiplying all 3 linear measurements. Images were analyzed and pixels per mm were calculated

using Photoshop CS7 (Adobe). Observers were blinded to genotype and after data collection larvae were recovered from agarose and genotyped.

Whole mount *in situ* hybridization (WISH)

Staged larvae were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline with 0.01% Tween-20 (PBST) at 4°C overnight and dehydrated stepwise into 100% methanol. Partial *f10* cDNA fragments were amplified with T7 overhangs (Table S1) to synthesize sense and antisense digoxin-labeled RNA riboprobes (DIG RNA labeling kit, Roche). The amplification of cDNA templates, synthesis of riboprobes, and WISH were performed essentially as described.[7-12]

Histologic examination

After WISH, larvae were embedded in JB-4 plastic resin for serial sectioning. Samples were stepwise dehydrated in an ascending gradient of ethanol to a final concentration of 100%, and equilibrated in catalyzed JB-4A solution and embedding medium containing JB-4B (Electron Microscopy Sciences, Hatfield, PA) as described.[10] Samples were sectioned at 5 µm using a Leica RM2265 ultra-microtome (Leica Biosystems) and mounted with Permount™ (Fisher Scientific) for microscopic examination. Adult zebrafish were humanely killed and subsequently fixed with 4% PFA/PBST at 4°C overnight, embedded in paraffin, sectioned at 4-5 µm, and stained with hematoxylin and eosin.

Whole transcriptome sequencing

Offspring from $f10^{+/-}$ incrosses were collected at 3 dpf, fin clipped, and placed in RNAlater (Qiagen) and stored at -80°C. Genomic DNAs from tail biopsies were isolated and genotyping performed as described above. Larvae were recovered from RNAlater, pooled, and RNA isolated using the PureLink RNA Micro kit (Invitrogen). Three biological replicates were collected for $f10^{+/+}$ and $f10^{-/-}$ genotypes, each containing pools of twenty-five larvae. At least 40 million paired end 100 base pair reads were generated from each pool using an Illumina HiSeq2000 (Otogenetics). Reads were aligned to the zebrafish reference genome (Zv9) using STAR v2.4.0 (Dobin ref) with default parameters and processed using the DNAnexus Platform Advanced RNAseq analysis. After alignment, estimation of transcript abundance measures as fragments per kilobase of exon per million aligned fragments (FPKM) values was performed using Cufflinks in the Tuxedo protocol (Trapnell et al. 2012). Differential gene expression (counts) FPKM values were determined using Cuffdif, a part of the Cufflinks package.

Intersegmental vessel measurement

Heterozygous *f10* zebrafish bred into a *Tg*(*flk1:mCherry/NTR)* background were incrossed. Intersegmental vessel (ISV) length was measured on larval offspring at 24 and 48 hours post fertilization (hpf), followed by genotyping. Three ISVs located between the edge of the yolk sac extension and tail were randomly selected for measurement. The "polyline tool" (Olympus cellSens Software) was used to trace and measure ISVs. The three measurements were averaged for each individual. Following measurement, larvae were lysed and genotyped as described (Methods).

Analysis of apoptosis

Two methods were used to assess apoptosis, acridine orange staining and immunohistochemistry against caspase 3. For the former, larvae were incubated in the dark for 30 minutes at room temperature in acridine orange solution (2 µg/mL) as previously described,[13] followed by washing 4 times for 5 minutes each in system water. Larvae were anesthetized and visualized immediately under a fluorescence microscope (Olympus SZX16), imaged, and genotyped. The latter method was done as previously described[14]. Larval zebrafish were fixed in 1% PFA for 4 hours at room temperature, then washed in PBST and blocked in SBS (saponin blocking solution: 0.2% saponin, 2 mg/ml BSA, 5% lamb serum, 1X PBS) overnight at 4°C. Following blocking, larvae were stained with 1:250 rabbit anti-human cleaved caspase 3 (BD Pharm, 559565) in SBS overnight at 4°C. Larvae were washed with PBST and incubated in 1:1000 anti-rabbit Alexa-488 (Invitrogen A27034) in SBS for 4 hours at room temperature. Imaging was performed using an Olympus DP73 camera on an Olympus IX73 microscope, followed by genotyping.

Assay for thrombin activity

Thrombin activity was measured in plasma using a method adapted from Jagadeewsaran and Liu[15]. Juvenile zebrafish were anesthetized at 1 month post fertilization and exsanguinated into 20 ul of solution consisting of 0.4% sodium citrate, 0.05% Tween-20, and 1X PBS. Following blood collection, this was mixed with 40 ul of buffer containing 10 mM cysteine, 10 mM Tris-HCl (pH 7.5), and 150 mM NaCl. Samples were centrifuged at 1000 RPM for 5 minutes to pellet cells and supernatants transferred to a polystyrene flat bottom 96 well plate, followed by addition of $CaCl_2$ and fibrinogen (Sigma F3879) to final concentrations of 4 mM and 10 mg/ml, respectively. The plate was immediately transferred to a spectrophotometer (Molecular Devices) for analysis. 10.2 units of bovine thrombin (Sigma T6200) was used as a positive control. Absorbance was measured every 2 minutes for a total of 90 minutes at room temperature and a wavelength of 405

nm. Two fish per genotype were pooled in each well. The average time to half maximal absorbance and maximum absorbance were calculated for each genotype.

SUPPLEMENTAL REFERENCES

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SUPPLEMENTAL DATA

Table S1. Comprehensive list of primers

Name	Forward 5'→3'	Reverse 5'→3'
***f10* genotyping**		
JS689, 729	cccattcacagctttactcattt	gagactggcaggatttcccg
qPCR		
18s	tcgctagttggcatcgtttatg	cggaggttcgaagacgatca
f2	aggaagctcgagaagtgtttg	ccctctgtagtcgcacatattc
f10	caagggttcaagggctacaa	cttctccatcacctcacagaaa
at3	actgtctatggagccaagttg	tggtgttggtgtctatggaac
fga	gttactcaaagaccaccctcac	ggatgtcgggcatatcttcttc
riboprobe generation		
f10-s	taatacgactcactatagggatgtcttgggttttctggaact	ccgccacagaagcccatgtt
f10-as	aaaaaaccatggatgtcttgggttttctggaact	taatacgactcactatagggccgccacagaagcccatgtt
efnb2a	cagacacgcctctactcaactg	taatacgactcactatagggatagatgtttgctgggctctgt
cdh5	agacaccgctgacctgaaactg	taatacgactcactatagggggcttgattttgaagaggtctg
ephB4	aggaggtcagtggtctggatga	taatacgactcactatagggggtaccagaacccatcttgaac
flk1	cctcagtcaaagccttcttcac	taatacgactcactatagggctcttggtctttctggttcgat
flt4	accatgtctggtttcagatcct	taatacgactcactataggggtagagagtgctgctggacctt
Primers for zebrafish f10 constructs		
pubi:*zf10-egfp*	tcatgaatgtcttgggttttc	tcatgacgactcttcttattg
pubi:zf10-p2A-egfp	caagacaccaataagaagagtggaagcggagctactaacttcag	gctcctcgcccttgctcaccat
Site-directed mutagenesis		
hR68C	gaggaggcctgctgcgaaatcttcgaagatgttaaaaagacgg	ataattgcagcgctcctcaatgcactcgcgctccatgttc
hG173W	caaatgttgggtcatttatccaaagaagaccagaagcatc	aatggatcctgagactggcaggatttcccgttgggggcca
hT176_Q186	cacacgccgaacatcaccgaaagcgagaacgaagaagagac	gacgccacatttgaatggatcctgagactggcaggatttc
G262D	ttctgtgatggaaccattctgagcgagttctacatcctaac	ggttccatcacagaaaccctcgttttcctcattgatgagc
C390F	acagcttcaagctgtccagcagcttcatcatcacccagaa	acagcttgaagctgttgcggtccacgtagggcacctccag
I323M	cctaccacaacgacatggccctcatcaagctctcgaaac	tgtcaggctggtagttcttgtggatcagaatctcatctac
Q416L	agaaagatgcatgtctgggggacagcggcgggccgca	cctcctggtcgtatccagcgcagaacatgcggccggagat
Sequencing		
JS952	agttcttgacagaggtgtaa	
JS942	tatttcctgaaaaaaattct	
JS943	tctcaaagcagtgccatctg	
JS944	aacgcaaatattaacggtaa	
JS945	tgtctatgtatgctctgtaa	
JS946	agacctttaaaaataaaaaa	
JS825	aaaaaaccatggatgtcttgggttttctggaact	
JS832	agaccgacagcatgttcggg	
JS847	ttacgtcgccgtccagctcg	
JS387	cgaccactaccagcagaaca	
JS1014	gatacgaccaggaggagaaaga	
JS1025	gctcctcgcccttgctcaccat	

Table S2. Expression vectors

No.	Backbone	Promoter	*p2A*	Note
191	Tol2	*ubi*	-	*zf10*_hG262D
196	Tol2	*ubi*	-	*zf10*
203	Tol2	*ubi*	-	*zf10*_hC390F
213	Tol2	*ubi*	+	*zf10*_hR68C
214	Tol2	*ubi*	+	*zf10*_hG173W
215	Tol2	*ubi*	+	*zf10*_hT176_Q186
216	Tol2	*ubi*	+	*zf10*_I323M
217	Tol2	*ubi*	+	*zf10*_Q416L
219	Tol2	*ubi*	+	*zf10*

Table S3. Hemorrhagic phenotypes identified by histologic analysis

Age	*Total*	*f10*$^{+/+}$	*f10*$^{+/-}$	*f10*$^{-/-}$	sites of hemorrhage in *f10*$^{-/-}$
27 dpf	6	3	0	3	brain, muscle, abdomen, fin
33 dpf	7	3	0	4	brain, abdomen
39 dpf	3	1	0	2	brain, abdomen, muscle
48 dpf	15	5	5	5	brain, muscle, abdomen, fin
268 dpf	3	1	1	1	fin

Table S4. Relative expression of vascular markers in wild type versus *f10*$^{-/-}$ mutants

Gene_ID	Gene	Locus	FPKM +/+	FPKM -/-	log2 (fold change)	p-value
ENSDARG00000037373	*ephb2a*	23:22097551-22240745	1.46867	1.34685	-0.124917	0.497
ENSDARG00000075549	*cdh5*	7:45433469-45568430	7.66435	7.68367	0.00363315	0.979
ENSDARG00000015815	*kdrl*	14:33838842-33899405	3.17005	3.30249	0.0590478	0.682
ENSDARG00000015717	*flt4*	14:19717037-19856641	1.95701	2.2628	0.209455	0.222
ENSDARG00000040346	*ephB4a*	5:70801142-70856493	0.824275	0.984351	0.256046	0.762
ENSDARG00000040346	*ephB4a*	5:70801142-70856493	7.72658	8.85288	0.196316	0.191
ENSDARG00000040346	*ephB4a*	5:70801142-70856493	1.65191	1.26684	-0.382896	0.550
ENSDARG00000027112	*ephB4b*	23:44476383-44528029	3.30656	3.62652	0.133255	0.574
ENSDARG00000027112	*ephB4b*	23:44476383-44528029	2.87297	2.71808	-0.0799556	0.756

FPKM, fragments per kilobase of exon per million aligned fragments.

Table S5. Clinical data of patients with previously unknown variants

Mutation	F10 antigen	F10 activity	Allelic status	Symptoms	Treatment
R68C	77	5	Compound heterozygous with FX Friuli (P383S)	Spontaneous ecchymosis and hematomas	ND
G173W	ND	<1	Compound heterozygous with frameshift mutation	Spontaneous petechiae, ecchymosis, hematoma	RBCs, FFP, PCCs
ΔT176_Q186	35	4	Homozygous	Spontaneous gingival bleeding, epistaxis, hemarthrosis, hematoma, hematuria	ND
I323M	<1	<1	Compound heterozygous with frameshift mutation	Bleeding 10 days after toungue bite	ND
Q416L	36	2	Compound heterozygous with frameshift mutation	Spontaneous epistaxis	Cauterization

ND, no data; RBCs, red blood cell transfusions; FFP, fresh frozen plasma transfuion; PCCs, prothrombin complex concentrates.

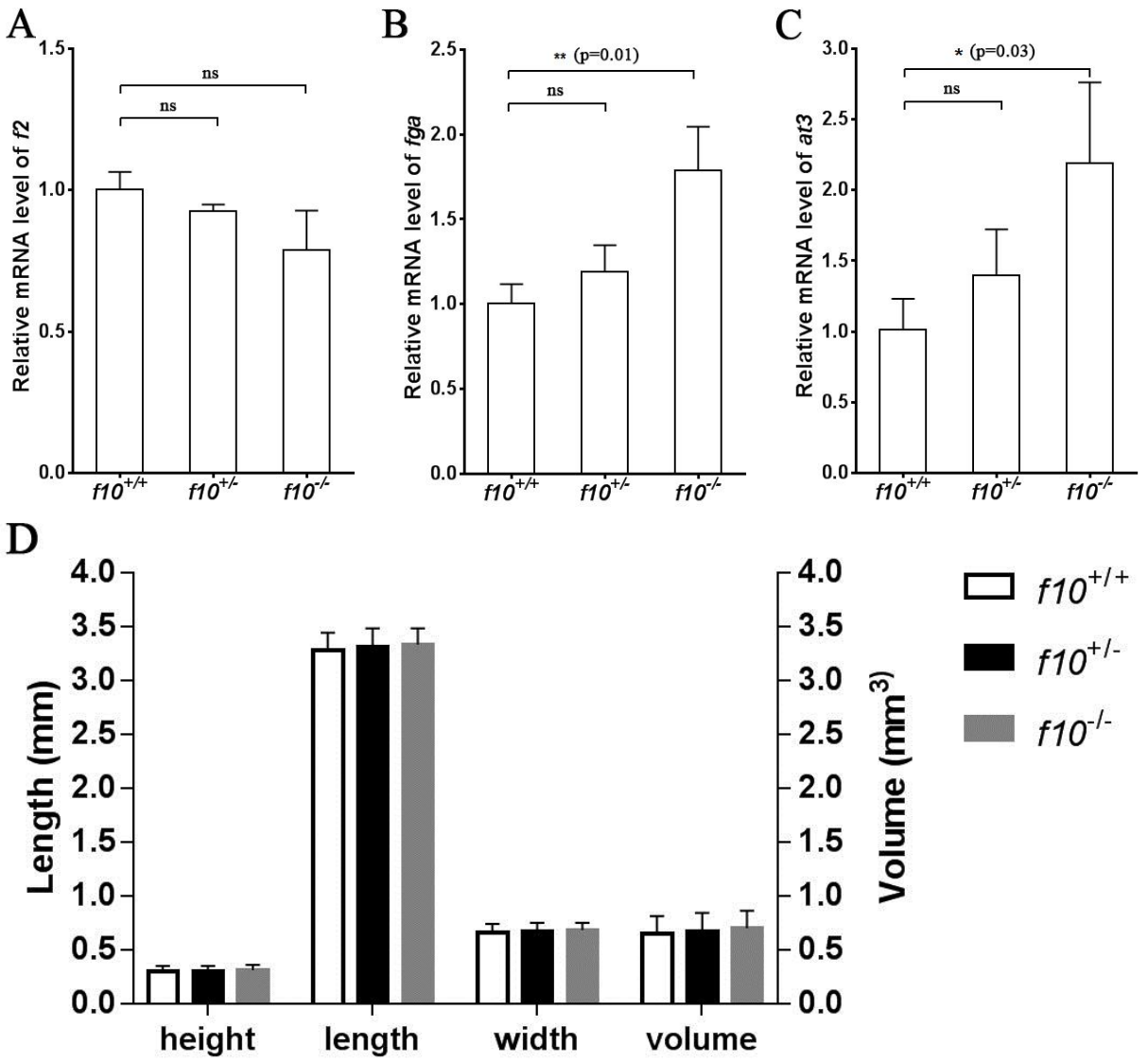

Figure S1. Expression of coagulation factors in *f10* mutants are altered in a dose dependent fashion. (A) *f2* mRNA was progressively downregulated in $f10^{+/-}$ and $f10^{-/-}$ mutants, although the results were not statistically significant (Student's t-test). (B) The expression level of coagulation factor *fga* was upregulated in both $f10^{+/-}$ and $f10^{-/-}$ mutants, although this effect was only significant for the homozygous mutants ($p<0.01$). (C) Expression of *at3* demonstrated nearly 1.5- and over 2-fold increases in $f10^{+/-}$ and $f10^{-/-}$ mutants, respectively, although the effect was only significant for the latter ($p<0.05$). mRNAs were normalized to *18s* rRNA. (D) Anesthetized 3 dpf zebrafish larvae were measured in mm at the yolk sac (height), rostrum to tail tip (length), eye-to-eye (width), and all 3 linear measurements multiplied to calculate volume (mm^3). Data were collected prior to genotyping. The numbers of embryos analyzed per genotype were as follows; $f10^{+/+}$ n=29, $f10^{+/-}$ n=57, $f10^{-/-}$ n=26. There were no statistically significant differences between mutant and wild type larvae (Student *t* test).

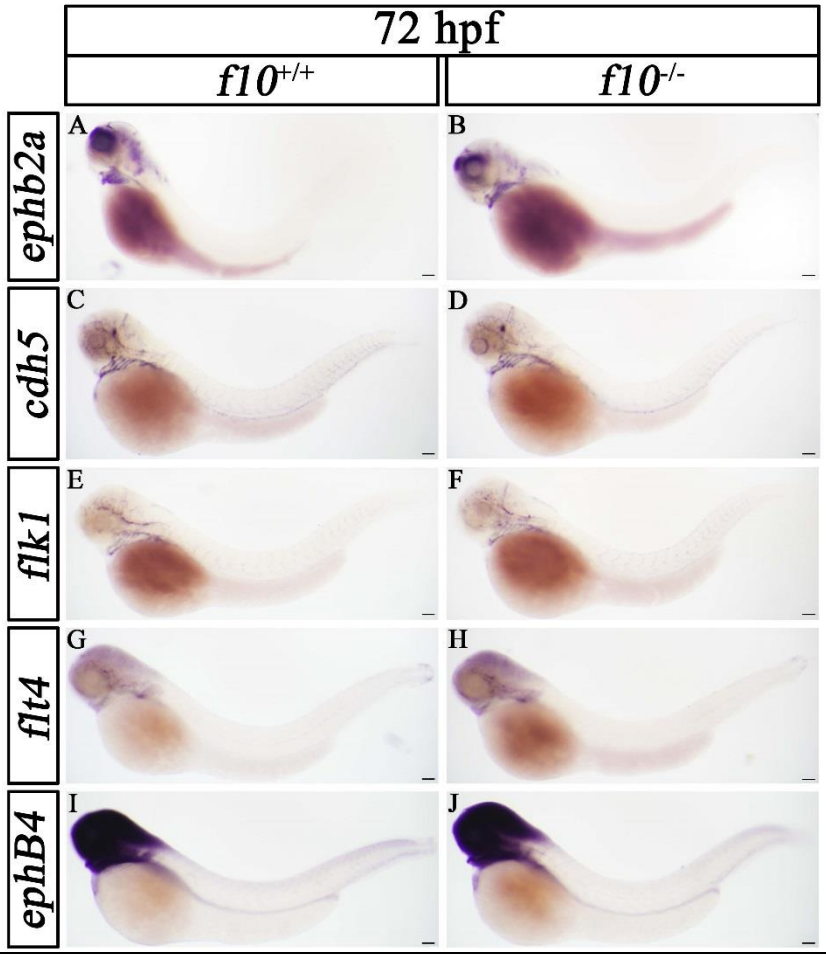

Figure S2. WISH reveals normal expression of arterial and venous markers in *f10* mutants. Offspring from *f10*$^{+/-}$ incrosses were subjected to WISH, phenotypically examined and categorized, and subsequently genotyped. The spatiotemporal expression patterns of *ephb2a* (A-B), *cdh5* (C-D), *flk1* (E-F), *flt4* (G-H), and *ephB4* (I-J) were indistinguishable between *f10*$^{-/-}$ mutants and *f10*$^{+/+}$ and *f10*$^{+/-}$ siblings. Anterior is to the left. Scale bar: 100 µm.

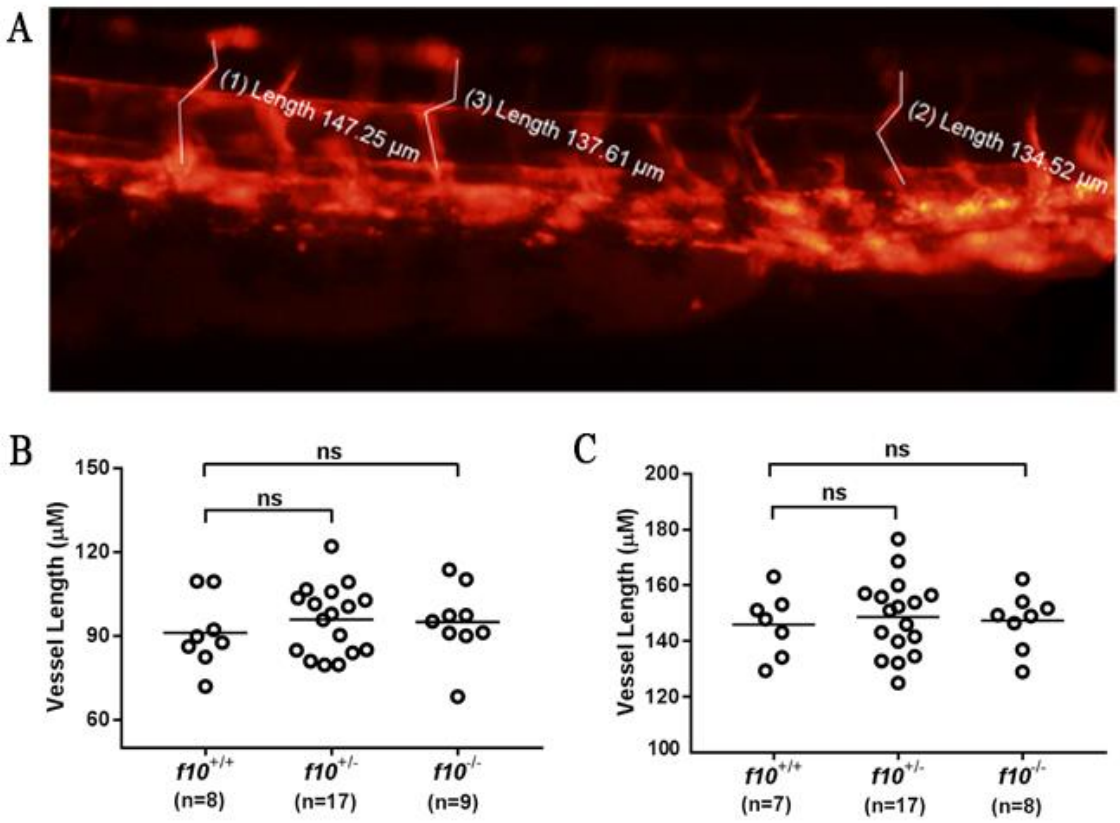

Figure S3. Intersegmental vessel (ISV) measurement does not reveal any significant abnormality in the vascular development of *f10* mutants. (A) ISVs were traced and measured in *f10* mutant and *Tg*(*flk1:mCherry/NTR)* embryos using cellSense Software (Olympus), followed by genotyping. (B-C) Averaged results are shown for 24 and 48 hpf embryos, respectively. ns, not significant.

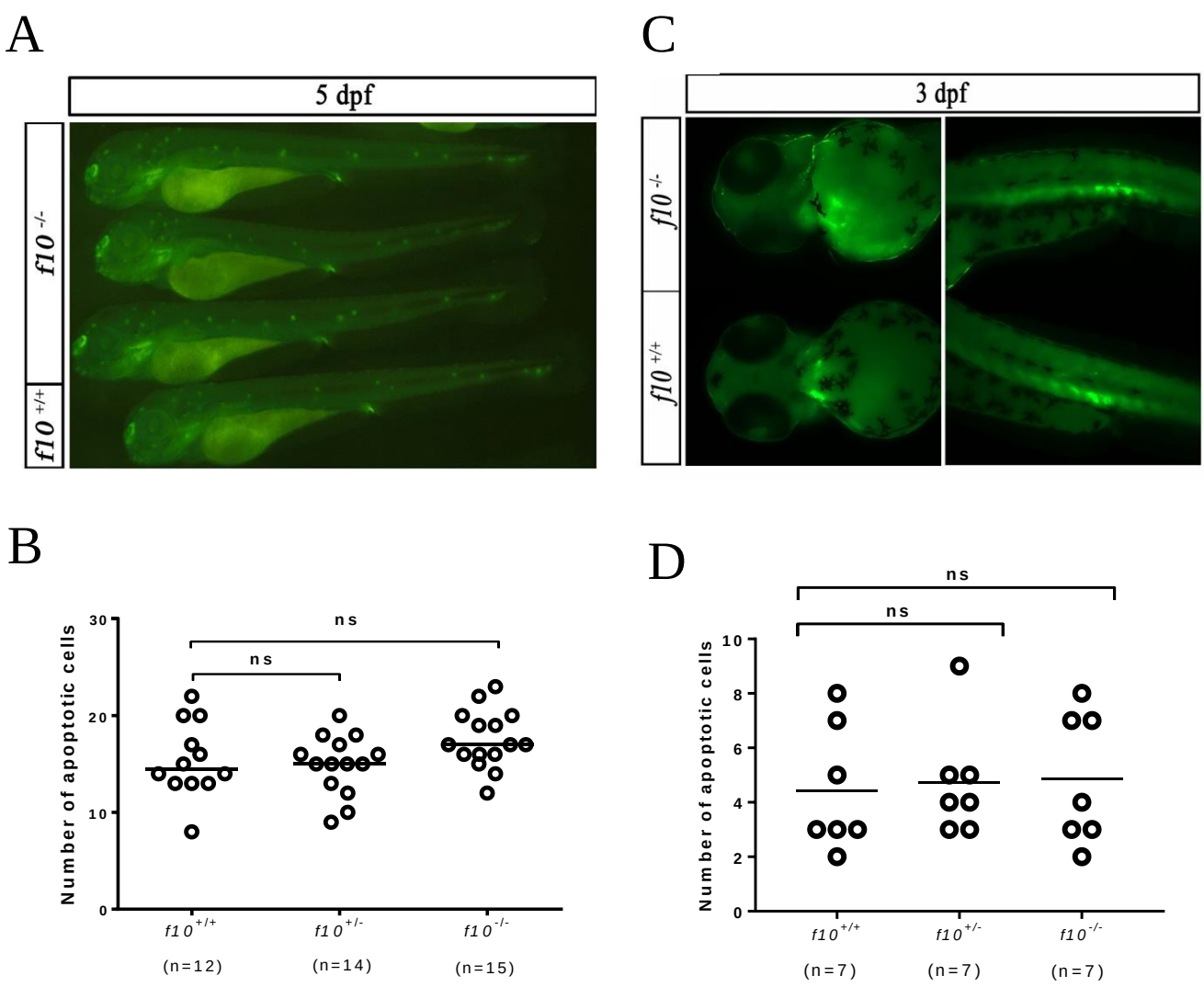

Figure S4. No increased apoptosis is detected in *f10* mutants. Larvae were stained with acridine orange (A-B) or probed with an antibody to caspase 3 (C-D) to detect apoptic cells, followed by blinded phenotypic evaluation and subsequent genotyping. Anterior is to the left. (B, D) The number of apoptotic cells were counted and no significant differences in fluorescent cells were observed between $f10^{+/+}$ and $f10^{-/-}$ mutant larvae. ns, not significant by Student *t* test.

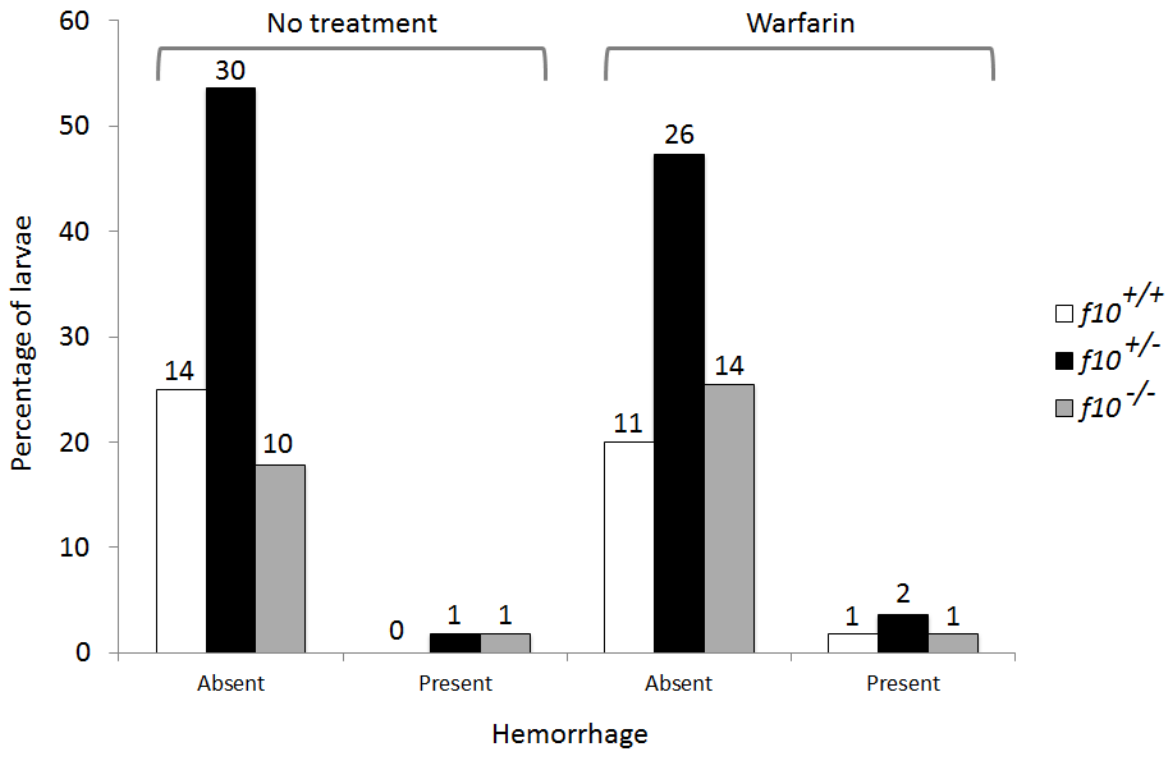

Figure S5. Treatment of *f10* mutant embryos with warfarin does not increase the incidence of grossly visible hemorrhage. Embryos from $f10^{+/-}$ incrosses were treated with either warfarin (10 µg/mL) or E3 medium as a control at 1 dpf and stained with o-dianisidine at 7 dpf. The presence of hemorrhage was evaluated by a blinded observer, followed by genotyping. There was no significant difference in hemorrhage between the treated and untreated groups across all 3 genotypes. The y-axis indicates percentage amongst each category (no treatment vs. warfarin) and the numbers above the bars indicate the quantity of larvae in that group.