ILLUSTRATED REVIEW

rpth
research & practice
in thrombosis & haemostasis

Fishing for answers to hemostatic and thrombotic disease: Genome editing in zebrafish

Azhwar Raghunath PhD[1] 🆔 🐦 | Allison C. Ferguson BS[1] 🆔 |
Jordan A. Shavit MD, PhD[1,2] 🆔 🐦

[1]Department of Pediatrics, University of Michigan School of Medicine, Ann Arbor, Michigan, USA

[2]Department of Human Genetics, University of Michigan School of Medicine, Ann Arbor, Michigan, USA

Correspondence
Jordan A. Shavit and Azhwar Raghunath, Department of Pediatrics, University of Michigan, Room 8301, Medical Science Research Building III, 1150 West Medical Center Dr, Ann Arbor, MI 48109, USA.
Email: jshavit@umich.edu and razh@umich.edu

Abstract

Over the past two decades, the teleost vertebrate *Danio rerio* (zebrafish) has emerged as a model for hemostasis and thrombosis. At genomic and functional levels, there is a high degree of conservation of the hemostatic system with that of mammals. Numerous features of the fish model offer unique advantages for investigating hemostasis and thrombosis. These include high fecundity, rapid and external development, optical transparency, and extensive functional homology with mammalian hemostasis and thrombosis. Zebrafish are particularly suited to genome-wide mutagenesis experiments for the study of modifier genes. They are also amenable to whole-organism small-molecule screens, a feature that is exceptionally relevant to hemostasis and thrombosis. Zebrafish coagulation factor knockouts that are in utero or neonatal lethal in mammals survive into adulthood before succumbing to hemorrhage or thrombosis, enabling studies not possible in mammals. In this illustrated review, we outline how zebrafish have been employed for the study of hemostasis and thrombosis using modern genome editing techniques, coagulation assays in larvae, and in vivo evaluation of patient-specific variants to infer causality and demonstrate pathogenicity. Zebrafish hemostasis and thrombosis models will continue to serve as a clinically directed basic research tool and powerful alternative to mammals for the development of new diagnostic markers and novel therapeutics for coagulation disorders through high-throughput genetic and small-molecule studies.
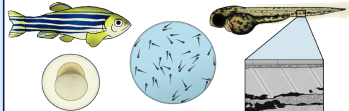
KEYWORDS
coagulation, genetics, genome editing, hemostasis, thrombosis, zebrafish

Fishing for answers to hemostatic and thrombotic disease: genome editing in zebrafish

Introduction to zebrafish

The zebrafish (*Danio rerio*), has numerous features that make it amenable to the study of hemostasis and thrombosis, including high fecundity, rapid and external development, optical transparency, and extensive functional and genomic homology with mammals.

Technologies for genetic studies in zebrafish

Cas9

sgRNA

Target Sequence

PAM
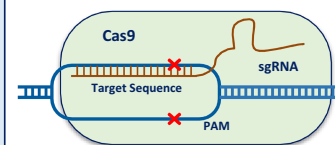
Zebrafish are suited to the application of modern powerful techniques for both targeted (eg, genome editing) and genome-wide genetic manipulation.

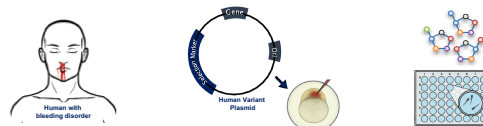**Zebrafish models of coagulation disorders**
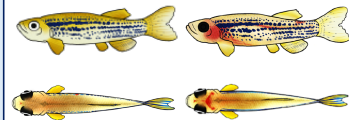
Unlike many mammalian counterparts that exhibit in utero or neonatal lethality, zebrafish coagulation factor mutants survive into early adulthood before succumbing to coagulopathy, enabling unique types of investigation.

Hemostasis/thrombosis in zebrafish

venous thrombosis

arterial thrombosis

fluorescently-tagged fibrin(ogen) rich thrombus
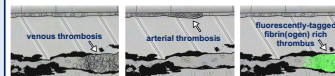
Coagulation in zebrafish shares many features with mammals. Hemostasis can be analyzed in zebrafish using an array of techniques, including vascular injury and labeling of coagulation factors and cells with fluorescent proteins.

Evaluation of patient coagulation factor mutations

Gene

Human with bleeding disorder

Human Variant Plasmid

Variants of uncertain significance can be studied rapidly in vivo using zebrafish models of coagulation disorders to demonstrate pathogenicity. The zebrafish is also an ideal model for the development of new diagnostic markers and novel therapeutics for coagulation disorders. Zebrafish hemostasis and thrombosis models will continue to serve as a clinically directed basic research model and powerful alternative to mammals.

Graphical Abstract

This graphical abstract outlines our review of the use of zebrafish to study disorders of hemostasis and thrombosis. We initially introduce the zebrafish and its associated technologies. This is followed by a review of the models of hemostatic and thrombotic disease that have been produced using genome editing. Finally, we review application of zebrafish to studies of patient coagulation factor variation.

**Essentials**

- The zebrafish hemostatic system is highly conserved with mammals genomically and functionally.
- Hemostatic disease models produced using genome editing show conservation with human disorders.
- Zebrafish coagulation factor mutants that are early lethal in mammals survive into adulthood.
- Potential disease-causing variants can be rapidly assessed in vivo using zebrafish knockouts.

Unique and relevant features of zebrafish for the study of hemostasis and thrombosis

Embryos and Larvae

- ❑ Development is external, rapid, and transparent.[1]
- ❑ The first week of life is sustained through yolk-based nutrition.
- ❑ Hundreds of larvae can be maintained in petri dishes at low cost.[2]

Development

venous thrombosis
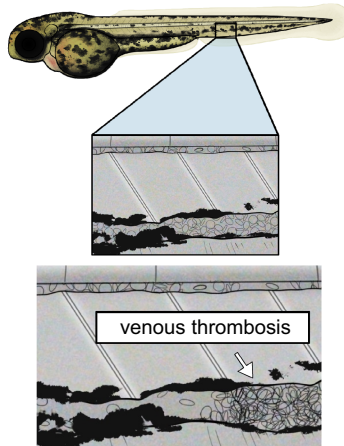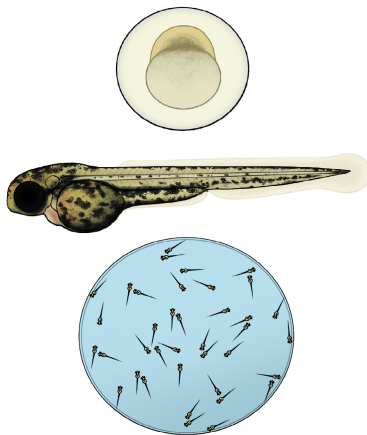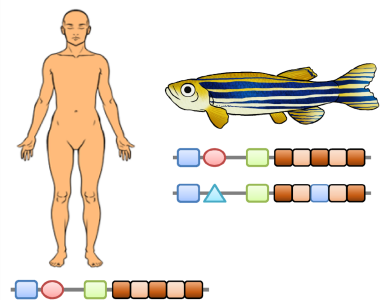
- ❑ Circulation and hemostasis develop by 2 days of life.[3,4]
- ❑ Hemostasis and thrombosis can be easily visualized under low power microscopy.[5]

Gene Duplication

- ❑ ~30% of the zebrafish genome is duplicated, including some coagulation factors.[6,7]
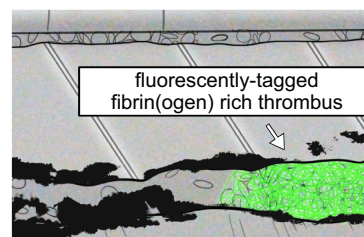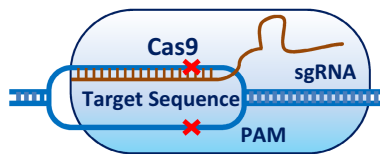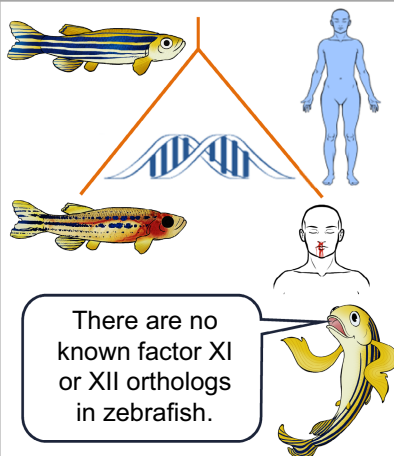- ❑ Duplications can reveal subfunctionalized roles not identified in mammals.[8]

Teleost whole genome duplication after divergence from mammals

Conservation

- ❑ Virtually all known coagulation factors have been identified in the sequenced genome.[7]
- ❑ Hemostasis and thrombosis have been shown to be functionally conserved.[4,9]

There are no known factor XI or XII orthologs in zebrafish.

Genome Engineering

Cas9
sgRNA
Target Sequence
PAM

fluorescently-tagged fibrin(ogen) rich thrombus

- ❑ Transgenic and genome edited models are easy and cheap to produce.[10]
- ❑ Fluorescently tagged proteins and structures (eg fibrin(ogen) and blood cells) enable visual assessment of disease states.[11,12]
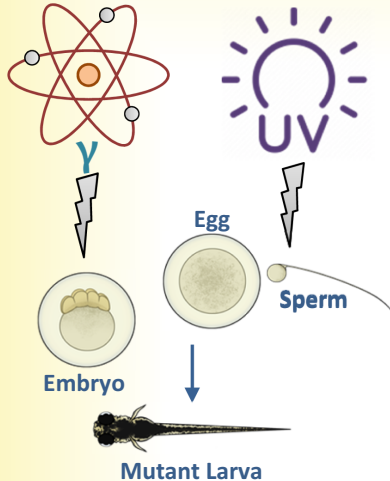
Drug Development

- ❑ Larvae with engineered coagulation disorders can be arrayed into multiwell plates and assayed with small molecule libraries or used for large scale mutagenesis studies.[13-15]
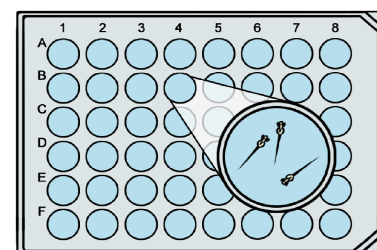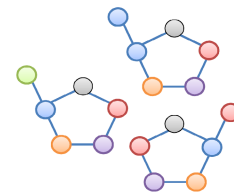
Evolution of technology for the generation of zebrafish disease models

Germline recessive lethal and specific locus mutations are induced by exposure to ultraviolet (UV) light or gamma rays.[16-18]

GCTTCTGACACAACTGTGTTGAGTCTATCGCTGAAACAGACACCATGGTGCATCTG
CGAAGACTGTGTTGACACAACTCAGATAGCGACTTTGTCTGTGGTACCACGTAGAC
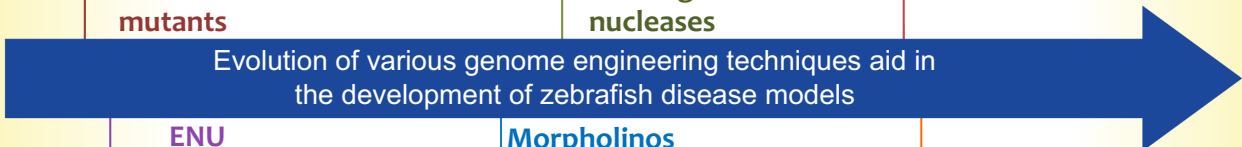
FokI

2011

TALENs

Zinc-finger nucleases (ZFNs) are the first successful application of genome editing to generate zebrafish disease models, but are limited in targeting, difficult to produce, and have a high failure rate.[22,23]
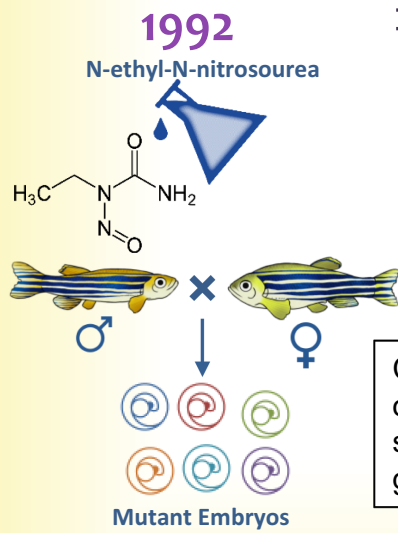
FokI

Egg

Sperm

Embryo

Mutant Larva

1983

1st zebrafish mutants
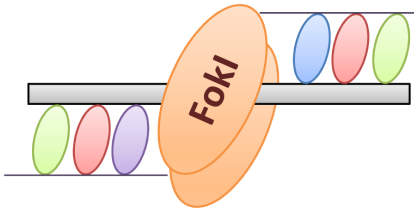
2008

Zinc-finger nucleases

TALEN genome editing nucleases (transcription activator-like effector) are much more efficient than ZFNs with broad targeting abilities.[24,25]

Evolution of various genome engineering techniques aid in the development of zebrafish disease models

ENU mutagenesis

1992

N-ethyl-N-nitrosourea

H_3C N NH_2
        |
        N
        ‖
        O

Mutant Embryos

Morpholinos

2000

Gene

Morpholino

mRNA

| Exon 1 | Intron 1 | Exon 2 |

Morpholino   **Splice block**

mRNA

AUG

Translation block

Mis-spliced or No protein

Chemically modified antisense oligonucleotides (morpholinos) block splicing and translation to produce gene specific knockdowns.[20,21]

CRISPR/Cas9 utilizes custom single guide RNAs (sgRNAs) that recruit a nuclease for specific targeting. These provide the efficiency and broad targeting of TALENS, but dramatically increase the ease of application.[26,27]
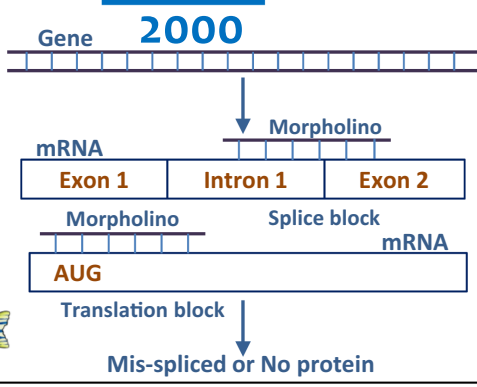
CRISPR/Cas9

2013

N-ethyl-N-nitrosourea (ENU), an alkylating agent, is used for the random generation of point mutations in phenotype-driven recessive screens.[19]
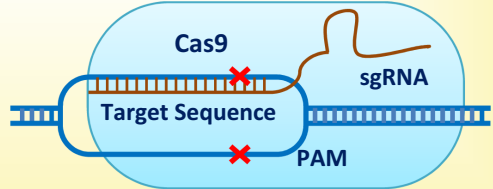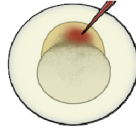
Cas9

sgRNA

Target Sequence

PAM

Modeling disorders of hemostasis and thrombotic disease in zebrafish

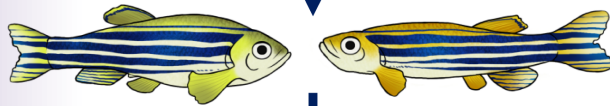Genome editing is used to develop models for known disorders of coagulation.[28]
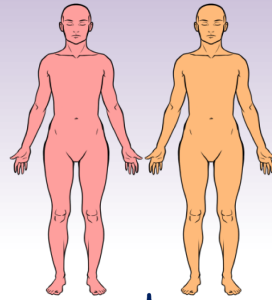
Patient specific variants identified in sequencing or association studies are modeled to determine pathogenicity in vivo.[29]

Sanger and next generation sequencing identifies variants in patients.[32,33]

Zinc finger nucleases, TALENS, and CRISPR/Cas9 are injected into single cell embryos to target known coagulation factors.[30,31]

Zebrafish develop from a single cell to a free swimming larva in 3 days, at which point nearly every organ system has developed, including flowing blood and an intact coagulation system.[3,5]
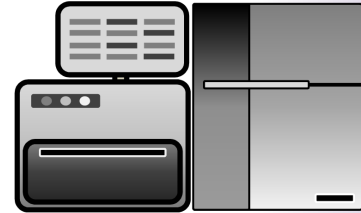
Deletion
Insertion
Substitution
Inversion

Coagulation disorder models are used to evaluate variants of uncertain significance. Variants are engineered into expression vectors, which are then injected into single cell embryos, and tested for the ability to rescue at the larval stage.[31,34,35]

+/+   +/m

+/+ X +/m   +/m X +/+   +/+ X +/+   +/m X +/m

Human Variant Plasmid
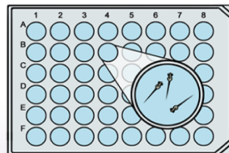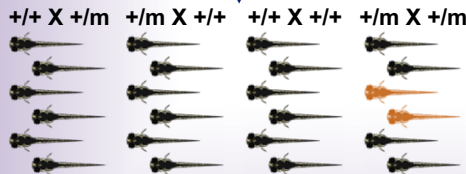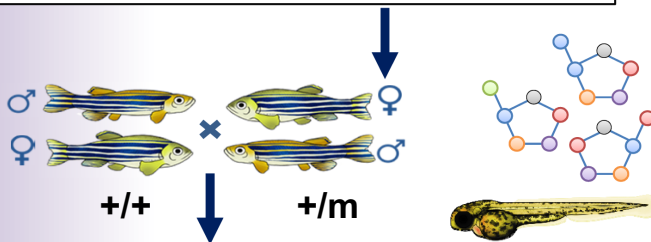
Disease models can be used to find modifier genes through unbiased genome-wide chemical mutagenesis[15,19] (left) or novel therapeutics using small molecule libraries (right).[13]
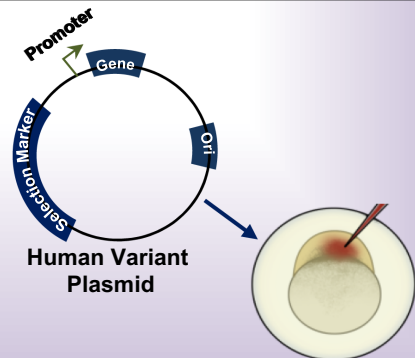
Zebrafish gene duplication

Ancestral gene

Duplication

1. Nonfunctionalization

2. Subfunctionalization

3. Neofunctionalization

Pak2⁻/⁻

Teleost fish underwent a whole genome duplication ~400 million years ago.[36] Acquired mutations in duplicate genes can result in inactivation (nonfunctionalization), partition of multifunctional genes (subfunctionalization) or acquisition of new function (neofunctionalization).[37] Gray boxes indicate loss of domain function.

Tissue Factor
- *tfa*
- *tfb*

Factor IX
- *f9a*
- *f9b*

Genes duplicated in hemostasis

Factor VII
- *f7a*
- *f7i*
- *f7l*

Protein C
- *proca*
- *procb*
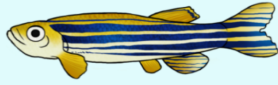
Whole genome duplication in zebrafish has resulted in the duplication of the *tf*, *f9*, *f7*, and *proc* genes.[7,38]

In zebrafish, a hypomorphic *pak2a* mutation causes the redhead (*rhd*) phenotype (intracranial hemorrhage). Only injection of *pak2a* mRNA rescues the *rhd* phenotype.[39]

Hemorrhage

redhead phenotype

pak2a

pak2b

Loss of p21-activated kinase 2 (*Pak2⁻/⁻*) in mice results in embryonic lethality secondary to developmental defects.[40] Examination of *pak2* duplication (*pak2a* and *b*) in zebrafish enabled dissection of Pak2 functions, and discovery of a role for Pak2a in vascular integrity.[39]
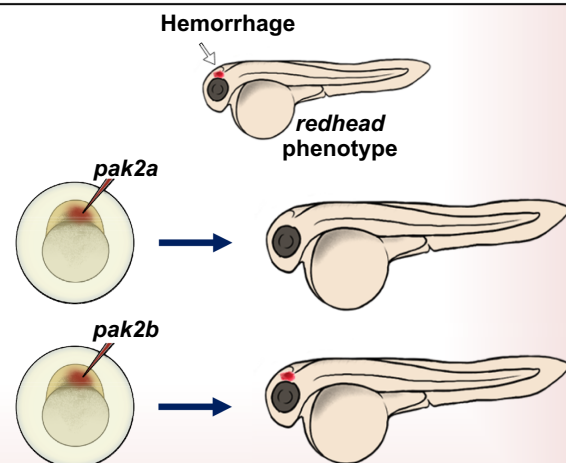
The teleost-specific genome duplication with the presence of duplicated paralogs gives a unique opportunity to identify previously undiscovered gene functions.[36]

Comparison of platelets and thrombocytes

Platelets

- Anucleate with granular cytoplasm[41]
- Produced in large numbers vastly out of proportion to nucleated blood cells
- Plasma membrane invades the platelet interior to make a surface-connected canalicular system[42]
- Receptors mediate hemostasis and other functions[43]
- Unique to mammals

Thrombocytes

- Nucleated with dense chromatin, cytoplasmic projections[44]
- Relative numbers similar to white cells[45]
- Function similar to platelets in adherence and aggregation[46]
- Stimulated by multiple platelet agonists: eg, ADP, arachidonic acid, collagen[47]
- Receptors for adhesion and aggregation are conserved[48]
- Present in fish, birds, reptiles, and amphibians

Megakaryocyte **Platelet**

Thrombocyte

Zebrafish thrombocyte spreading on fibrinogen is similar to human platelets.[49] During activation, human platelets form filopodia and lamellipodia extensions,[50] zebrafish thrombocytes also develop pseudopod-like protrusions after activation.

Platelet Spreading in Humans

Inactive **Early activation** **Late activation** **Active**
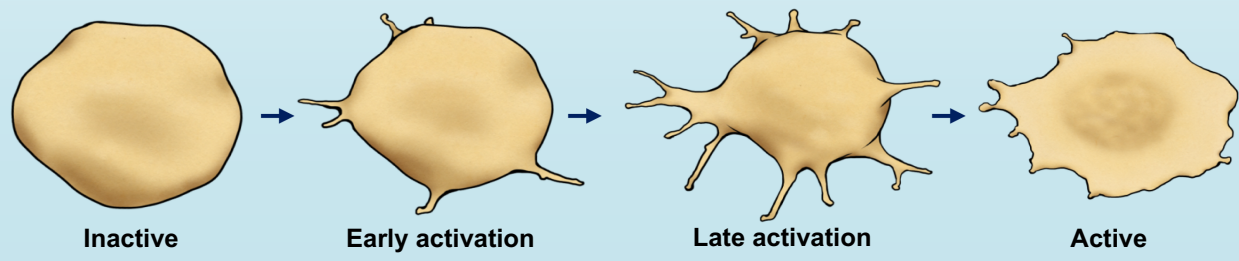
Thrombocyte Spreading in Zebrafish

Inactive **Early activation** **Late activation** **Active**
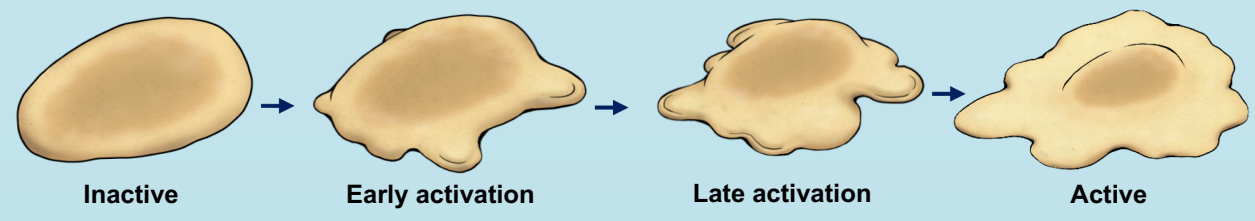
Thrombosis assays in zebrafish

Ferric chloride

- [ ] FeCl$_3$ is layered over larvae immobilized in agarose[51]
- [ ] FeCl$_3$ produces free radicals causing endothelial injury and vessel occlusion
- [ ] Time to occlusion (TTO) measured in caudal vessels
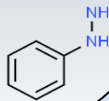
Phenylhydrazine

- [ ] Phenylhydrazine is layered over caudal vessels of immobilized larvae[51,52]
- [ ] Thought to activate flippase and externalize phosphatidylserine in erythrocytes and thrombocytes, resulting in vessel occlusion

Laser injury

- [ ] Endothelium injured in agarose immobilized larvae using a pulsed nitrogen laser, resulting in specific venous or arterial vessel occlusion[53]
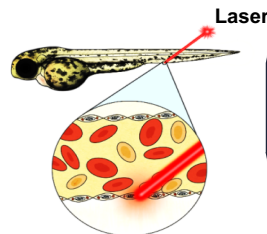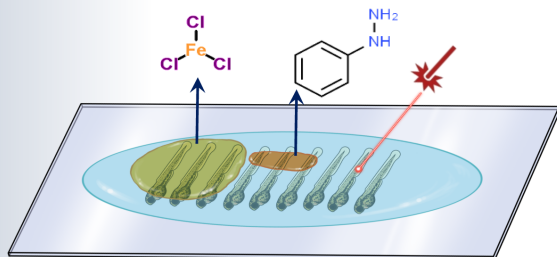
Laser

Unlike mammals, erythrocytes and thrombocytes are nucleated.
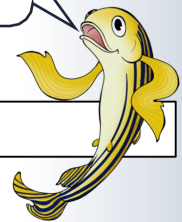
Outcomes of laser-mediated endothelial injury in various transgenic lines

Normal circulation

Venous injury

venous thrombosis

Arterial injury

arterial thrombosis

Occlusive thrombi at the site of laser-mediated endothelial injury.[53]

Occlusive thrombi in a *cd41-eGFP* transgenic[12] (green thrombocytes) shows arterial thrombi to be thrombocyte-rich, analogous to human pathology.[5] Thrombocytes appear at 48 hours post fertilization (hpf).[12,54]

Occlusive thrombi from double *cd41-eGFP* and *gata1-DsRed* transgenics[55] (latter labels erythrocytes red). These are fibrin-rich akin to human venous thrombosis (see fibrinogen illustration below).[51,55] After circulation initiates at 24 hpf, primitive erythrocytes enter the vasculature.[56]

Zebrafish *at3* deficiency demonstrates overlapping and distinct phenotypes with mammals
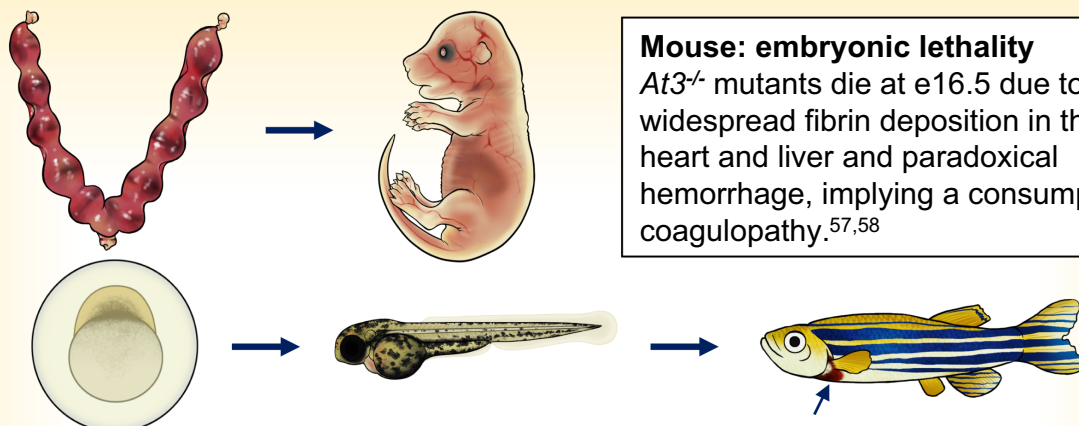
Mouse: embryonic lethality
At3−/− mutants die at e16.5 due to widespread fibrin deposition in the heart and liver and paradoxical hemorrhage, implying a consumptive coagulopathy.[57,58]

Zebrafish: lethality in adulthood
Genome editing with zinc finger nucleases was used to produce *at3* null mutants. *at3*−/− juveniles tolerate spontaneous thrombosis and uncontrolled disseminated intravascular coagulopathy early in development, but adults succumb to intracardiac thrombosis. Some adults exhibit visible hemorrhage (arrow).[59]
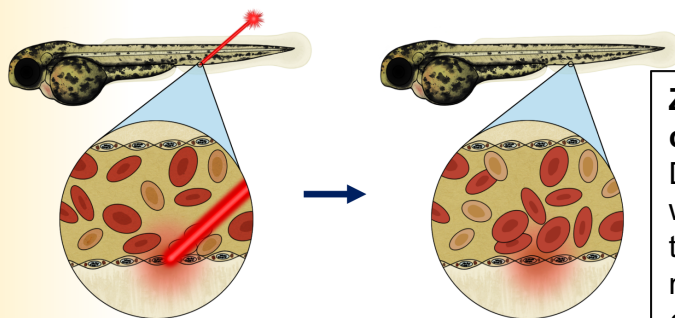
Zebrafish: consumptive coagulopathy
Disseminated intravascular coagulation with consumption of fibrinogen results in the absence of occlusion following laser-mediated venous endothelial injury in *at3*−/− larvae at 3 dpf.[59]

Zebrafish: spontaneous thrombosis
at3−/− mutant zebrafish larvae labeled with fluorescently-tagged fibrinogen reveal fibrin deposition consistent with thrombosis secondary to disseminated intravascular coagulation.[59]

at3+/+, *at3*+/−

at3−/−
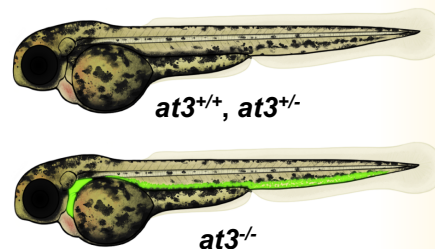
Human and zebrafish *at3* cDNA transgene
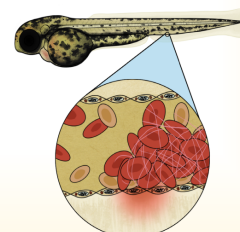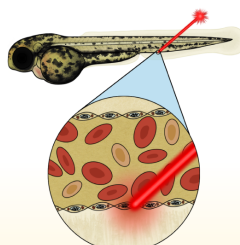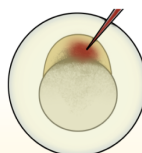
cDNA rescue: Both human and zebrafish *at3* cDNA injected into *at3*−/− single-cell embryos rescue the delayed occlusion after laser-mediated venous endothelial injury.[59]

Common pathway gene knockout in zebrafish results in unexpected survival

Loss of *f2*, *f5*, and *f10* in fish are compatible with development to adulthood
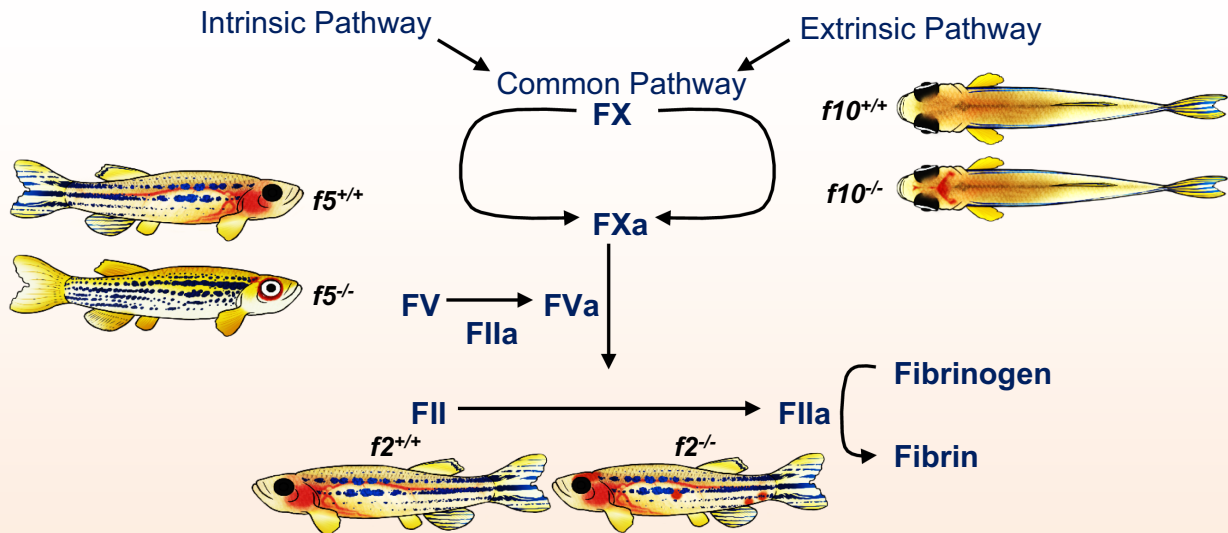
Genome editing with TALENs and CRISPR/Cas9 was used to produce *f2*, *f5*, and *f10* mutant embryos/larvae, which show no overt hemorrhage. They develop normally into the mid-juvenile stage but exhibit spontaneous hemorrhage beginning around 2 months of age, with complete lethality by 4 months.[31,34,60]

Bleed

Common pathway mouse knockouts exhibit *in utero* lethality due to hemorrhage versus possible vascular defects.[61-65]

In zebrafish, common pathway knockouts survive into early adulthood before succumbing to lethal hemorrhage.
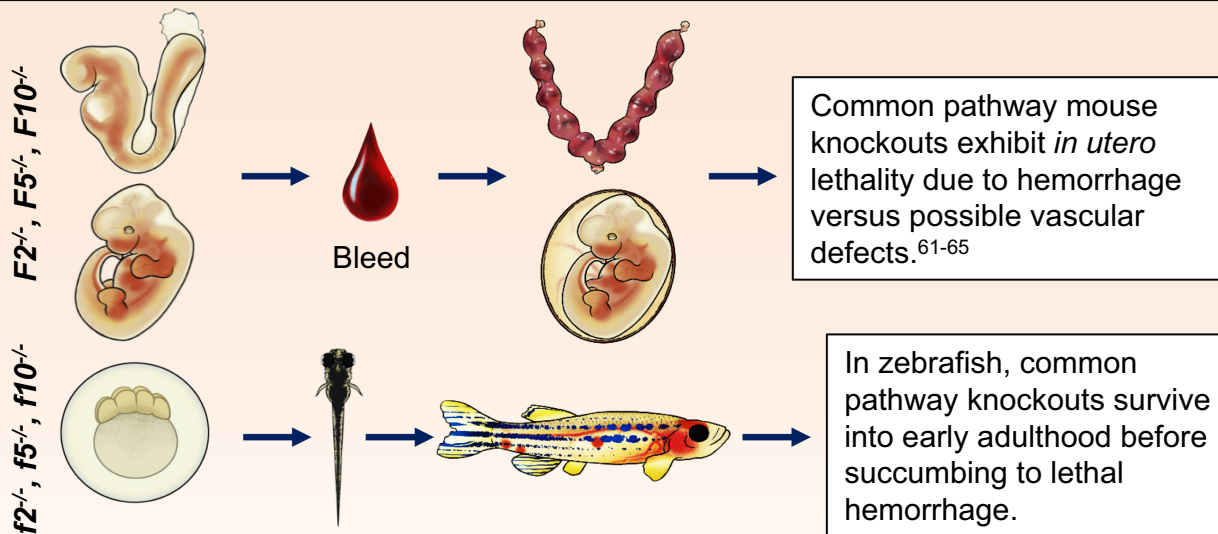
Based on analysis of the zebrafish common pathway mutants, angiogenesis and vasculogenesis appear to be normal. These data suggest that thrombin generation does not play a role in these processes, and in utero death in mammals is likely due to hemorrhage.

Thrombin

Coagulopathy

The role of fibrinogen in zebrafish hemostasis

Fibrin clot
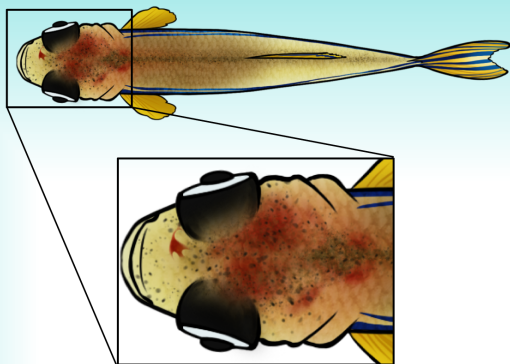
Loss of fibrinogen results in hemorrhage in multiple tissues

fga mutants exhibit spontaneous hemorrhage in the jaw, abdomen, muscle, and fin, as well as the forebrain, midbrain, and hindbrain (inset).[35,66] Hemorrhage occurs later than common pathway mutations, with partial lethality by one year of age.
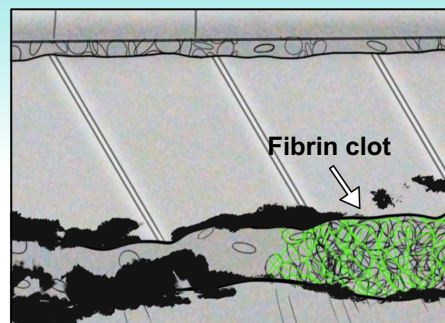
Fibrin(ogen) incorporates into a developing clot in zebrafish larvae

Fluorescently tagged fibrinogen demonstrates that fibrin is the primary component of thrombi produced through endothelial injury in the venous circulation.[11]

fga⁻/⁻;nfe2⁻/⁻

fga⁻/⁻;nfe2⁻/⁻

Incubation in ε-aminocaproic acid

fga cDNA/human fibrinogen infusion

fga⁻/⁻

fga⁻/⁻
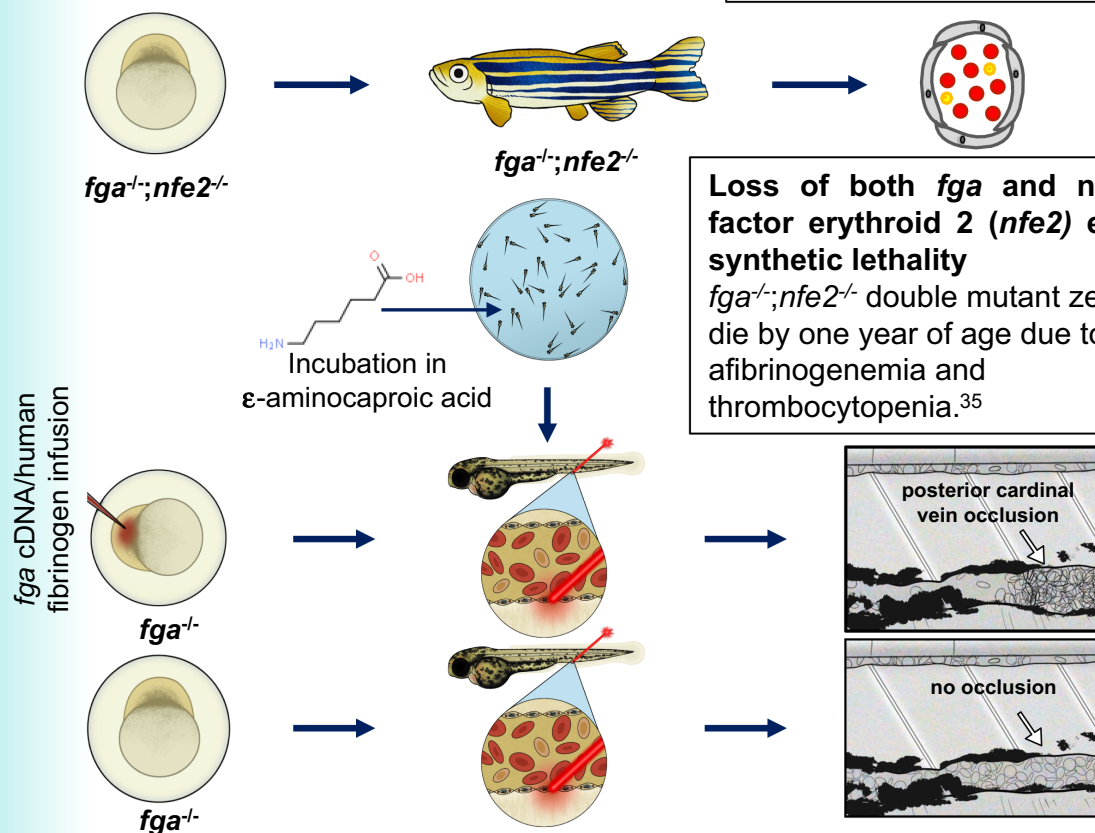
Loss of both *fga* and nuclear factor erythroid 2 (*nfe2*) exhibit synthetic lethality

fga⁻/⁻;nfe2⁻/⁻ double mutant zebrafish die by one year of age due to afibrinogenemia and thrombocytopenia.[35]

posterior cardinal vein occlusion

no occlusion

fga mutant larval fish exhibit a hemostatic defect

As in mammals, targeted mutation of *fga* results in loss of fibrinogen, and absence of thrombus formation following endothelial injury.[35,67] This can be rescued by the expression of zebrafish *fga* cDNA or human fibrinogen infusion. Surprisingly, incubation with ε-aminocaproic acid also partially reverses this hemostatic defect.[35]

Loss of nuclear factor erythroid 2 (Nfe2) results in defective adult but normal embryonic/larval thrombopoiesis in zebrafish
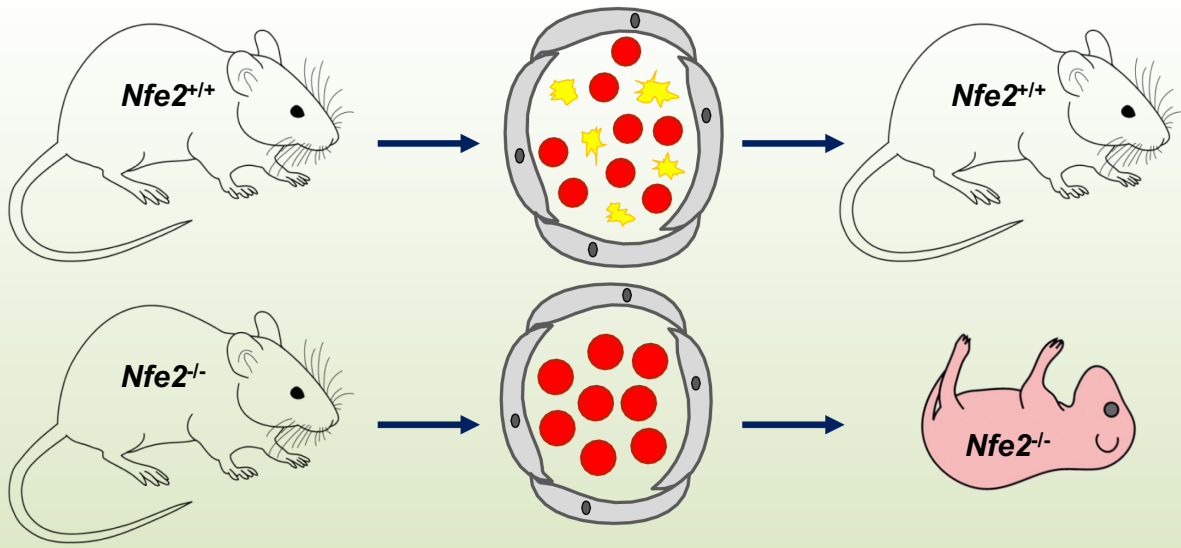
Mouse: thrombocytopenia and neonatal hemorrhage
Nfe2-/- mutants develop normally except for severe, nearly absolute thrombocytopenia. Most homozygous mutants die in the neonatal period secondary to hemorrhage.[68]

Zebrafish: *nfe2-/-* **larvae exhibit normal thrombocyte function**
TALENs were used to engineer a null mutation in *nfe2*.[49] Homozygous mutants display normal thrombocyte-rich clots (arrow, right) in the arterial circulation following laser-mediated endothelial injury of the dorsal aorta at 6 dpf.
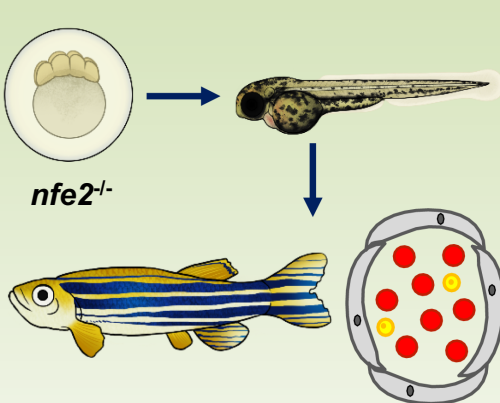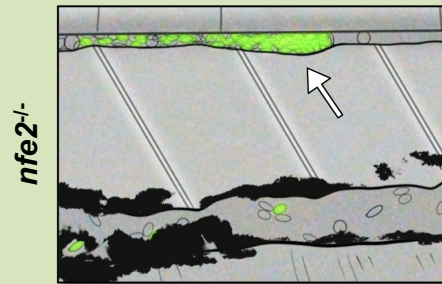
Zebrafish: larval thrombopoiesis
nfe2-/- mutant larva are indistinguishable from wild type siblings. They have normal numbers of thrombocytes and aggregate in response to laser-mediated arterial endothelial injury.[49]

Zebrafish: adult thrombopoiesis
nfe2-/- adults display severe thrombocytopenia, but no apparent hemorrhage and normal long-term survival. Mutant thrombocytes have demonstrably decreased spreading on fibrinogen.[49]
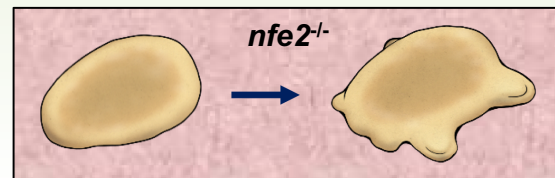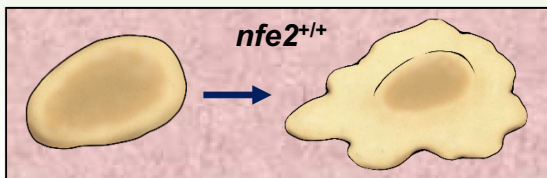
adamts13 and *vwf* in zebrafish

Wild-type zebrafish

lysine-rich histone

Normal Blood Flow

Thrombocyte

VWF

Cleaves large multimers of VWF

ADAMTS13

Weibel-Palade body

H2A H2B H3 H4

Endothelial cell

adamts13⁻/⁻ zebrafish

lysine-rich histone

Thrombotic thrombocytopenic purpura

Neutrophil

No cleavage of VWF

ADAMTS13

H2A H2B H3 H4

VWF

Endothelial cell
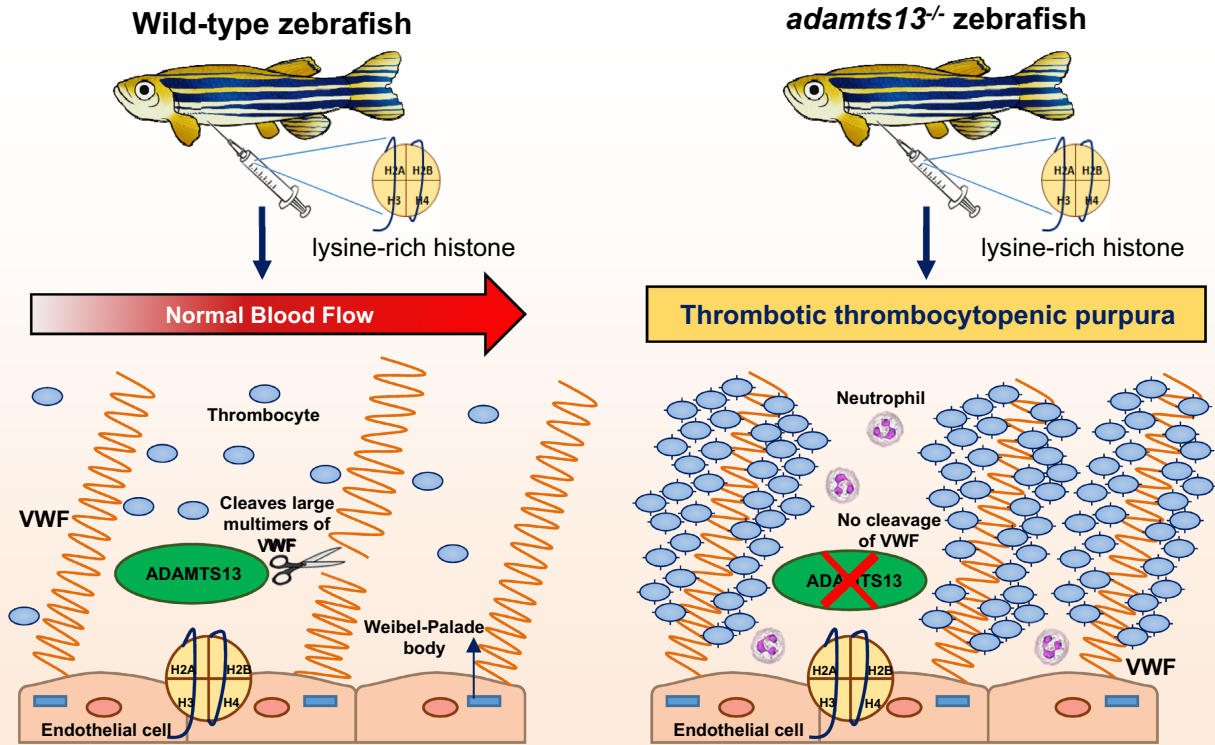
Adamts13 deficiency alone does not cause acute thrombotic thrombocytopenic purpura

In *adamts13*⁻/⁻ zebrafish mutants, intraperitoneal injection of lysine-rich histone triggers severe thrombotic thrombocytopenic purpura (TTP) and causes death.[69] This is mediated through Vwf, as *adamts13*⁻/⁻;*vwf*⁻/⁻ zebrafish mutants do not develop TTP.

vwf⁻/⁻

Validated fish antibodies are scant. Zheng et al[69] had to produce their own anti-Vwf antibody to visualize multimers.

Arterial laser injury

arterial thrombosis

vwf⁻/⁻

Venous laser injury

venous thrombosis
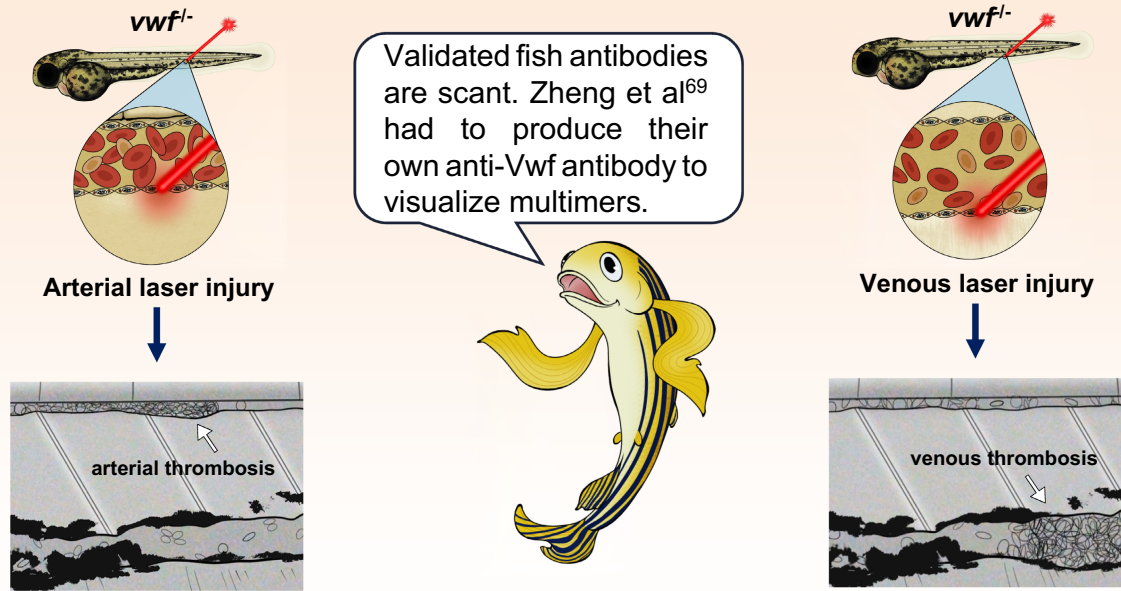
Vwf in zebrafish: zebrafish Vwf forms high molecular multimers and is packaged into Weibel-Palade bodies.[70] Loss of Vwf results in a higher time to occlusion in *vwf*⁻/⁻ mutant fish than the wild type larvae in both the arterial and venous circulation.[71]

Extended survival of fish common pathway mutants relative to mammals
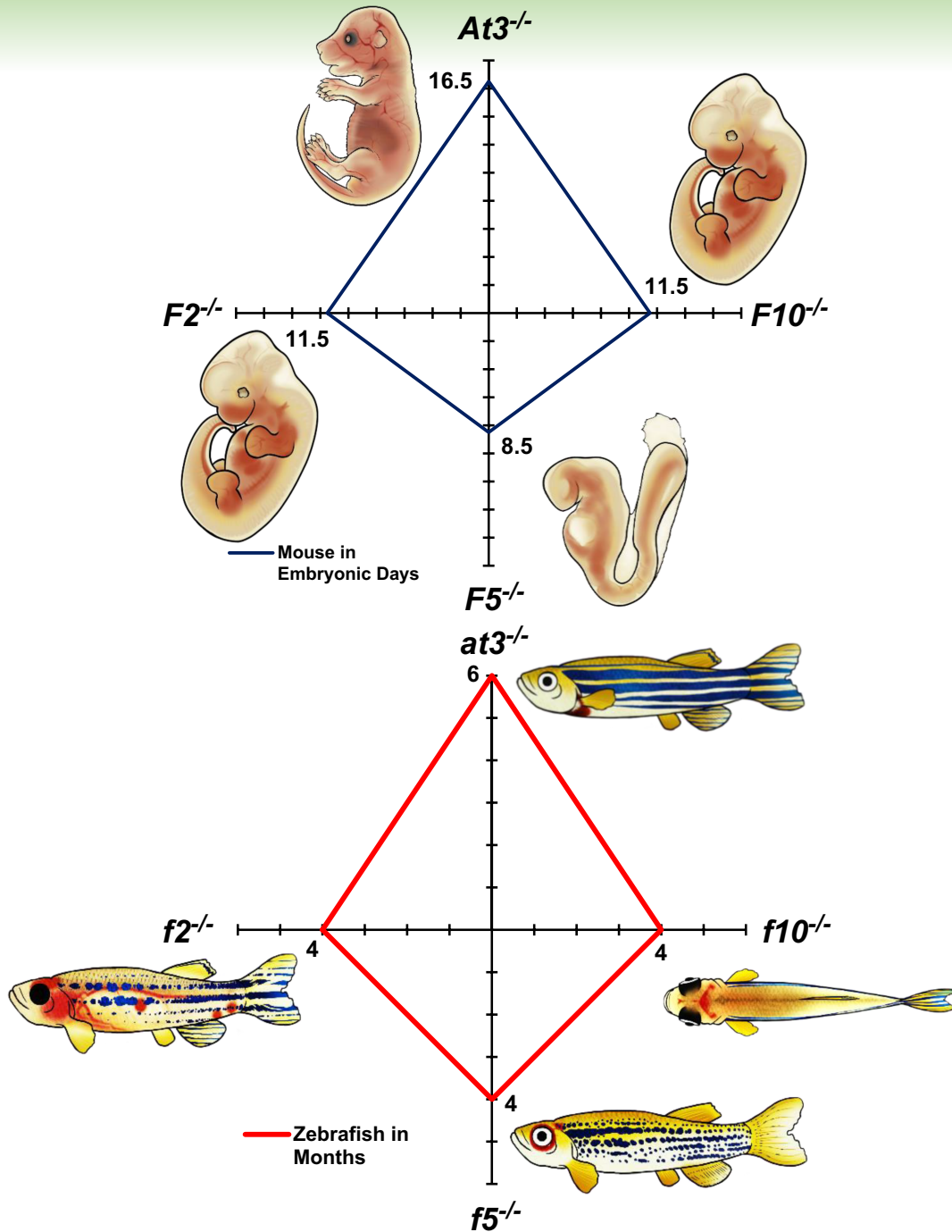
Complete loss of common pathway factors is embryonic lethal in mouse models and presumed lethal in humans.[31,34,60] Hypotheses for the differences between mammals and zebrafish include:

- ❑ absence of birth trauma
- ❑ differential hemostatic challenges in an aquatic environment
- ❑ lower blood pressures in zebrafish
- ❑ species-specific genetic differences resulting in a differential baseline hemostatic balance[60,72]

(black and red lines indicate average age of death for each mutant line)

Evaluation of human genetic variants in zebrafish hemostasis and thrombosis knockout models

AT3 thrombosis variants	Affected domain(s)	Ability to rescue *at3* mutant DIC phenotype[59]
Leu99Phe[73,74]	heparin binding domain	complete
Lys114Glu[75,76]	heparin binding domain	complete
Arg393His[77,78]	reactive center loop (RCL) : P1 Arg mutation	none
Ala404Asp[79]	pleiotropic effect on RCL and heparin binding domain	partial

F5 deficiency variants	Affected domain(s)	Ability to rescue *f5* mutant phenotype[31]
Ser83Arg	A1	complete
Gly97Asp	A1	none
Tyr1702Cys[80]	A3	none
Arg2074Cys[81]	C2	complete
Arg2187Cys	C2	complete

F10 deficiency variants	Affected domain(s)	Ability to rescue *f10* mutant phenotype[34]
Arg68Cys	EGF1	none
Gly173Typ	activation peptide	none
ΔT176_Q186 deletion	activation peptide	none
Gly262Asp	protease	none
Ile323Met	protease	partial
Cys390Phe	protease	none
Gln416Leu	protease	partial

FGA variants	Affected domain(s)	Ability to rescue *fga* mutant phenotype[35]
Met1Val[82]	pre-peptide	none
Cys55Gly[83]	N-terminus	complete
Cys64Tyr[84]	N-terminus	complete
Tyr809Cys[85]	α_EC domain	none

Since hemostasis must occur within a closed circulatory system, consisting of flowing blood, proteins, cells, and endothelium, the zebrafish is superior to cell culture systems for assessing variants.

AUTHOR CONTRIBUTIONS

AR and JS developed the concepts and wrote the manuscript. AR and AF produced the illustrations. AR, AF, and JS approved the final content.

RELATIONSHIP DISCLOSURE

JAS has been a consultant for Sanofi, Takeda, CSL Behring, HEMA Biologics, and Bayer. AR and ACF report no conflicts of interest.

ORCID

Azhwar Raghunath https://orcid.org/0000-0001-5217-0341
Allison C. Ferguson https://orcid.org/0000-0003-3195-8572
Jordan A. Shavit https://orcid.org/0000-0002-2874-4904

TWITTER

Azhwar Raghunath @raghugeek
Jordan A. Shavit @Clot1

REFERENCES

1. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev Dyn*. 1995;203(3):253-310.
2. Matthews M, Trevarrow B, Matthews J. A virtual tour of the guide for zebrafish users. *Lab Anim (NY)*. 2002;31(3):34-40.
3. Isogai S, Horiguchi M, Weinstein BM. The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. *Dev Biol*. 2001;230(2):278-301.
4. Weyand AC, Shavit JA. Zebrafish as a model system for the study of hemostasis and thrombosis. *Curr Opin Hematol*. 2014;21(5):418-422.
5. Rost MS, Grzegorski SJ, Shavit JA. Quantitative methods for studying hemostasis in zebrafish larvae. *Methods Cell Biol*. 2016;134:377-389.
6. Howe K, Clark MD, Torroja CF, et al. The zebrafish reference genome sequence and its relationship to the human genome. *Nature*. 2013;496(7446):498-503.
7. Hanumanthaiah R, Day K, Jagadeeswaran P. Comprehensive analysis of blood coagulation pathways in teleostei: evolution of coagulation factor genes and identification of zebrafish factor VIII. *Blood Cells Mol Dis*. 2002;29(1):57-68.
8. Meyer A, Schartl M. Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. *Curr Opin Cell Biol*. 1999;11(6):699-704.
9. Jagadeeswaran P, Cooley BC, Gross PL, Mackman N. Animal models of thrombosis from zebrafish to nonhuman primates: use in the elucidation of new pathologic pathways and the development of antithrombotic drugs. *Circ Res*. 2016;118(9):1363-1379.
10. Rafferty SA, Quinn TA. A beginner's guide to understanding and implementing the genetic modification of zebrafish. *Prog Biophys Mol Biol*. 2018;138:3-19.
11. Vo AH, Swaroop A, Liu Y, Norris ZG, Shavit JA. Loss of fibrinogen in zebrafish results in symptoms consistent with human hypofibrinogenemia. *PLoS One*. 2013;8(9):e74682.
12. Lin HF, Traver D, Zhu H, et al. Analysis of thrombocyte development in CD41-GFP transgenic zebrafish. *Blood*. 2005;106(12):3803-3810.
13. Lam PY, Peterson RT. Developing zebrafish disease models for in vivo small molecule screens. *Curr Opin Chem Biol*. 2019;50:37-44.
14. Patton EE, Zon LI, Langenau DM. Zebrafish disease models in drug discovery: from preclinical modelling to clinical trials. *Nat Rev Drug Discov*. 2021;20(8):611-628.
15. Santoriello C, Zon LI. Hooked! Modeling human disease in zebrafish. *J Clin Invest*. 2012;122(7):2337-2343.
16. Walker C, Streisinger G. Induction of mutations by gamma-rays in Pregonial germ cells of zebrafish embryos. *Genetics*. 1983;103(1):125-136.
17. Chakrabarti S, Streisinger G, Singer F, Walker C. Frequency of gamma-ray induced specific locus and recessive lethal mutations in mature germ cells of the zebrafish, *Brachydanio rerio*. *Genetics*. 1983;103(1):109-123.
18. Streisinger G, Walker C, Dower N, Knauber D, Singer F. Production of clones of homozygous diploid zebra fish (Brachydanio rerio). *Nature*. 1981;291(5813):293-296.
19. Grunwald DJ, Streisinger G. Induction of recessive lethal and specific locus mutations in the zebrafish with ethyl nitrosourea. *Genet Res*. 1992;59(2):103-116.
20. Nasevicius A, Ekker SC. Effective targeted gene 'knockdown' in zebrafish. *Nat Genet*. 2000;26(2):216-220.
21. Kim S, Radhakrishnan UP, Rajpurohit SK, Kulkarni V, Jagadeeswaran P. Vivo-morpholino knockdown of alphaIIb: a novel approach to inhibit thrombocyte function in adult zebrafish. *Blood Cells Mol Dis*. 2010;44(3):169-174.
22. Doyon Y, McCammon JM, Miller JC, et al. Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat Biotechnol*. 2008;26(6):702-708.
23. Meng X, Noyes MB, Zhu LJ, Lawson ND, Wolfe SA. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nat Biotechnol*. 2008;26(6):695-701.
24. Sander JD, Cade L, Khayter C, et al. Targeted gene disruption in somatic zebrafish cells using engineered TALENs. *Nat Biotechnol*. 2011;29(8):697-698.
25. Bedell VM, Wang Y, Campbell JM, et al. In vivo genome editing using a high-efficiency TALEN system. *Nature*. 2012;491(7422):114-118.
26. Jao LE, Wente SR, Chen W. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proc Natl Acad Sci USA*. 2013;110(34):13904-13909.
27. Hwang WY, Fu Y, Reyon D, et al. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol*. 2013;31(3):227-229.
28. Hwang WY, Fu Y, Reyon D, Gonzales AP, Joung JK, Yeh JR. Targeted mutagenesis in zebrafish using CRISPR RNA-guided nucleases. *Methods Mol Biol*. 2015;1311:317-334.
29. MacArthur DG, Manolio TA, Dimmock DP, et al. Guidelines for investigating causality of sequence variants in human disease. *Nature*. 2014;508(7497):469-476.
30. Kretz CA, Weyand AC, Shavit JA. Modeling disorders of blood coagulation in the zebrafish. *Curr Pathobiol Rep*. 2015;3(2):155-161.
31. Weyand AC, Grzegorski SJ, Rost MS, et al. Analysis of factor V in zebrafish demonstrates minimal levels needed for early hemostasis. *Blood Adv*. 2019;3(11):1670-1680.
32. Baudhuin LM, Lagerstedt SA, Klee EW, Fadra N, Oglesbee D, Ferber MJ. Confirming variants in next-generation sequencing panel testing by sanger sequencing. *J Mol Diagn*. 2015;17(4):456-461.

33. Wei X, Ju X, Yi X, et al. Identification of sequence variants in genetic disease-causing genes using targeted next-generation sequencing. *PLoS One*. 2011;6(12):e29500.

34. Hu Z, Liu Y, Huarng MC, et al. Genome editing of factor X in zebrafish reveals unexpected tolerance of severe defects in the common pathway. *Blood*. 2017;130(5):666-676.

35. Hu Z, Lavik KI, Liu Y, et al. Loss of fibrinogen in zebrafish results in an asymptomatic embryonic hemostatic defect and synthetic lethality with thrombocytopenia. *J Thromb Haemost*. 2019;17(4):607-617.

36. Glasauer SM, Neuhauss SC. Whole-genome duplication in teleost fishes and its evolutionary consequences. *Mol Genet Genomics*. 2014;289(6):1045-1060.

37. Braasch I, Bobe J, Guiguen Y, Postlethwait JH. Reply to: 'Subfunctionalization versus neofunctionalization after whole-genome duplication'. *Nat Genet*. 2018;50(7):910-911.

38. Iyer N, Al Qaryoute A, Kacham M, Jagadeeswaran P. Identification of zebrafish ortholog for human coagulation factor IX and its age-dependent expression. *J Thromb Haemost*. 2021;19(9):2137-2150.

39. Buchner DA, Su F, Yamaoka JS, et al. pak2a mutations cause cerebral hemorrhage in redhead zebrafish. *Proc Natl Acad Sci USA*. 2007;104(35):13996-14001.

40. Arias-Romero LE, Chernoff J. A tale of two paks. *Biol Cell*. 2008;100(2):97-108.

41. Thon JN, Italiano JE. Platelets: production, morphology and ultrastructure. *Handb Exp Pharmacol*. 2012;210:3-22.

42. Lee D, Fong KP, King MR, Brass LF, Hammer DA. Differential dynamics of platelet contact and spreading. *Biophys J*. 2012;102(3):472-482.

43. Hartwig JH. Mechanisms of actin rearrangements mediating platelet activation. *J Cell Biol*. 1992;118(6):1421-1442.

44. Khandekar G, Kim S, Jagadeeswaran P. Zebrafish thrombocytes: functions and origins. *Adv Hematol*. 2012;2012:857058.

45. Jagadeeswaran P, Sheehan JP, Craig FE, Troyer D. Identification and characterization of zebrafish thrombocytes. *Br J Haematol*. 1999;107(4):731-738.

46. Lloyd-Evans P, Barrow SE, Hill DJ, et al. Eicosanoid generation and effects on the aggregation of thrombocytes from the rainbow trout, *Oncorhynchus mykiss*. *Biochim Biophys Acta*. 1994;1215(3):291-299.

47. Hill DJ, Hallett MB, Rowley AF. Effect of prostanoids and their precursors on the aggregation of rainbow trout thrombocytes. *Am J Physiol*. 1999;276(3):R659-R664.

48. Hughes CE, Radhakrishnan UP, Lordkipanidzé M, et al. G6f-like is an ITAM-containing collagen receptor in thrombocytes. *PLoS One*. 2012;7(12):e52622.

49. Rost MS, Shestopalov I, Liu Y, et al. Nfe2 is dispensable for early but required for adult thrombocyte formation and function in zebrafish. *Blood Adv*. 2018;2(23):3418-3427.

50. Sandmann R, Köster S. Topographic cues reveal two distinct spreading mechanisms in blood platelets. *Sci Rep*. 2016;6:22357.

51. Gregory M, Hanumanthaiah R, Jagadeeswaran P. Genetic analysis of hemostasis and thrombosis using vascular occlusion. *Blood Cells Mol Dis*. 2002;29(3):286-295.

52. Zhu XY, Liu HC, Guo SY, et al. A zebrafish thrombosis model for assessing antithrombotic drugs. *Zebrafish*. 2016;13(4):335-344.

53. Jagadeeswaran P, Carrillo M, Radhakrishnan UP, Rajpurohit SK, Kim S. Laser-induced thrombosis in zebrafish. *Methods Cell Biol*. 2011;101:197-203.

54. Huarng MC, Shavit JA. Simple and rapid quantification of thrombocytes in zebrafish larvae. *Zebrafish*. 2015;12(3):238-242.

55. Traver D, Paw BH, Poss KD, Penberthy WT, Lin S, Zon LI. Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants. *Nat Immunol*. 2003;4(12):1238-1246.

56. Long Q, Meng A, Wang H, Jessen JR, Farrell MJ, Lin S. GATA-1 expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene. *Development*. 1997;124(20):4105-4111.

57. Ishiguro K, Kojima T, Kadomatsu K, et al. Complete antithrombin deficiency in mice results in embryonic lethality. *J Clin Invest*. 2000;106(7):873-878.

58. Kojima T. Targeted gene disruption of natural anticoagulant proteins in mice. *Int J Hematol*. 2002;76(Suppl 2):36-39.

59. Liu Y, Kretz CA, Maeder ML, et al. Targeted mutagenesis of zebrafish antithrombin III triggers disseminated intravascular coagulation and thrombosis, revealing insight into function. *Blood*. 2014;124(1):142-150.

60. Grzegorski SJ, Hu Z, Liu Y, et al. Disruption of the kringle 1 domain of prothrombin leads to late onset mortality in zebrafish. *Sci Rep*. 2020;10(1):4049.

61. Cui J, O'Shea KS, Purkayastha A, Saunders TL, Ginsburg D. Fatal haemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V. *Nature*. 1996;384(6604):66-68.

62. Sun WY, Witte DP, Degen JL, et al. Prothrombin deficiency results in embryonic and neonatal lethality in mice. *Proc Natl Acad Sci USA*. 1998;95(13):7597-7602.

63. Xue J, Wu Q, Westfield LA, et al. Incomplete embryonic lethality and fatal neonatal hemorrhage caused by prothrombin deficiency in mice. *Proc Natl Acad Sci USA*. 1998;95(13):7603-7607.

64. Dewerchin M, Liang Z, Moons L, et al. Blood coagulation factor X deficiency causes partial embryonic lethality and fatal neonatal bleeding in mice. *Thromb Haemost*. 2000;83(2):185-190.

65. Mullins ES, Kombrinck KW, Talmage KE, et al. Genetic elimination of prothrombin in adult mice is not compatible with survival and results in spontaneous hemorrhagic events in both heart and brain. *Blood*. 2009;113(3):696-704.

66. Fish RJ, Di Sanza C, Neerman-Arbez M. Targeted mutation of zebrafish fga models human congenital afibrinogenemia. *Blood*. 2014;123(14):2278-2281.

67. Fish RJ, Freire C, Di Sanza C, Neerman-Arbez M. Venous thrombosis and thrombocyte activity in zebrafish models of quantitative and qualitative fibrinogen disorders. *Int J Mol Sci*. 2021;22(2):655.

68. Shivdasani RA, Rosenblatt MF, Zucker-Franklin D, et al. Transcription factor NF-E2 is required for platelet formation independent of the actions of thrombopoietin/MGDF in megakaryocyte development. *Cell*. 1995;81(5):695-704.

69. Zheng L, Abdelgawwad MS, Zhang D, et al. Histone-induced thrombotic thrombocytopenic purpura in adamts13 (−/−) zebrafish depends on von Willebrand factor. *Haematologica*. 2020;105(4):1107-1119.

70. Ghosh A, Vo A, Twiss BK, et al. Characterization of zebrafish von Willebrand factor reveals conservation of domain structure, multimerization, and intracellular storage. *Adv Hematol*. 2012;2012:214209.

71. Iyer N, Tcheuyap VT, Schneider S, Marshall V, Jagadeeswaran P. Knockout of von Willebrand factor in zebrafish by CRISPR/Cas9 mutagenesis. *Br J Haematol*. 2019;186(4):e76-e80.

72. Shavit JA, Ginsburg D. Hemophilias and other disorders of hemostasis. In: Rimoin DL, Pyeritz RE, Korf BR, eds. *Emery and Rimoin's Principles and Practice of Medical Genetics*. 6th ed. Elsevier Science; 2013:1-33.

73. Olds RJ, Lane DA, Boisclair M, Sas G, Bock SC, Thein SL. Antithrombin Budapest 3. An antithrombin variant with reduced heparin affinity resulting from the substitution L99F. *FEBS Lett*. 1992;300(3):241-246.

74. Kuhle S, Lane DA, Jochmanns K, et al. Homozygous antithrombin deficiency type II (99 leu to Phe mutation) and childhood thromboembolism. *Thromb Haemost*. 2001;86(4):1007-1011.

75. Mushunje A, Zhou A, Huntington JA, Conard J, Carrell RW. Antithrombin 'DREUX' (Lys 114Glu): a variant with complete loss of heparin affinity. *Thromb Haemost*. 2002;88(3):436-443.

76. Picard V, Susen S, Bellucci S, Aiach M, Alhenc-Gelas M. Two new antithrombin variants support a role for K114 and R13 in heparin binding. *J Thromb Haemost*. 2003;1(2):386-387.

77. Bauer KA, Ashenhurst JB, Chediak J, Rosenberg RD. Antithrombin "Chicago": a functionally abnormal molecule with increased heparin affinity causing familial thrombophilia. *Blood*. 1983;62(6):1242-1250.

78. Owen MC, Beresford CH, Carrell RW. Antithrombin Glasgow, 393 Arg to his: a P1 reactive site variant with increased heparin affinity but no thrombin inhibitory activity. *FEBS Lett*. 1988;231(2):317-320.

79. Luxembourg B, Delev D, Geisen C, et al. Molecular basis of antithrombin deficiency. *Thromb Haemost*. 2011;105(4):635-646.

80. Castoldi E, Lunghi B, Mingozzi F, et al. A missense mutation (Y1702C) in the coagulation factor V gene is a frequent cause of factor V deficiency in the Italian population. *Haematologica*. 2001;86(6):629-633.

81. Duga S, Montefusco MC, Asselta R, et al. Arg2074Cys missense mutation in the C2 domain of factor V causing moderately severe factor V deficiency: molecular characterization by expression of the recombinant protein. *Blood*. 2003;101(1):173-177.

82. Smith N, Bornikova L, Noetzli L, et al. Identification and characterization of novel mutations implicated in congenital fibrinogen disorders. *Res Pract Thromb Haemost*. 2018;2(4):800-811.

83. Zhang JZ, Redman CM. Role of interchain disulfide bonds on the assembly and secretion of human fibrinogen. *J Biol Chem*. 1994;269(1):652-658.

84. Hanss M, Pouymayou C, Blouch MT, et al. The natural occurrence of human fibrinogen variants disrupting inter-chain disulfide bonds (a{alpha}Cys36Gly, a{alpha}Cys36Arg and a{alpha}Cys45Tyr) confirms the role of N-terminal a{alpha} disulfide bonds in protein assembly and secretion. *Haematologica*. 2011;96(8):1226-1230.

85. https://varsome.com/variant/hg19/