SUPPLEMENTARY APPENDIX

Histone-induced thrombotic thrombocytopenic purpura in *adamts13*$^{-/-}$ zebrafish depends on von Willebrand factor

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Supplemental Materials

Methods

Generation of *adamts13*$^{-/-}$, *vwf*$^{-/-}$ and *adamts13*$^{-/-}$*vwf*$^{-/-}$ zebrafish by CRISPR-cas9: Zebrafish (*danio rerio*) were used according to the protocol approved by our Institutional Animal Care and Use Committee. (1) Wild type Zebrafish (AB strain) and genetically modified zebrafish were maintained in a housing system that has ultraviolet light and dechlorinated and carbon filtered tap water. The water was maintained at temperature of 28 ± 0.5 °C, hardness of 150–250 mg/L $CaCO_3$, dissolved oxygen >7 mg/L, and pH 7.2–7.8. Lighting was controlled to provide a constant light/dark (14/10 hours) cycle. Zebrafish were fed twice daily with commercial dry pellets or brine shrimp. The guide RNA (gRNA) targeting the signal peptide region of zebrafish *adamts13* (*a13*) or the propeptide region of zebrafish *vwf* was designed using the CRISPR design tool (http://crispr.mit.edu/). A 69-nt oligonucleotide (for *a13*: GCGGCCTCTAATACGACT-CACTATAGG**GGCCTCCCTTT-GAGATAGTGT***GTTTTAGAGCTAGAAATAGCA;* for *vwf*: GCGGCCTCTAATACGAC-TCACTATAGG**GCAGCGAAGCTTCTCCATACT-***GTTTTAGAGCTAGAAATAGCA*) was synthesized (ThermoFisher, Waltham, MA), which consists of a T7 promoter (underlined), a target sequence (in **bold**), and a gRNA scaffold (*italic*). The oligonucleotide was used to assemble a gRNA-encoding template by annealing and extension with the gRNA core sequence. The gRNA was then generated using a Guide-it sgRNA transcription kit (Takara-Clontech, Mountain View, CA). The *Cas9* mRNA was synthesized from pT3TS-nCas9n using the mMESSAGE mMACHINE T3 kit (Life Technologies, Carlsbad, CA). Both the gRNA and Cas9 mRNA

were purified with the MEGAclear RNA purification kit (ThermoFisher, Waltham, MA) and dissolved in RNase-free water before use. The final products (2 nL, containing 12.5 pg/nL gRNA and 300 pg/nL Cas9 mRNA) were co-injected into one-cell stage embryos of double transgenic zebrafish (*gata1*-dsRed and *fli1*-eGFP). (2, 3)

Identification of a desired mutant in zebrafish: Zebrafish embryos injected with gRNA and Cas9 mRNA were hatched and grown to adulthood. Genomic DNA was extracted from a fin clip. The targeted regions of *a13* or *vwf* locus were amplified by PCR with a gene specific primer pair (for *a13*: forward 5'-AGATTCACTGAAACC-TCAAG-3' and reverse 5'-CAGCTGCTCGCAACACATATC-3'; for *vwf*: forward 5'-GATTTGCTGTTGGTCGAGGG-3' and reverse 5'-AGCAGCTGATGTGATGAAGTATG-3'). The potential mutations in *F0* founders were identified by PCR and followed by a T7 endonuclease I digestion, (4) which were then outcrossed with *wt* fish to generate more advanced (F1, F2, and F3*)* progenies. The $a13^{-/-}$ and $vwf^{-/-}$ zebrafish were crossed to generate a double null ($a13^{-/-}vwf^{-/-}$) zebrafish.

Off-target assessment: A CRISPR tool was used to identify the potential off-target sites listed in **Table S1**. All regions were amplified with specific primer pairs from F0, F1, and F2 zebrafish embryos and digested with T7 endonuclease I.

Capillary-based Western blotting assay: Plasma of $a13^{-/-}$ and *wt* zebrafish at age 3 months and embryo cell lysate (ten embryos in each group) from $a13^{-/-}$ and *wt* zebrafish were prepared for Western blotting. The embryos were lysed with T-PER™ tissue protein extraction reagents (ThermoFisher). A capillary-based Western blotting (WES) (ProteinSimple, San Jose, CA) was used to determine ADAMTS13 protein. The primary

antibody was generated commercially (ABmart, Shanghai, China) by immunization of mice with nine synthetic peptides of zebrafish ADAMTS13 protein (**Table S2)**. A peroxidase-conjugated anti-mouse IgG and chemiluminescent reagent kits were provided from the manufacturer for detection (Protein Simple, San Jose, CA).

Agarose gel electrophoresis for multimers: Plasma VWF multimers in zebrafish with various genotypes were determined by Western blotting after separation of the proteins on 1.5% SDS-agarose gel. (5) Primary anti-VWF antibody was produced by immunization of rabbits with two synthetic peptides of zebrafish VWF listed in **Table S2** and affinity-purified (ABclonal, Woburn, MA). IRDye800-conjugated anti-rabbit IgG (1:5,000) and Odyssey imaging system (LI-COR, Lincoln, Nebraska) were used for the detection. NIH image/ImageJ was used to determine the multimer intensity and distribution.

Intravital microscopy: Zebrafish larvae (5-dpf) with various genotypes were immobilized in 0.8% low-melt agarose gel containing 10 mM HEPES and 0.04% anesthetic tricaine (Sigma-Aldrich). A drop (2.5 μL) of 0.3% $FeCl_3$ was placed on the tail region. Digital images were obtained under AZ100 fluorescent microscope (Nikon, Melville, NY) and analyzed offline. A laboratory assistant without prior knowledge of the genotype manually determined the time to complete cessation of blood flow in the tail venules. Additionally, the larvae were imaged for high resolution under an A1 3D confocal microscope (Nikon, Melville, NY).

Microfluidic assays: Microchannels (Fluxion Bioscience, San Francisco, CA) were coated with a fibrillar collagen (100 μg/mL) in 0.01N hydrochloric acid. The surface was

then blocked with 0.5% BSA. Pooled whole blood (~50μL) collected from 10 adult zebrafish of each genotype, and anticoagulated with PPACK (100 μM), was diluted with 50 μL of PBS and perfused under arterial shear (15 dyne/cm^2) over the collagen-coated surfaces. The time-lapse digital images were collected every 3 seconds for a total 120 seconds. The relative increase in fluorescent intensity and the length of thrombocyte-decorated strings were determined offline using the Montage software.

FRETS-rVWF73 assay: Zebrafish or human plasma (1-2 μL) was added into a well containing 50 μL of assay buffer (5 mM Bis-Tris, pH 6.0, 25 mM $CaCl_2$, 0.005% tween-20, and 4% of pefabloc) (Sigma- Aldrich, St. Louis, MO) and 50 μL FRETS-rVWF73 (2 μM) prepared as described previously. (6, 7) The rate of fluorescence generation was determined using the SpectraMax microtiter plate reader (Molecular Devices, Sunnyvale, CA).

Effect of histone on release of VWF from cultured endothelial cells under flow. Microfluidic channels were seeded with immortalized murine endothelial cells (SVEC4-10) for 24 hours. After washed with DMEM containing 10% FBS, cells were treated without (control) or with a lysine-histone (10 μg/mL) for 10 min. A PPACK-anticoagulated whole blood from *a13*$^{-/-}$ mice was perfused over the endothelial surfaces and the coverage and the rate of accumulation of fluoresceinated platelets were determined under an inverted microscope equipped with a high-speed CCD camera every 2 seconds for 3 minutes.

Administration of a lysine-rich histone into zebrafish: Lysine-rich histone (or H5505) (Sigma-Aldrich) (200 mg/kg body weight) or an equal volume of PBS was

injected via the intraperitoneal cavity into adult zebrafish (3-4 months). On day 1, 2, 3, and 7 (or 14 in some experiments) after H5505 administration, whole blood (2 μL each) was collected into a tube containing EDTA (5 mM) and analyzed using flow cytometry.

Plasma levels of histone-DNA in zebrafish: Plasma levels of histone-DNA complexes were determined using a cell death detection kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. This ELISA-based assay uses a capturing antibody against an epitope shared by all histones and a detecting antibody against single or double DNA fragments, conjugated with horseradish peroxidase. The unit of nucleosomes refers to the average amount of histone-DNA complexes in plasma from a pool of healthy human controls, which was defined as having 1 AU per mL.

Total and differential blood cell counts: Zebrafish whole blood was diluted (1:51) with PBS (containing 4 mM EDTA). The total cell counts per unit (μL) of blood were measured using Hemavet 950FS Hematology Analyzer (Drew Scientific, Miami Lakes, FL). The cell number in PBS was set as zero for the negative control. Flow cytometry was performed to differentiate erythrocytes (red), immature thrombocytes (red and green), and mature thrombocytes (green) using the FACS Vantage flow cytometer (BD Biosciences, San Jose, CA). The percentage of erythrocytes and thrombocytes were determined in 50,000 cells per sample. The number of thrombocytes per liter whole blood were calculated using a formula: thrombocyte count= total cells counts per liter X thrombocyte percentage X dilution factor). The erythrocyte count was calculated in the same way. The blood from a non-transgenic (wild-type) zebrafish that does not express

any fluorescent protein markers was used for the negative control for flow cytometry gating purpose.

Blood smear analysis: Whole blood from adult zebrafish was used for preparation of a thin blood smear. After fixation and staining with Giemsa, cell images were obtained using a light microscope (Carl Zeiss, Göttingen, Germany) with an oil lens (100x). Fins of zebrafish were collected for genotyping if unknown. The number of fragmented erythrocytes was determined by manual count of cells in three separate fields in each individual sample. The assistant who counted the cells was blinded to genotype and treatment. The mean and standard error of fragmented erythrocytes from 10 fish in each group were determined.

Histological and immunohistochemical analyses*:* Zebrafish with various genotypes and treatments that died or were sacrificed were then fixed in 4% paraformaldehyde in PBS. The whole fish were then embedded in paraffin and sectioned using a microtome (6 μm, each) for hematoxylin and eosin (H&E) staining. The fixed fish tissues were also deparaffinized, rehydrated, and used for immunohistochemistry. After being blocked with 3% normal goat serum, the tissue sections were incubated with a polyclonal anti-zebrafish VWF IgG, followed by HRP-conjugated secondary antibody at 4 °C overnight. The bound secondary antibody was visualized by incubation with DAB (3, 3 - diaminobenzidine) substrate. The control slides were processed and treated the same way with the exception of being incubated with primary antibody. The images were obtained using a light microscope equipped with a digital camera (Carl Zeiss). The

number of occlusive microvascular thrombi was determined under a high power len (100x) from n=10 zebrafish in each group.

Statistical analysis: All data are presented as the mean ± standard errors of mean (SEM) or the median in a Box-whisker plot (min to max values) unless specified in the figure legends. Kaplan-Meier survival analysis was determined by a Log-rank test. Mann-Whitney test was used to determine the difference between two groups, while Krüskal-Wallis one-way analysis of variance was used to test the significance for more than three groups. All statistical analyses were carried out using Prism7 software.

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Suppl. Tables and Figures

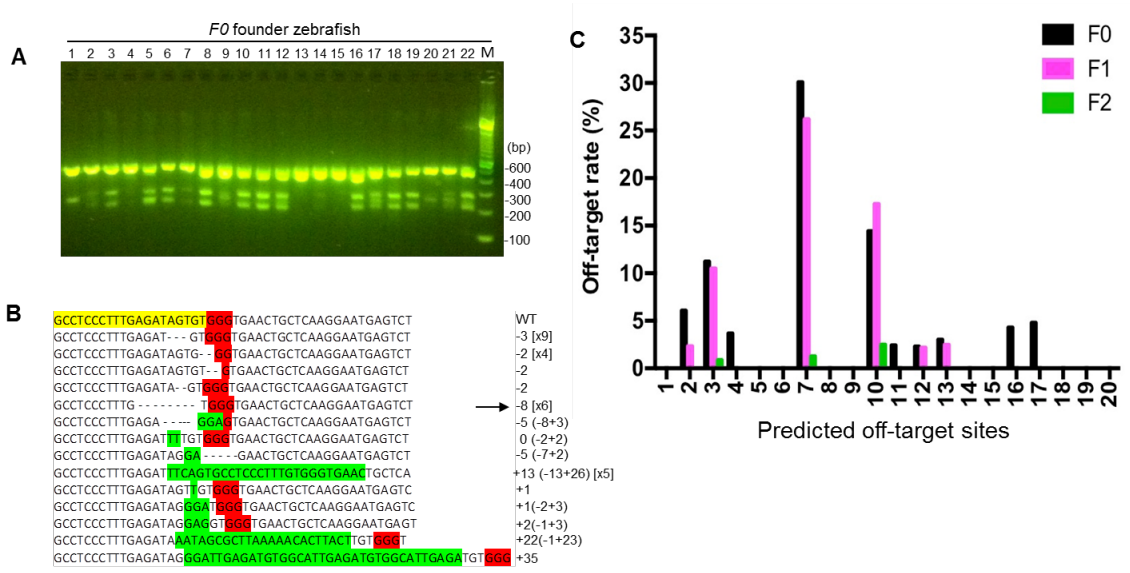
Suppl. **Table 1**. Primer sequences used to amplify 20 predicted off-target sites, their score, and genomic locus are listed below.

Off-target Sites	Sequence	Score	Genomic Locus	Primers for detection (5'to 3')
OT-1	CGCTCCATTGGAGATAGTGTGAG	0.9	chr11:-36815719	F: TTACAGCTGCACACATTCTCAGAT R: TGTCGAGATGATTGACAGGGGTT
OT-2	TCCCCCCCTTGTGATAGTGTGGG	0.7	chr8:-39104423	F: ATTTCACTCCCCCACAGAATCA R: GCATCTCGCCATCTGAGAGC
OT-3	ACCTACCTTTGAGATAGCGTCAG	0.7	chr23:+41007038	F: AGAGCGTATTGATGACGGGG R: AATAAGCTGCCCACTGCTTCA
OT-4	ATCTCAGTTTGAGATAGTGTTAG	0.6	chr15:+15008668	F: CTGTCATCATGGCAAAGATA R: CCACTCCTCAGTGCATC
OT-5	GCCAACATTTTAGATAGTGTTGG	0.5	chr17:-8364356	F: TGTGGTGAATGTTCTGGGCG R: GTTTGAATTGTGCACATCTTTCCT
OT-6	AACTCACTCTGAGATAGTGTAAG	0.5	chr12:-39925190	F: TCTGCATTGAGTTTCCGCAC R: CGGTTTCAGTGGAGGTGGAA
OT-7	CTCTCTCTTTAAGATAGTGTAAG	0.5	chr16:+34084701	F: CGGATCCATCAGGGGGTGTA R: TGAAACCACAACCCATTTGCTC
OT-8	AACTCCCTCTGAGACAGTGTGAG	0.3	chr9:+29458518	F: ACAGCTAATGTCGACGAGCAA R: CTCTGGTTTAGGGTCTGGGAC
OT-9	GCCTTCTTTTCATATAGTGTGAG	0.2	chr22:-21148113	F: TAGGGAGGGTCGGATTTTGT R: TGGCACAGTATTTGCATACCAC
OT-10	TCCTCCGTTTGAGATACTGCTGG	0.2	chr25:+31028137	F: CACTTACCGCAGTACAGGACC R: CTACAGGCGTCAGACGAAAC
OT-11	TCCTGCGTTTGAGATTGTGTTGG	0.2	chr23:+1106681	F: ACACTTGTAAAACATGTGCTTTGG R: CCCGTTGTCTCTAGTGAGGC
OT-12	GACTGCTTTTGAGATTGTGTGGG	0.2	chr21:-40288792	F: GGAATCGCACACATCGCTTG R: CCTAGACGTCAACCCTGAACG
OT-13	GACTGCTTTTGAGATTGTGTGGG	0.2	chr21:+40432057	F: GGAATCGCACACATCGCTTG R: CCTAGACGTCAACCCTGAACG
OT-14	GCCTCGCTGTGAGATTGTGTTGG	0.2	chr25:-10380881	F: TTGTATGGCTGCGTCACGTC R: ACTACAGGGTGAAACTGGTGA
OT-15	GCCTCCCATAGAGAGAGTGGGAG	0.2	chr7:-43323190	F: CAGGCTGGTCATGTGTCTGT R: CGTCAGTGACACAGCCTTCA
OT-16	CCTTCCCTTTGAGAAAGTATAAG	0.1	chr24:-41555505	F: ATCACACTTGTGGGCTAGGTT R: TCTTGTGGTCTAATGGCTTTTGAG
OT-17	TCCTCCCCATGAGTTAGTGTTAG	0.1	chr6:-51086496	F: ATTGATTTCCAGCAGGGCGA R: ACTCTGCATATGCCTTCAGCA
OT-18	GCCTACCTTTTAAATAATGTGAG	0.1	chr22:-19504784	F: TGTTACAAAAGGCTGCACTGTC R: GCACCTCTGATCCAGGCCAAT
OT-19	GCCTCCACTTGAGACAATGTGGG	0.1	chr24:-4541397	F: GAGTGCACTTGACAGGCTGG R: GACGAGAAGGCATCCTGACT
OT-20	GCCTCTCTTTGAGTTAATGTCAG	0.1	chr17:-28651892	F: GTGTCCTGAGGAACCAGATGA R: CAGATACCCTTGCAGGTCGT

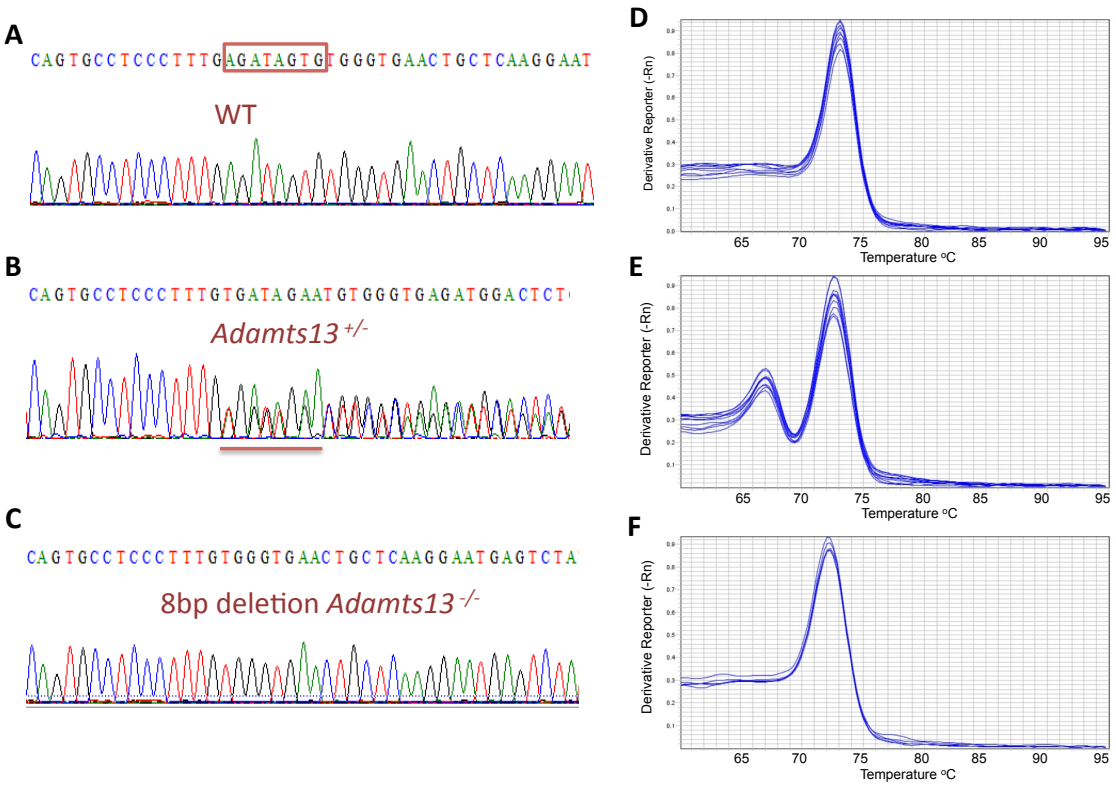
Suppl. **Table 2. Synthetic peptides used to generate zebrafish specific anti-ADAMTS13 and anti-VWF IgG.**

Peptides used for adamts13 antibody		
Region	Sequence	Position
N	VGPDVYEVHRQDTE	10 - 23
N	YSFFRAGKAECVQD	192 - 205
N	PAISYSSGDSQCKL	389 - 402
M	RKYGQEYGDLTNPN	576 - 589
M	KSGKVKGQLSAPLT	686 - 699
M	VYVWSPRTGECSKT	811 - 824
C	ERDVEEEKCESETK	966 - 979
C	CERMTGKMVKSRTN	1168 - 1181
C	GEYQQIKGMFRAEY	1305 - 1318
Peptides used for VWF antibody generation		
A1	CTLGLDPEPQKKPPTAKPSTTS	1431-1452
D4	CVDDEGNERKPGET	1880-1893

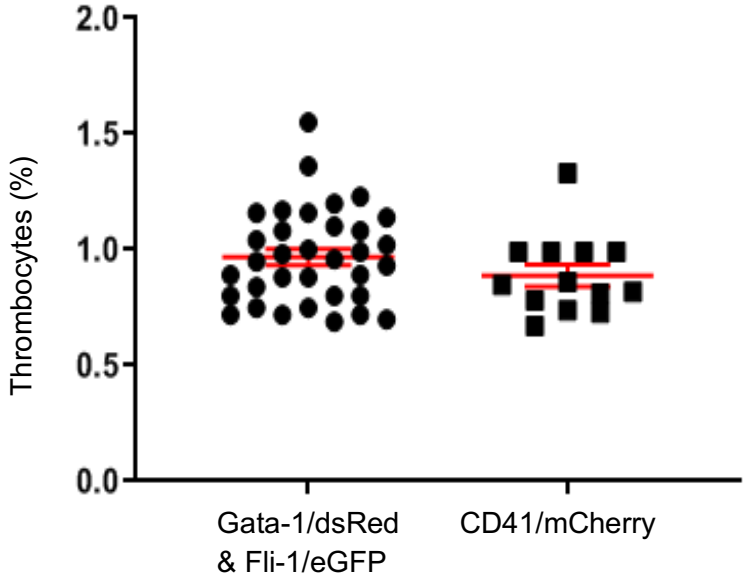
N, M, and C indicate the amino terminal, middle portion, and carboxyl terminal domains of zebrafish adamts13; A1 and D4 indicate the peptides derived from A1 and D4 domains of zebrafish vwf.



Suppl. **Fig. 1. Verification of mutations in *a13* gene and off-target effects in F0, F1 and F2 zebrafish. A**. T7E1 digestion indicates the presence of mutations, deletions, or insertions in the target region in 19 of 22 zebrafish embryos; **B**. Sanger sequencing demonstrates *wt* allele (top) and 14 mutated sequences from 33 F1 founders. The number of nucleotides inserted (+) or deleted (-) is enumerated in parentheses and the number of zebrafish that possessed the same mutation is denoted in the bracket. Arrow (→) indicates the chosen mutant predicted to introduce a premature stop codon and used to produce F2 and F3 progenies; **C**. Detection of the off-targets in genetically modified zebrafish. All predicted off-target regions were amplified with specific primer pairs listed in Table 1 in the DNA isolated from F0, F1, and F2 founder zebrafish embryos and digested with T7 endonuclease I. All off-targeted sites are located in the introns.



Suppl. **Fig. 2. Determination of *a13* genotypes by real-time PCR plus a melting curve analysis**. **A**. Sequence in the target region of *wt adamts13* gene; box indicates the 8 nucleotides that were deleted in *a13*$^{-/-}$ zebrafish; **B**. Heterozygous mutations produce double-peaks in the sequencing chromatograms (underlined). **C**. Sequencing confirmed that 8 nucleotides were deleted in *a13*$^{-/-}$ zebrafish. **D-F.** The genotyping method based on the melt curve analysis of amplified PCR products (54 bp) that surround the 8 bp deleted region using SYBR™ Green PCR Master Mix (Applied Biosystems) and primers (Forwarding: atatttttcagtgcctccct and Reversing: tcattccttgagcagttcac). Here, *wt* (**D**) and *a13*$^{-/-}$ (**F**) samples have specific melt curves, which can be differentiated by Tm. The *a13*$^{+/-}$ samples (**E**) exhibit 2 melt curves as the heteroduplexes are formed following PCR, which are melt at lower temperature.



Suppl. **Fig. 3. Quantitation of thrombocytes using different transgenic markers in zebrafish.** The percentage of thrombocytes were determined by flow cytometry based on expression of eGFP in *Gata-1*/dsRed & *Fli-1*-eGFP and mCherry in *cd41*/mCherry transgenic zebrafish, respectively. The data are shown as individual values (dots) and mean ± SEM.

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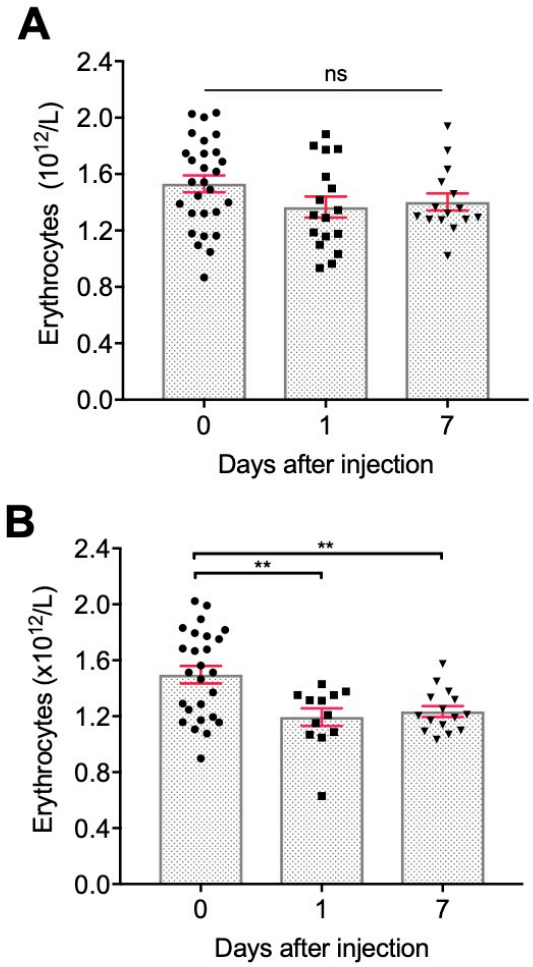
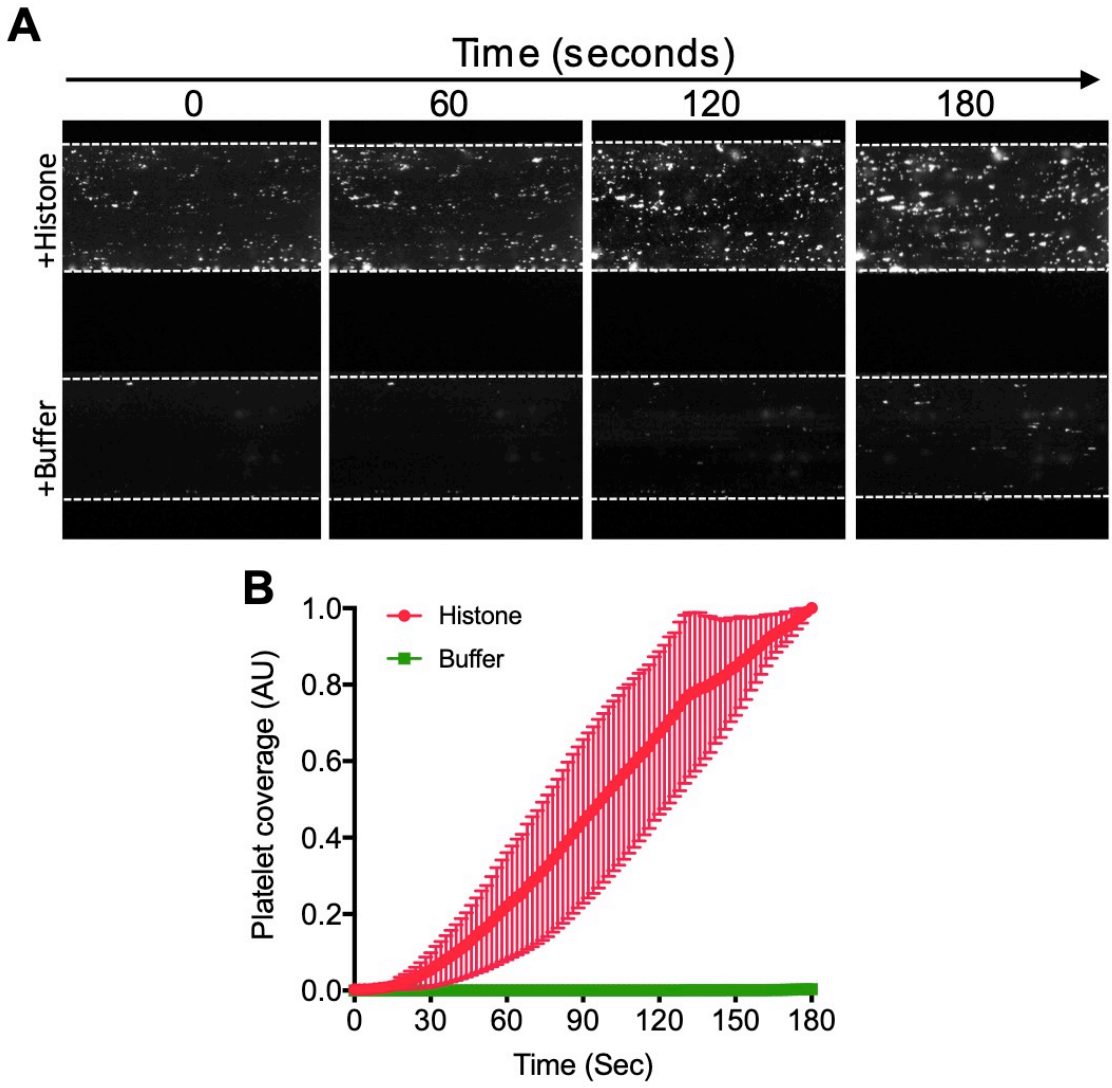


Suppl. **Fig. 4. Histone challenge results in reduction in the number of erythrocytes in *a13*$^{-/-}$, but not in *wt* zebrafish**. The number of erythrocyte (red) in *wt* (**A**) and *a13*$^{-/-}$ (**B**) zebrafish was determined prior to, 1, and 7 days following a lysine-rich histone challenge. The data shown are the individuals (dots) and mean ± SEM. Kruskal-Wallis analysis determined the statistical significance among three groups. Here, ns and ** denote *p* values > 0.05 and <0.01, respectively.



Suppl. **Fig. 5. Lysine-rich histone stimulates rapid release of VWF from cultured endothelial cells.** Murine endothelial cells were cultured in a microfluidic channel and stimulated with a lysine-rich histone (10 μg/mL) or DMEM for 10 min. A PPACK-anticoagulated whole blood from *a13*$^{-/-}$ mice was perfused over a histone-activated and non-activated endothelial surface over 180 seconds. The final coverage of fluorescein-labeled platelets (anti-CD41) (**A**) and the rate of platelet accumulation over time (**B**) were determined using an inverted fluorescent microscope. The results in B are the mean ± SEM from three independent experiments (N=3).