LIM-only protein FHL2 attenuates vascular tissue factor activity, inhibits thrombus formation in mice and *FHL2* genetic variation associates with human venous thrombosis

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Received: July 25, 2018.

Accepted: August 26, 2019.

Pre-published: August 29, 2019.

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Supplementary Appendix

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This document includes supplementary methods and three supplementary figures and their legends.

Supplementary methods

In vivo thrombosis in mesenteric veins

Five-week old male FHL2-KO mice and respective littermate wild-type (WT) mice (C57BL/6; n=8 per group) were anesthetized by isoflurane (2% for induction, 1.6-1.8% to maintain anaesthesia during imaging) and thrombus formation in the mesenteric veins was provoked as described previously.(1) Briefly, DyLight488-labeled anti-GPIbβ antibody (Emfret Analytics) was injected intravenously (i.v., 0.5 µg/g body weight) for fluorescent labelling of circulating platelets. In addition, Alexa Fluor 546-labeled fibrinogen (Invitrogen, per mouse 25 µl of 1.5 mg/ml) was administered i.v. to visualize fibrin formation. A segment of the ileum was exteriorized through a right side abdominal incision. The mesentery was spread out on a polydimethylsiloxane (PDMS) pad and fixed with Vaseline and minutien pins to prevent excessive peristaltic motion. A standardized vascular injury was induced by placement of a filter paper soaked with 5% $FeCl_3$ for 5 minutes. Platelet deposition, thrombus formation and fibrin formation in mesenteric venules were monitored by two-photon laser scanning microscopy. Imaging was performed on a Leica SP8-MP equipped with a Coherent Insight Deepsee laser tuned to 770nm excitation wavelength and a 25x/NA0.95 Fluotar VISIR objective. Time to occlusion was defined as the arrest of blood flow for at least one minute. When occlusion had not occurred within 30 minutes, the experiment was terminated. Animal handling was in accordance with national and European animal experimental protocols.

Quantification of thrombus size in mesenteric vessels

To quantify thrombus size after specific time intervals, fluorescence images were processed using Fiji software (Laboratory for Optical and Computational Instrumentation, University of Wisconsin-Madison WI, USA). Within the image of a mesenteric venule, two similar ROIs were defined, representing the thrombus area and an adjacent area representing the background. Subsequently, a threshold level was set by eliminating all pixels with intensity lower than 99.0% of the pixels of the background ROI. Intensities (gray levels) of all pixels in the thrombus ROI were then integrated. No image processing was applied.

Real-time PCR, Western Blot, Protein stability and Co-immunoprecipitation Assays

Real-time PCR, Western Blot, protein stability and co-immunoprecipitation assays were performed as described previously.(2, 3) Primers used in this study: TF forw: 5′- CTGCTCGGCTGGGTCTTC -3′, TF rev: AATTATATGCTGCCACAGTATTTGTAGTG-3′. Antibodies applied in this study were anti-HA (12CA5; Roche Applied Science), anti-TF (American Diagnostica), anti-β-actin (Sigma), and anti-tubulin (Cedarlane laboratories).

FHL2 Knockdown

Recombinant lentiviral particles encoding FHL2 and short hairpin RNAs (shRNAs) targeting FHL2 were produced, concentrated, and titrated as described previously. (24)

Western blot analysis

Western blot was performed as described previously.(2) Antibodies applied in western blot analysis were HA antibody (12CA5; Roche Applied Science), TF (American Diagnostica), and FHL2 (Abcam).

Immunofluorescence

Immunofluorescence was performed as described previously.(4) Cells were seeded on cover slips and serum-starved for 4 h. After starvation, cells were stimulated with TNFα (50 ng/ml) for 6 h. Cells were fixed with 4 % (w/v) Formal-Fix (Thermo Scientific), washed and incubated with FHL2

antibody (Abcam). Following repeated washing steps with PBS, protein localization was visualized by secondary antibodies coupled to fluorescent dyes Alexa Fluor-568 or −488 (Molecular Probes). Nuclei were counterstained with DAPI.

TF activity assay

TF activity in HUVECs and SMCs was assayed as previously described.(5) Briefly, the cells were seeded in 24-well plates and were washed with HBS before incubation with 1 nM human Factor VIIa and 100 nM human Factor X (FX) at 37^{0}C. After 10, 15 and 30 min of incubation, samples containing FXa were quenched in 50 mM Tris solution (pH 7.4) containing 100 mM EDTA and FXa activity was measured in a spectrophotometer using the chromogenic substrate Spectrozyme FXa (Bioconnect Diagnostics).

Luciferase Assays

Luciferase assays were performed as described previously using TF-promoter luciferase reporter plasmids and full-length FHL2 or FHL2-variants.(2, 3) Briefly, HEK293T cells were transiently transfected with human TF-promoter luciferase reporter plasmids (bp -227 to bp +121 bp) (Addgene plasmid # 15442) or TF-promoter luciferase reporter plasmids in which either the AP-1 (Addgene plasmid # 15443) or the NFκB (Addgene plasmid # 15444) response element was mutated together with plasmids encoding full-length FHL2 or FHL2-variants(2, 6).

SNP association in venous thromboembolism (VTE) patients

To study the association between FHL2 and VTE, a two-step validation study was designed, consisting of a discovery and replication phase. For the discovery phase, 18 FHL2 SNPs (listed in Table 1) were tested. In collaboration with the International Network against VENous Thrombosis (INVENT) consortium, the association between these candidate SNPs and venous thrombosis was assessed. Details about the design of the INVENT GWAS have been previously published (7). In short, the INVENT GWAS consists of 7,507 VTE case subjects and 52,632 control subjects from European-ancestry adults. In all 12 studies, VTE (pulmonary embolism or deep vein thrombosis) was objectively diagnosed and VTE events related to cancer, autoimmune disorders, or natural anticoagulant inhibitor deficiencies (protein C, protein S, antithrombin) were excluded in most studies. Genotyping arrays differed between studies. All participating studies were approved by their institutional review board and informed consent was obtained from studied individuals (7). From the 19 requested SNPs, 18 SNPs were available in the INVENT resources. The other (FHL2_rs137869171) didn't pass the QC for entering the meta-analysis (either MAF<0.005 or poor imputation quality). For the remaining18 SNPs we obtained summary statistics. To obtain replication evidence, SNP rs4851770 ($P < 0.000201$) was selected for genotyping in The Thrombophilia, Hypercoagulability and Environmental Risks in Venous Thromboembolism (THE-VTE) case-control study including 676 patients with a first objectively diagnosed episode of deep venous thrombosis aged 18-70 years and 368 control subjects. This two-center, case–control study has been previously described (8). Testing was performed in a blinded fashion with TaqMan (Applied Biosystems).

References

1. Kuijpers MJ, de Witt S, Nergiz-Unal R, van Kruchten R, Korporaal SJ, Verhamme P, et al. Supporting roles of platelet thrombospondin-1 and CD36 in thrombus formation on collagen. Arteriosclerosis, thrombosis, and vascular biology. 2014 Jun;34(6):1187-92.
2. Kurakula K, Sommer D, Sokolovic M, Moerland PD, Scheij S, van Loenen PB, et al. LIM-only protein FHL2 is a positive regulator of liver X receptors in smooth muscle cells involved in lipid homeostasis. Mol Cell Biol. 2015 1/2015;35(1):52-62.
3. Kurakula K, van der Wal E, Geerts D, van Tiel CM, de Vries CJ. FHL2 protein is a novel co-repressor of nuclear receptor Nur77. J Biol Chem. 2011 12/30/2011;286(52):44336-43.

4. Kurakula K, Hamers AA, van Loenen P, de Vries CJ. 6-Mercaptopurine reduces cytokine and Muc5ac expression involving inhibition of NFkappaB activation in airway epithelial cells. Respiratory research. 2015 Jun 19;16:73.
5. van den Hengel LG, Osanto S, Reitsma PH, Versteeg HH. Murine tissue factor coagulant activity is critically dependent on the presence of an intact allosteric disulfide. Haematologica. 2013 1/2013;98(1):153-8.
6. Mackman N, Fowler BJ, Edgington TS, Morrissey JH. Functional analysis of the human tissue factor promoter and induction by serum. Proceedings of the National Academy of Sciences of the United States of America. 1990 Mar;87(6):2254-8.
7. Germain M, Chasman DI, de Haan H, Tang W, Lindstrom S, Weng LC, et al. Meta-analysis of 65,734 individuals identifies TSPAN15 and SLC44A2 as two susceptibility loci for venous thromboembolism. American journal of human genetics. 2015 Apr 2;96(4):532-42.
8. van Hylckama Vlieg A, Baglin CA, Luddington R, MacDonald S, Rosendaal FR, Baglin TP. The risk of a first and a recurrent venous thrombosis associated with an elevated D-dimer level and an elevated thrombin potential: results of the THE-VTE study. Journal of thrombosis and haemostasis : JTH. 2015 Sep;13(9):1642-52.

Supplemental Figures

Supplemental Figure S1: Quantification of thrombus size

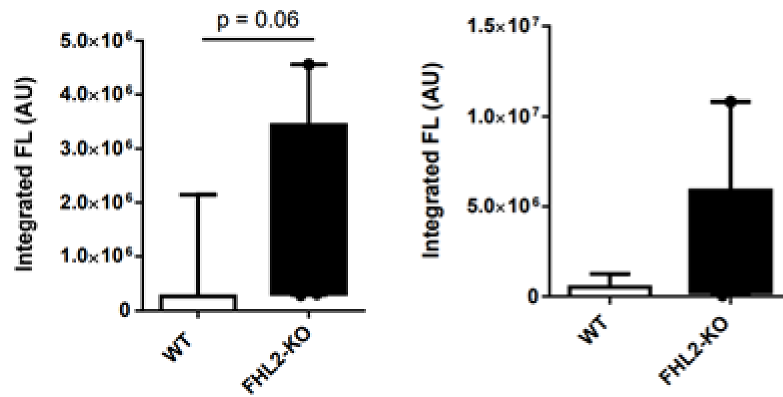

Figure S1: Quantification of thrombus size from platelets labelled with DyLight488-labeled anti-GPIbβ antibody (left panel) and based on fibrin formed from Alexa Fluor 546-labeled fibrinogen. Data are expressed as integrated fluorescence intensities for multiple vessels after 15 min (box- and whiskerplots, n = 6–9 veins).

Supplemental Figure S2: TF and FHL2 expression levels in HUVECs and SMCs

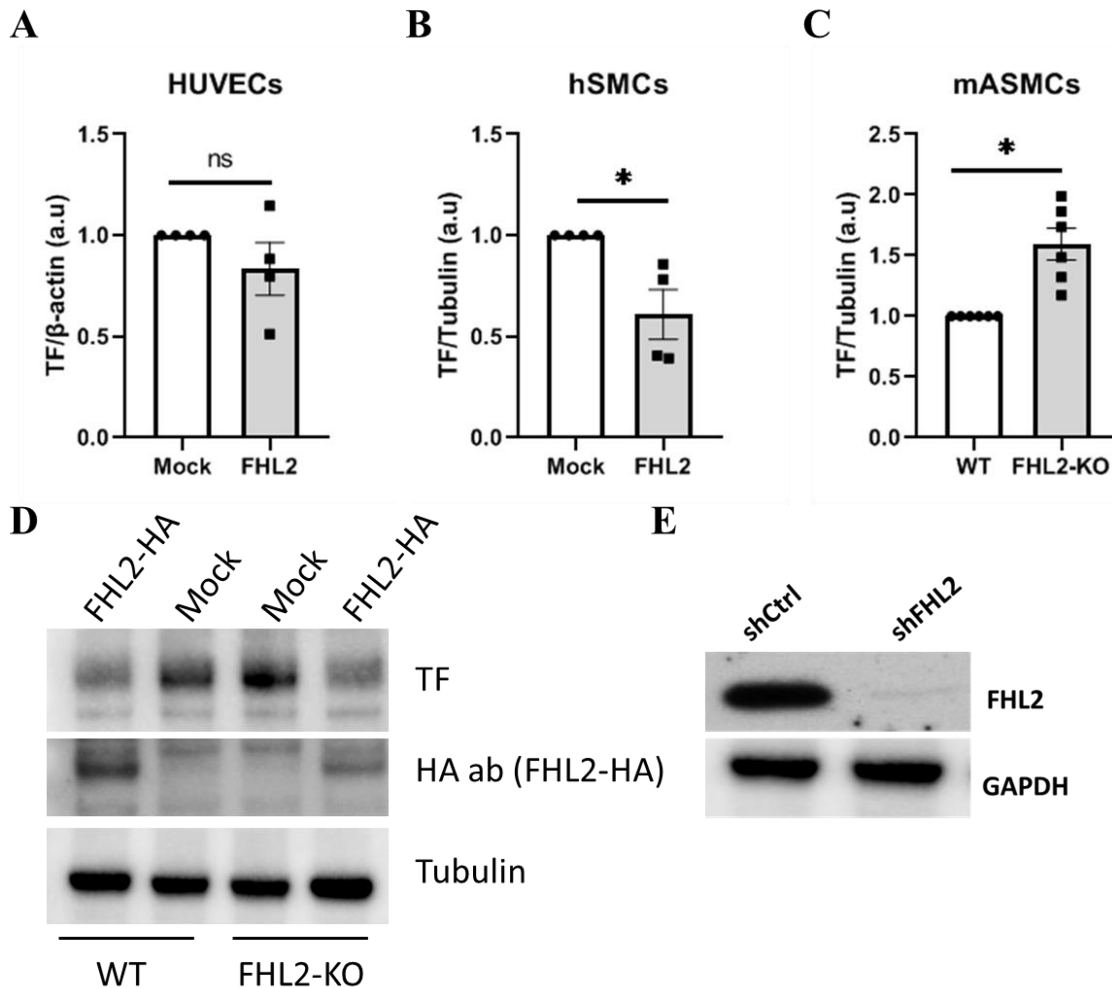

Figure S2. A-B) Relative densitometric analyses of TF protein in western blot in HUVECs (A) and human SMCs (hSMCs) (B) following lentiviral transduction with Mock or HA-FHL2 was performed. β-actin for HUVECs and Tubulin for hSMCs served as loading controls. C) Relative densitometric analyses of TF protein in western blot in WT and FHL2-KO SMCs. Tubulin served as a loading control. D) Western blot analysis in WT and FHL2-KO SMCs following lentiviral transduction with Mock or HA-FHL2 was performed to determine the levels of overexpressed FHL2 and TF using antibodies directed against TF or HA-epitope-tag. Tubulin served as a loading control. E) Western blot analysis of WT SMCs following knock-down of FHL2 using shFHL2 lentivirus was performed to determine the levels of FHL2. GAPDH served as a loading control.

Supplemental Figure S3: FHL2 deficiency induces TF expression carotid artery after carotid artery ligation, aortas and also in bone-marrow derived macrophages

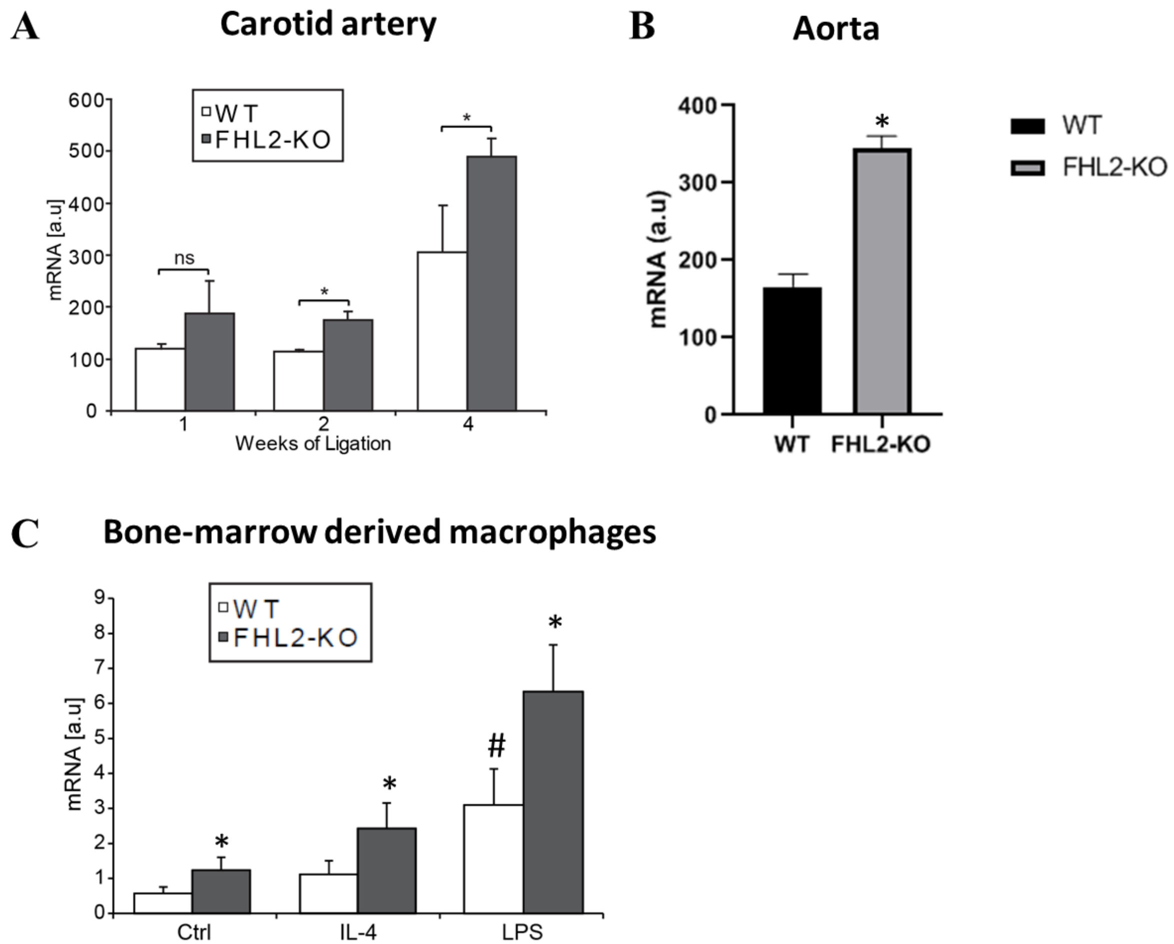

Figure S3. A) qRT-PCR was performed to assess mRNA expression of TF in the carotid arteries from WT and FHL2-KO mice after 1, 2 and 4 weeks of ligation. Data are means±SD. *P<0.05 for FHL2-KO versus WT mice. n=7 for 1 and 2 weeks and n=14 for 4 weeks. B) qRT-PCR was performed to assess mRNA expression of TF in the uncultured intact aortas from WT and FHL2-KO mice. Data are means±SEM. *P<0.05 for FHL2-KO versus WT mice (n=3/group). C) Bone-marrow derived macrophages from FHL2-KO mice showed increased mRNA levels of TF under basal or LPS (100 ng/mL) and IL-4 (50 ng/ml) for 6h stimulated conditions than macrophages derived from WT mice. *WT vs FHL2-KO; #WT ctrl vs WT LPS.

Supplemental Figure S4: Cellular localization of FHL2 in endothelial cells

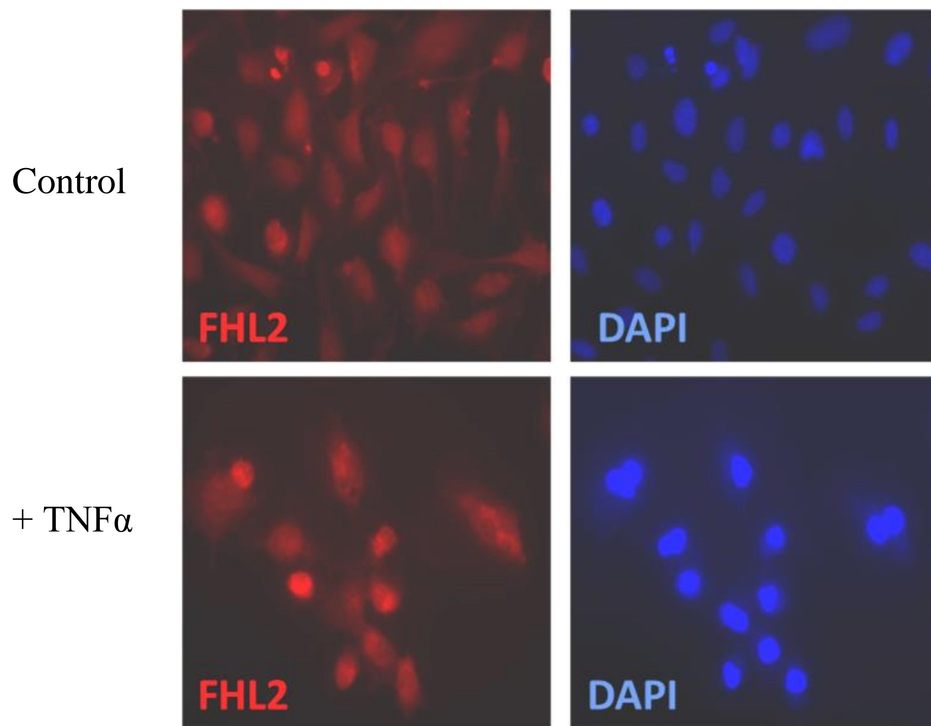

Figure S4: Cultured HUVECs were fixed and stained for FHL2 expression by immunofluorescence using FHL2 Ab. TNFα (50ng/ml) was used to stimulate the cells for 6h. DAPI was applied to counterstain the nuclei.

Supplemental Figure S5: FHL2 deficiency increases TF expression and activity in human microvascular endothelial cells (HMECs).

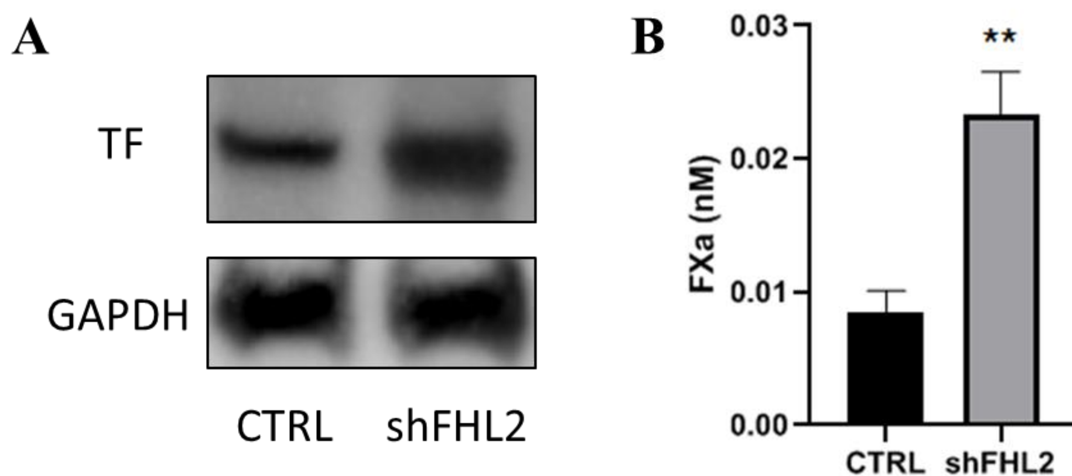

Figure S5: A) Western blot analysis in HMECs following FHL2 knock-down and TNFα stimulation for 3h was performed to determine the protein levels of TF using antibody directed against TF. GAPDH served as a loading control. B) FXa generation was measured in HMECs following FHL2 knock-down and TNFα stimulation for 3h.