SUPPLEMENTARY APPENDIX

Murine tissue factor disulfide mutation causes a bleeding phenotype with sex specific organ pathology and lethality

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Supplemental material: Methods

Construction of the C213G TF targeting vector

The vector generated for murine TF (mTF) targeting is a replacement-type vector containing two long arms of homology. The coding sequence of the murine full length TF isoform containing a T721G mutation in its open reading frame was inserted into KpnI/NotI restriction sites of a LNTK vector (PolyGene AG, Ruemlang, Switzerland) located 5' to a neomycin resistance gene, flanked by two loxP sites (floxed neo). A 3 kb genomic sequence 5' upstream of the first exon of the TF gene, and a 5.2 kb Bsp120I/NotI restriction fragment located 3' downstream of the last exon of the TF gene, were used as the left and the right arms of homology, respectively. A diphtheria toxin alpha chain minigene (dtα) under the control of the DNA polymerase II promoter served as a negative selection marker; dtα was inserted into the targeting vector on its 3'-end as a 1.2 kb Bsp120I/NotI restriction fragment. The targeting vector was linearized on the NotI restriction site located on its 3'-end prior to electroporation. Embryonic stem cell transfection (129P2/OlaHsd), screening, neomycin excision as well as blastocyst injection was conducted by PolyGene AG (Ruemlang, Switzerland). Chimeric mice were bred with C57BL/6J mice.

Animals

One mouse colony was maintained by breeding of heterozygous animals on a mixed 129P2/OlaHsd-C57BL/6J background with a mean of 50% from each background strain. In parallel, mice were backcrossed to C57BL/6J (B6) by a speed congenic approach: from each generation the two offspring with the highest percentage of B6 background was chosen for further backcrossing. The genetic background was analyzed screening 96 polymorphic microsatellite markers (Elchrom Scientific AG,

Cham, Switzerland). After 5 backcrosses 3 mice had reached 100% B6 background and founded a B6 colony, which was maintained by breeding of heterozygous animals. After the discovery of a strain phenotype with increased lethality, the animals were checked daily and euthanized, when they developed pronounced affliction (apathy, dyspnea, neurological symptoms). Dead and euthanized strained animals were both included in the survival analysis with their age at death or euthanasia. Two cohorts of mice derived from the B6-colony were used for systemic phenotyping including hematological analyses and blood pressure measurement. Mice were maintained in individually ventilated cages with water and standard mouse chow (Altromin no. 1314) according to the institutional housing conditions and local laws. All tests performed were approved by the responsible authority of the local government.

Embryonic analysis

For embryonic analyses heterozygous C213G/+ TF mice were bred and females were controlled for plugs every morning. The day of the plug was considered to be E0.5.

At E9.5, uteri were fixed with 4% formalin in PBS for at least 4 days. Using a razor blade, individual implantation sites were separated and each implantation site was halved transversely to the uterus. A piece of embryonic or yolk sac tissue was taken from one half and washed with PBS before genotyping, the other half was put back to 4% formalin in PBS. After genotyping, the remaining half was embedded to paraffin and sectioned to do immunohistochemical staining of smooth muscle α-actin (Sigma, F3777).

At E14.5 uteri were dissected, a piece of yolk sac was taken from every implantation site for genotyping, while fetus and placenta were fixed in 4% formalin in PBS.

Pathological analysis

C213G/C213G TF (11 female and 10 male) and control (5 female and 5 male) mice were sacrificed according to approved protocols and all organs were examined macroscopically during necropsy. For microscopical analysis, 30 organs (see www.mouseclinic.de for more info) were immersion-fixed in 4% neutral buffered formalin and subsequently embedded in paraffin. Four-micrometer thick sections were either stained with hematoxylin and eosin (H&E) for general histological analysis, Picro-sirius Red for better visualization of collagen or Prussian Blue for iron deposits. Finally, slides were scanned using a Hamamatsu NanoZoomer 2.0HT digital scanner and analysed with NDP.view2 software (Hamamatsu Photonics, Japan).

Expression profiling

Total RNA was isolated from organs just before microarray hybridization. Organs (heart, lung and brain) were thawed in Trizol Reagent (Sigma), homogenized using a Polytron homogenizer (Heidolph) and total RNA was extracted from individual samples using RNeasy Midi kits (Qiagen) following the manufacturer's protocol. 2 µg RNA aliquots were run on a formaldehyde agarose gel to check for RNA integrity and concentrations were calculated from OD260/280 measurement. The RNA was stored at -80°C in RNase free water (Qiagen).

Four biological replicates for each genotype group were performed (four mutants and four controls). Therefore, 500 ng of total RNA was amplified and biotinylated in a single round using the Illumina TotalPrep RNA Amplification Kit (Ambion). 750 ng of albelled and amplified RNA was hybridized on Illumina MouseRef8 v2.0 Expression

Bead Chips containing about 25K probes. Staining and scanning (Illumina HiScan Array Reader) was done according to the Illumina expression protocol.

Bleeding time

After onset of anesthesia, tails were prewarmed in a 37°C water bath for 10 min. Then 0.5 mm of the tail tip was amputated and the tail was immediately put back to 37°C PBS. The time to cessation of bleeding was measured.

Blood Withdrawal and Storage

Blood samples were collected from isoflurane-anesthetized mice from the retro-bulbar sinus with non-heparinized glass capillaries (1.0 mm in diameter; Neo-lab; Munich, Germany). A proportion of 50µl was collected in an EDTA-coated end-to-end capillary and diluted 1:5 with CellPack buffer in prefilled capillary tubes (Sysmex, Art.No 99940020) and mixed thoroughly for hematological measurements. Samples were placed on a rotary agitator at room temperature for until analysis.

Hematology

Diluted samples were used to determine complete blood cell counts using a Sysmex XT2000iV device (Sysmex Deutschland GmbH, Norderstedt, Germany) by applying the capillary mode according to the instructions of the manufacturer as described previously[1]

Tail-cuff blood pressure measurement

Blood pressure was measured in non-anesthetized mice with a non-invasive tail-cuff method using the MC4000 Blood Pressure Analysis Systems (Hatteras Instruments Inc., Cary, North Carolina, USA). Four animals were restrained on a pre-warmed

metal platform in metal boxes. The tails were looped through a tail-cuff and fixed in a notch containing an optical path with a LED light and a photosensor. The blood pulse wave in the tail artery is detected as transformed into an optical pulse signal by measurement of light extinction. Pulse detection, cuff inflation and pressure evaluation are automated by the system software. After five initial inflation runs for habituation, 12 measurement runs are performed for each animal in one session. Runs with movement artifacts are excluded. After one day of training, in which the animals are habituated to the apparatus and protocol, the measurements are performed on four consecutive days between 8:30 and 11:30 AM.

TF expression analysis

RNA was extracted from heart, lung or brain using TRIzol Reagent (Molecular Research Center), according to the manufacturer's protocol. Five µg of total RNA were used to synthesize cDNA using reverse transcriptase (Stratagene, La Jolla, CA) and random hexamer primers. Transcript levels were quantified by Real Time PCR using SyBr Green Master Mix (Applied Biosystems) on an Applied Biosystems 7300 System using primers specific for flTF: 5' TCAAGCACGGGAAAGAAAAC (TF forward) and 5' CTGCTTCCTGGGCTATTTTG (TF reverse) and normalized for β-actin levels. Relative expression levels represent multiples of ten of $2^{(Ct[flTF]-Ct[\beta\text{-actin}])}$. Protein was extracted by manual grinding of organ tissue in lysis buffer containing 50 mM octylglucoside, 50 mM Tris, 150 NaCl, 10 µg/µl aprotinin and 10 µg/µl leupeptin pH 7.4. Lysates were precipitated with -20°C aceton overnight and resuspended in non-reducing sample buffer with careful sonication. Samples were separated by 10% SDS-PAGE and transferred to a PVDF membrane (Immobilion®-FL, Millipore). The membrane was stained with anti-mouse TF rabbit antiserum (R8084) [2] and anti-human GAPDH (cross-reactive to mouse) mouse monoclonal (MAB374, Millipore)

primary antibodies, followed by staining with goat anti-rabbit 800CW and donkey anti-mouse 680 LT (both LI-COR® Biosciences) secondary antibodies. Staining was visualized on an Odyssey imager and quantified using the corresponding application software (version 3.0; both LI-COR® Biosciences)

TF activity

At E9.5 uteri were dissected. Tissues were snap-frozen and yolk sacs were used for genotyping. Tissues were lysed in HEPES-saline containing 0.02% sodium azide (HBS) and diluted in HBS containing 1mg/ml BSA and 50 µM phospholipid vesicles (70% phosphatidylcholine (PC) and 30% phosphatidylserine (PS), Avanti polar lipids) to measure TF activity using a plasma clotting assay. Human and mouse citrated plasma was mixed 9:1 (50µl) and prewarmed together with the sample (50µl) or a reference (recombinant lipidated human TF, American Diagnostica). After addition of 25 mM $CaCl_2$ (50µl) clotting times were measured. Clotting times were normalized to a standard curve and the total protein concentration of the sample.

Color coded gene regulation profiles

Illumina Genomestudio 2011.1 software was used for background correction and normalization of the data (quantile algorithm). The remaining negative expression values were corrected by introducing an offset. The identification of significant gene regulation was performed using SAM (Significant Analysis of Microarrays) included in the TM4 software package. Genes were ranked according to their relative difference value d(i), a score assigned to each gene on the basis of changes in gene expression levels relative to the standard deviation. Genes with d(i) values greater than a threshold were selected as significantly differentially expressed in a one class analysis. The percentage of such genes identified by chance is the false discovery

rate (FDR). To estimate the FDR, nonsense genes were identified by calculation 1000 permutations of the measurements. The selection of the top differentially expressed genes with reproducible up- or down-regulation includes less than 10% false positives (FDR) in combination with fold change > 1.5.
Functional enrichment analyses were generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) and using GePS software (Genomatix, Germany)

Macrophage experiments

Isolation and freezing of bone marrow cells: Femura and tibiae were dissected and cleaned from soft tissue. After removing the epiphyses, bones were flushed with RPMI. Bone marrow was suspended by pipetting and cells were counted. After centrifugation at 200 g, cells were resuspended in 90% FCS, 10% DMSO and slowly frozen to -80 and then liquid nitrogen on the next day.
Bone marrow-derived macrophages (BMDM): BMDM were generated from total bone marrow cells cultured for 7 days in DMEM, 10% FCS, 20% L cell medium, 1 mM L-Glutamine, penicillin/streptomycin as described previously[3]. Cells were plated at 1 x 10^6 cells/well in a 12-well plate and cultured overnight in the presence of 100 ng/ml IFNγ (Peprotech). Macrophages were typically stimulated with 1 µg/ml LPS (S.abortus equi, Enzo Life Sciences) for 4 hours prior to functional characterization. Cells were rinsed once and stimulated in BSS buffer (0.13 M Na-gluconate, 0.02 M HEPES, 5 mM glucose, 5 mM glycine, 5 mM KCl, 1 mM MgCl2, pH 7.5) with 5 mM ATP (Roche) for 30'. Cell supernatants were cleared from debris in an Eppendorf centrifuge at 1000 rpm at 4ºC for 10' and MP were recovered by centrifugation at 16,000 g at 4ºC for 1 hour. Pellets were resuspended in 150 µl HBS (10 mM Hepes,

pH 7.4, 137 mM NaCl, 5.3 mM KCl, 1.5 mM CaCl2) for FXa generation or prothrombinase assays and in SDS-sample buffer for Western Blotting.

Functional assays: Cell surface TF activity was determined with 0.5 nM FVIIa (kindly provided by L. Petersen, Novo Nordisk) and 50 nM FX (Haematologic Technologies) in HBS by measuring a time course of FXa generation. TF MP activity was measured in HBS with 2 nM VIIa and 100 nM FX by determining a time course of FXa generation that was quantified after quenching in EDTA with the chromogenic substrate (Spectrozyme FXa, American Diagnostica). MP prothrombinase activity was measured in HBS with 10 nM FVa, 5 nM FXa and 500 nM prothrombin (Haematologic Technologies) at ambient temperature, and quantification of thrombin generation with the chromogenic substrate Spectrozyme TH (American Diagnostica).

Western blotting: We used the following antibodies for detection of proteins associated with MP by Western blotting: polyclonal rabbit anti-mouse TF (R8084) and polyclonal anti-integrin β1 [2], and anti-β-actin (Sigma-Aldrich). For detection of cell-associated TF, we prepared membrane fractions from cells using repeated Triton-X114 phase separation of cell lysed with 1% Triton-X114 (0.1 M Tris pH 8.5, 10 mM EDTA, 1 mM PMSF) and solubilized the acetone washed detergent pellets in SDS-sample buffer (Invitrogen).

RNA extraction and qPCR: RNA was isolated from macrophages using TRIzol Reagent (Life Technologies) and 1 µg RNA was transcribed into cDNA with SuperScript III First-Strand Synthesis System (Invitrogen). Transcript levels were quantified by Real Time PCR using SyBr Green Master Mix (Applied Biosystems) on an Applied Biosystems 7300 System and normalized for β-actin levels. We used an intron-spanning primer pair that was anchored in Exon 5 and only amplified flTF cDNA.

Statistical analyses

Data are indicated as mean ± SEM. Unpaired Student's t-test was performed for comparison of two groups, and two-way ANOVA was used to compare 4 groups of two genotypes and both sexes. For comparison of genotype distributions a X^2-test was applied. A p value <0.05 was considered as significant. Hematology data were analyzed using R-Scripts. Depending on the distribution of the respective data parametric or non-parametric statistical methods were used.

References

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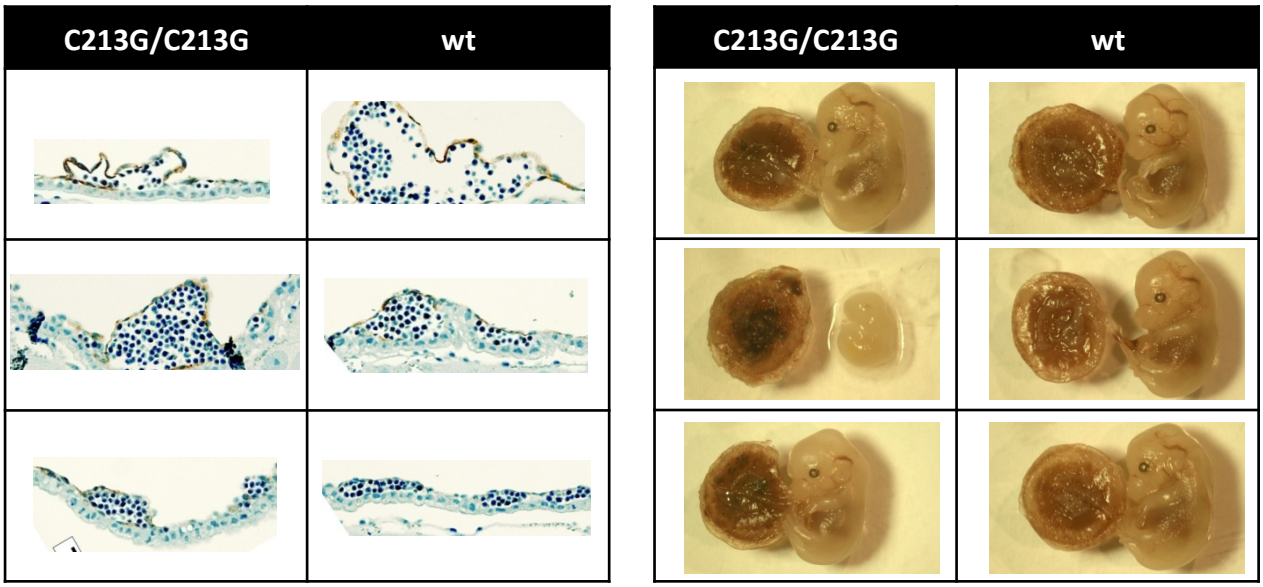
2. Furlan-Freguia C, Marchese P, Gruber A, Ruggeri ZM, Ruf W. P2X7 receptor signaling contributes to tissue factor-dependent thrombosis in mice. J Clin Invest. 2011;121(7):2932-2944.
3. Rothmeier AS, Marchese P, Petrich BG, et al. Caspase-1-mediated pathway promotes generation of thromboinflammatory microparticles. J Clin Invest. 2015;125(4):1471-1484.

Supplemental Table 1: data table of significantly regulated genes in heart as well as lung tissue from male and female homozygous C213G/C213G TF mice compared to wt TF mice. Data provided as an Excel file.

Supplemental Figure 1

A



E9.5 yolk sac histology showed similar erythrocyte filling and α-SMA expression in yolk sac vessels of homozygous C213G/C213G TF and wt embryos. At E14.5 dead homozygous C213G/C213G TF embryos were identified. Placentae of homozygous C213G/C213G TF embryos showed various degrees of blood pools. Placentas of 7 wt and 8 C213G/C213G TF mice were examined.

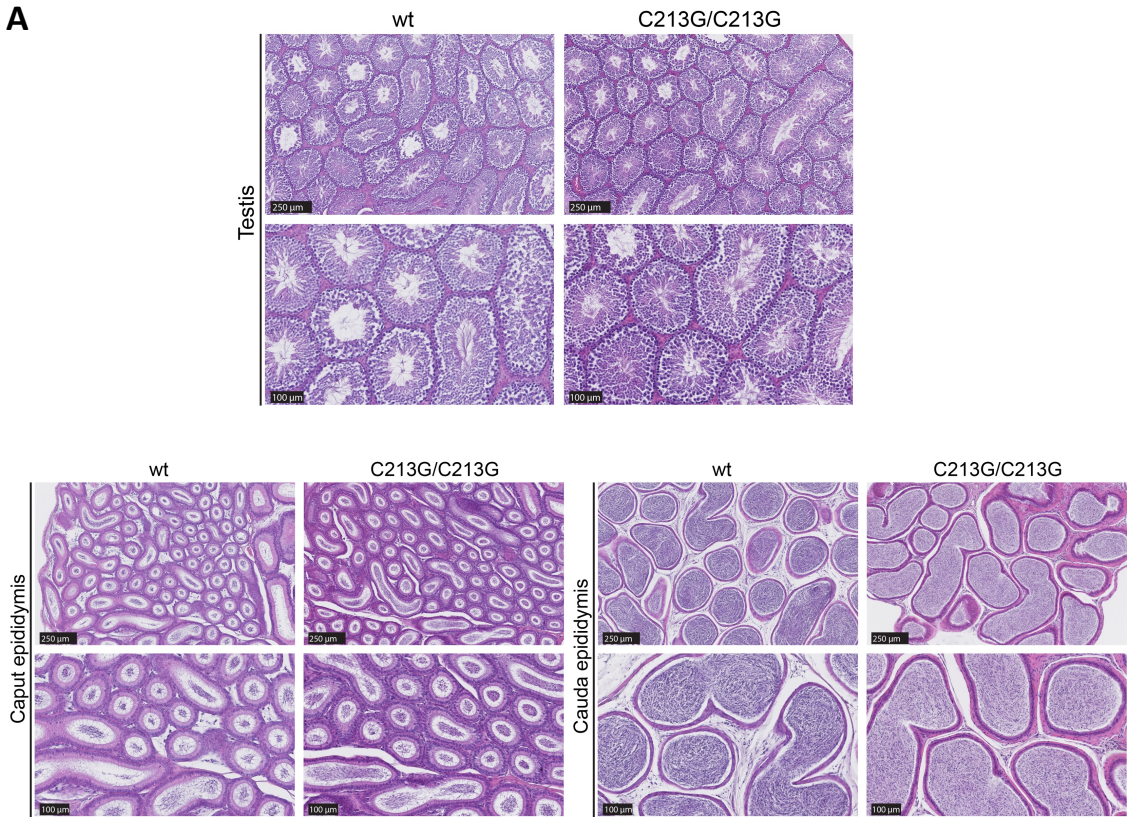
B

	wt		C213G/C213G	
	male	female	male	female
Hemorrhages in heart	0/5	0/5	5/10	7/11
Hemorrhages in lung	1/5	0/5	5/10	1/11
Hemorrhages in brain	0/5	0/5	2/10	2/11

Incidence of organ bleedings in male and female wt and homozygous C213G/C213G TF mice.

Supplemental Figure 2

A



Normal morphology of seminiferous tubules of the testes and sperm density in the epididymides of C213G/C213G TF mice. Representative pictures of hematoxylin and eosin (H&E)-stained sections of (upper panel) testis and (lower panel) epididymis (caput and cauda) from wt and homozygous C213G/C213G TF mice are displayed. 10x (picture above) and 20x (below) magnifications are shown.

B

fITF	**female**		**male**		**ANOVA**	**ANOVA**	**ANOVA**	**Post-Hoc**	**Post-Hoc**
	control	mutant	control	mutant	genotype	sex	genotype : sex	female	male
	n=10	n=10	n=10	n=9					
	mean ± sd	mean ± sd	mean ± sd	mean ± sd	p-value	p-value	p-value	p-value	p-value
Diastolic blood pressure [mm Hg]	125.77 ± 9	116.86 ± 11.5	120.25 ± 15.4	121.95 ± 11.4	0.358	0.955	0.179	0.142	1
Mean arterial pressure [mm Hg]	129.33 ± 8.6	121.3 ± 11.1	124.3 ± 15.2	126.41 ± 11.6	0.442	0.991	0.191	0.176	1
Pulse [bpm]	607.16 ± 33.7	594.18 ± 49.3	596.11 ± 70.8	607.6 ± 34.7	0.963	0.941	0.449	1	1
Systolic blood pressure [mm Hg]	137.38 ± 7.7	131.11 ± 10.7	133.38 ± 15	136.29 ± 12.3	0.657	0.876	0.23	0.304	1

Blood pressure and heart rate. Blood pressure and heart rate are unchanged in homozygous C213G/C213G TF as compared to wt TF mice, irrespective of gender.