TMEM16F-Mediated Platelet Membrane Phospholipid Scrambling Is Critical for Hemostasis and Thrombosis but not Thromboinflammation in Mice—Brief Report

Ayesha A. Baig,* Elizabeth J. Haining,* Eva Geuss, Sarah Beck, Frauke Swierenga, Podchanart Wanitchakool, Michael K. Schuhmann, David Stegner, Karl Kunzelmann, Christoph Kleinschnitz, Johan W.M. Heemskerk, Attila Braun, Bernhard Nieswandt

Objective—It is known that both platelets and coagulation strongly influence infarct progression after ischemic stroke, but the mechanisms and their interplay are unknown. Our aim was to assess the contribution of the procoagulant platelet surface, and thus platelet-driven thrombin generation, to the progression of thromboinflammation in the ischemic brain.

Approach and Results—We present the characterization of a novel platelet and megakaryocyte-specific TMEM16F (anoctamin 6) knockout mouse. Reflecting Scott syndrome, platelets from the knockout mouse had a significant reduction in procoagulant characteristics that altered thrombin and fibrin generation kinetics. In addition, knockout mice showed significant defects in hemostasis and arterial thrombus formation. However, infarct volumes in a model of ischemic stroke were comparable with wild-type mice.

Conclusions—Platelet TMEM16F activity contributes significantly to hemostasis and thrombosis but not cerebral thromboinflammation. These results highlight another key difference between the roles of platelets and coagulation in these processes. (Arterioscler Thromb Vasc Biol. 2016;36:2152-2157. DOI: 10.1161/ATVBAHA.116.307727.)

Key Words: blood platelets • fibrin • stroke • thrombin • thrombosis

During the process of thrombus formation, platelets interact with the coagulation system, providing a so-called procoagulant surface that facilitates thrombin generation. The major component of this procoagulant platelet surface is negatively charged aminophospholipids, which are exposed after platelet activation and sustained Ca2+ signaling. These lipids serve as anchoring sites for vitamin K-dependent coagulation factors, thus supporting the assembly of the tenase and prothrombinase complexes, which jointly enhance the rate of thrombin generation by 5 to 10 orders of magnitude.1 In addition to aminophospholipid exposure, procoagulant platelets also dramatically increase their size through a regulated process termed membrane ballooning2 or swelling,3 which increases the membrane surface area for coagulation factor binding. The importance of this procoagulant surface for the crosstalk between platelet activation and coagulation is beginning to be more fully understood through the analysis of patients who have the rare bleeding disorder Scott syndrome.1,4 This disorder, characterized by incidences of provoked bleeding and a decrease in prothrombin consumption measurements, has been attributed to mutations in the gene encoding the Ca2+-activated channel and scramblase TMEM16F (anoctamin 6).5,6 The primary defects in the platelets from the 3 known Scott syndrome patients are significant decreases in Ca2+-induced phosphatidylserine exposure, as well as microparticle formation, and diminished platelet membrane ballooning after activation highlighting TMEM16F as a potential key regulator of the procoagulant platelet surface.7

It is known that both platelets and coagulation strongly influence infarct progression after ischemic stroke.8–10 Our aim was to assess the contribution of the procoagulant platelet surface, and thus platelet-driven thrombin generation, to the progression of thromboinflammation in the ischemic brain using a newly generated TMEM16F knockout mouse model.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

TMEM16F expression in platelets and megakaryocytes was abolished by targeted deletion of exon 3, which affected...
all splice variants of the \( \text{Ano6} \) gene.\textsuperscript{11,12} Reverse transcription polymerase chain reaction using platelet mRNA from TMEM16F knockout mice (\( \text{Ano6}\text{fl/fl} \), conditional knockout [cKO]) showed a complete absence of the exon 3 coded region at the mRNA level (Figure 1Ai). This strategy resulted in abolished protein expression as demonstrated by the Western blotting of wild-type (WT) and cKO platelet lysates (Figure 1Aii). Platelet count and size were not significantly different between cKO and WT mice and no alterations, either in the surface expression of the major platelet glycoproteins or in coagulation parameters prothrombin time and activated partial thromboplastin time were observed (Tables I and II in the online-only Data Supplement). Also, cKO platelets were not significantly different from WT in terms of integrin activation and P-selectin exposure after stimulation with agonists that activate the major platelet signaling pathways (Figure IIA and IIB in the online-only Data Supplement) and also showed no activation defects in aggregometry studies (Figure IIC in the online-only Data Supplement).

However, after stimulation with a combination of collagen-related peptide and thrombin (to costimulate both GPCR [G-protein–coupled receptor] and ITAM [immunoreceptor tyrosine-based activation motif]-coupled signaling pathways) or the calcium ionophore, ionomycin, significantly less cKO platelets exposed phosphatidylserine than WT (as measured by Annexin A5 binding in flow cytometry). The cKO platelets that did expose phosphatidylserine bound less Annexin A5, which resulted in the appearance of an intermediate population described as low Annexin A5 binding (Figure 1D and 1E; Figure III in the online-only Data Supplement). Importantly, the differences observed in phosphatidylserine exposure were not because of differential activation of platelets as WT and cKO platelets showed comparable degranulation when costimulated with collagen-related peptide/thrombin or ionomycin (Figure III in the online-only Data Supplement). Platelet membrane ballooning (observed as an increase in light transmission after ionomycin stimulation of nonaggregating platelets or within platelet aggregates formed on a collagen and tissue factor microspot under flow) was also severely impaired in cKO platelets (Figure 1F and 1G). These in vitro and ex vivo data confirmed that the generated TMEM16F cKO mice had reduced platelet procoagulant potential. This is in line with observations of platelets from patients with Scott syndrome and other recently published TMEM16F knockout mouse models.\textsuperscript{11,13,14}

Given the reductions in procoagulant transformation, we assessed the ability of cKO platelets to promote thrombin generation. Measurement of thrombin generation over time using WT and cKO platelet-rich plasma revealed that the in vitro defects observed in cKO platelets led to significantly decelerated, but not abolished, thrombin generation. In all conditions tested, the time to initiation of thrombin generation (lagtime) and the time taken to reach peak thrombin concentrations were significantly delayed in cKO samples. Also, the peak thrombin concentrations reached were significantly lower in cKO samples than in WT samples (Figure 2A and 2B). This significant alteration in thrombin generation kinetics did not lead to a reduction in the integrated amount of thrombin produced, with endogenous thrombin potential values comparable between WT and cKO (Figure IV in the online-only Data Supplement). The delay in thrombin generation in cKO platelet-rich plasma translated into significantly delayed fibrin generation in an ex vivo whole-blood flow chamber model, with many samples not supporting fibrin generation within the observation window (Figure 2C).

To evaluate the consequence of the reduced procoagulant potential of cKO platelets for hemostasis, cKO mice were challenged in a tail bleeding assay and were found to have markedly prolonged bleeding times compared with WT mice (Figure 2D). Furthermore, when cKO mice were subjected to an FeCl\(_3\) injury in the carotid artery to assess thrombosis, \( \approx 50\% \) did not form a stable occlusion within the observation period and the remaining cKO animals formed a stable vessel occlusion significantly later than WT mice. This result closely reflected the results of the ex vivo fibrin generation assay in Figure 1C. In the thrombosis model, the defects in the cKO mice appeared to be because of frequent incidences of embolization (Figure 2E; Figure V in the online-only Data Supplement). This clear influence of platelet TMEM16F function on hemostasis and thrombosis was not the case for thromboinflammatory processes. In the transient middle cerebral artery occlusion model of ischemic stroke, cKO mice had similar brain infarct volumes (Figure 2F), neurological outcomes (tested by the Bederson score, Figure 2G) and motor function, and coordination outcomes (tested by the grip test, Figure 2H) as WT mice.

Discussion

It has been recently shown that 2 parameters are of relevance in determining the contribution that platelets make to thrombin generation and, thus, coagulation. Not only is the ability of platelets to present negatively charged phosphatidylserine on their surface important but also their capacity to increase the surface area over which phosphatidylserine is presented, by controlled membrane ballooning/swelling.\textsuperscript{2} As observed in patients with Scott syndrome, platelets from cKO mice had a severe reduction in both procoagulant characteristics.\textsuperscript{11} However, residual phosphatidylserine exposure remained, with some agonists stimulating more phosphatidylserine exposure in cKO platelets than others, suggesting that the activity of TMEM16F is not the only mechanism behind membrane phospholipid scrambling after platelet activation. These observations have been previously made in Scott syndrome platelets and also in platelets of constitutive TMEM16F knockout mice. The mechanism behind the residual phosphatidylserine exposure and the differential effects of ionophores and other agonists on its levels remains incompletely understood.\textsuperscript{11,13,15} However, a growing body of evidence suggests that the level of intracellular Ca\(^{2+}\) mobilized strongly influences the bifurcation between TMEM16F-dependent and TMEM16F-independent phosphatidylserine exposure pathways.\textsuperscript{15,16} Despite the
Figure 1. TMEM16F knockout platelets have reduced procoagulant characteristics. A, Reverse transcription polymerase chain reaction of platelet cDNA (i) and Western blot of platelet lysates from wild-type (WT) and conditional knockout (cKO) mice (ii), showing the loss of TMEM16F at the mRNA and protein level in cKO platelets. B, Normal circulating levels of platelets in cKO mice as measured by flow cytometry (platelet/μL±SD, n=15) and (C) normal cKO platelet size as measured by a hematology analyzer (MPV, mean platelet volume in femtolitre [fL]±SD, n=15). D, Measurement of phosphatidylserine exposure by flow cytometry. The platelet population was gated into 3 groups, Annexin A5 negative (white bars; 99% of events in WT resting condition), Annexin A5 high (black bars; 90% of events in the WT ionomycin-treated condition) and an intermediate population (gray bars), Annexin A5 low (percentage positive cells±SD, significance shown for Annexin A5 high populations). E, Representative histograms of Annexin A5–binding analyses (WT histogram nonfilled, cKO histogram gray-fill) i. Collagen-related peptide (CRP)/thrombin stimulation, ii. Ionomycin stimulation. F, Increase in light transmission (turbidity clearance) because of platelet membrane ballooning after ionomycin stimulation. JON/A F(ab)’2 fragments were used to block αIIbβ3 integrins, and thus aggregation. Although WT platelets showed a rapid increase in light transmission, associated with a change to a ballooned morphology, cKO platelets did not. G, Count of platelets with ballooned membranes, formed on collagen/tissue factor microspots under flow. Alexa Fluor 568 Annexin A5 labeled, citrated whole blood was perfused over a collagen/tissue factor microspot for 4.5 min to allow aggregate formation and the number of ballooned platelets in the field of view were counted (number of ballooned platelets/image±SD, n=10) with representative images from WT and cKO samples (scale bar, 2 μm) *P<0.05, **P<0.01, ***P<0.001; unpaired Student t test.
Figure 2. TMEM16F-mediated acceleration of thrombin generation is important for hemostasis, thrombosis and infarct growth in the ischemic brain. A. Quantification of tissue factor initiated thrombin generation measurements at 37°C. Conditional knockout (cKO) samples showed significantly increased time to initiation of thrombin generation (lagtime, left), time taken to reach peak thrombin (Continued)
residual phosphatidylserine exposure in cKO platelets, the kinetics of thrombin generation were significantly altered in thrombin generation assays, which translated into defects in ex vivo fibrin generation, thrombosis, and hemostasis. This confirmed the importance of platelet TMEM16F function for thrombus formation, despite a recent publication implicating a greater role for the procoagulant endothelium using a laser injury model. Importantly, our data indicate that this influence of platelet TMEM16F is not because of a reduction in the overall levels of thrombin generated over a prolonged period, as previously reported, but rather because the rate at which it is produced, a factor on which classical thrombosis appears to be critically dependent.

Unexpectedly, this does not seem to be the case for thrombin-activation, as the absence of platelet TMEM16F did not affect infarct progression or functional outcomes post ischemic stroke, despite the clear relevance of thrombin and coagulation in this setting. The overall difference between the loss of platelet TMEM16F on thrombosis and thromboinflammation may reflect the different timescales over which these processes occur. As infarct development and progression occurs over hours and thrombosis over minutes, the altered kinetics of cKO platelet-driven thrombin generation may not be relevant to the thromboinflammatory process. Furthermore, over longer time periods, other cell types such as red blood cells, activated endothelium, lymphocytes, and neurons are also known to become procoagulant, and so could contribute to thrombin levels within infarcted brain tissue.

These data suggest that TMEM16F is not the sole mediator of agonist-induced phosphatidylserine exposure. However, its activity is critical for the acceleration of this response and, thus, platelet-driven thrombin formation. This modulation of thrombin generation kinetics by TMEM16F in platelets is critical for hemostasis and thrombosis, but not during thromboinflammation reflecting the different roles of platelets and coagulation in these processes.

Acknowledgments
We thank Dr Deya Cherpokova for helpful scientific discussions and Dr Katharina Remer for support with all documentation and management of animal experimentation and welfare.

Sources of Funding
This work was supported by the Deutsche Forschungsgemeinschaft (SFB688 to B. Nieszwander, A. Braun, and C. Kleinschnitz). A.A. Baig was supported by a grant of the German Excellence Initiative to the Graduate School of Life Sciences, University of Würzburg.

Disclosures
None.

References
14. Fujii T, Sakata A, Nishimura S, Eto K, Nagata S. TMEM16F is required for phosphatidylserine exposure and microparticle release in activated...
TMEM16F (anoctamin 6) is not the sole mediator of the platelet procoagulant response. TMEM16F activity accelerates the platelet procoagulant response and platelet-driven thrombin formation. The modulation of thrombin generation kinetics by TMEM16F in platelets is important for hemostasis and arterial thrombosis, but not during thromboinflammation.
TMEM16F-Mediated Platelet Membrane Phospholipid Scrambling Is Critical for 
Hemostasis and Thrombosis but not Thromboinflammation in Mice—Brief Report

Ayesha A. Baig, Elizabeth J. Haining, Eva Geuss, Sarah Beck, Frauke Swieringa, Podchanart Wanitchakool, Michael K. Schuhmann, David Stegner, Karl Kunzelmann, Christoph Kleinschnitz, Johan W.M. Heemskerk, Attila Braun and Bernhard Nieswandt

Arterioscler Thromb Vasc Biol. 2016;36:2152-2157; originally published online September 15, 2016;
doi: 10.1161/ATVBAHA.116.307727

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved. Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/36/11/2152

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2016/09/22/ATVBAHA.116.307727.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
MATERIALS AND METHODS

Animals
The mouse Anoctamin6 (Ano6) gene has 20 exons spanning 184 kb on chromosome 15. For the generation of platelet and megakaryocyte specific TMEM16F knockout mice (conditional knockout, cKO), a targeting strategy was designed to conditionally delete exon 3 of the Ano6 gene by Cre-loxP system. Therefore, exon 3 was flanked by loxP sites and the floxed Ano6 mice were inter-crossed with mice expressing Cre under the Pf4 promoter. This resulted in the generation of a frameshift mutation and the introduction of a premature stop codon. The resulting truncated protein was predicted to be non-functional. Animals on a C57BL/6 background, between 8-12 weeks of age were used for experiments. Pf4-Cre negative littermates were used as wildtype controls. Animal studies were approved by the district government of Lower Frankonia (Bezirksregierung Unterfranken).

Antibodies and reagents
Prostacycline, apyrase grade III, human fibrinogen, 2,3,5-triphenyltetrazolium chloride were all from Sigma-Aldrich. High-molecular-weight heparin (Ratiopharm), U-46619 (Enzo Life Sciences), thrombin (Roche), TRlZol (Invitrogen), SuperScript III reverse transcriptase (Thermo Fischer Scientific), Ionomycin free acid, A23287 free acid (Merck), Sphero AccuCount fluorescent particles (Spherotech), medetomidine (Pfizer), midazolam, mini protease inhibitor cocktail (Roche), fentanyl (Janssen-Cilag), JON/A-PE antibody (Emfret Analytics), recombinant human tissue factor (Innovin, Dade Behring), Horm type I collagen (Nycomed Pharma), DiOC₆ (Anaspec), Alexa Fluor 647-fibrinogen (Molecular probes, Life Technologies), were all purchased as indicated. Rabbit anti-mouse Ano6 antibody was kindly provided by Karl Kunzelmann.¹ Collagen-related peptide (CRP) was generated as previously described.² Annexin A5 was generously provided by Jonathan F. Tait, University of Washington Medical Center and conjugated to DyLight 488 by standard methods. Rhodocytin was isolated as described.³ All other antibodies were generated and modified in our laboratory as previously described.⁴

Platelet preparation
Mice were bled from the retro-orbital plexus under isoflurane anesthesia. Blood was collected in a tube containing 6 U heparin. The platelet rich plasma (PRP) was fractionated from whole blood by 2 cycles of centrifugation at 300 g for 6 min at room temperature (RT). For the preparation of washed platelets, PRP was centrifuged at 800 g for 5 min at RT obtaining platelets as a pellet.
After re-suspension in modified Tyrodes-HEPES buffer (134 mM NaCl, 0.34 mM Na$_2$HPO$_4$, 2.9 mM KCl, 12 mM NaHCO$_3$, 5 mM HEPES, 1 mM MgCl$_2$, 5 mM glucose, 0.35 % BSA, pH 7.4) in the presence of prostacyclin (0.1 µg/mL) and apyrase (0.02 U/mL), the platelet pellet was washed twice by centrifugation at 800 g for 5 min at RT. Platelet count was measured by hematology analyzer (Sysmex KX-21™) and adjusted to $5 \times 10^5$ platelets/µL by re-suspension in modified Tyrodes-HEPES buffer containing 0.02 U/mL apyrase.

**RT-PCR**

Platelets were pooled from three mice per genotype, and the mRNA was isolated using TRIzol reagent (Invitrogen). cDNA was generated from the mRNA template using SuperScript III reverse transcriptase (ThermoFischer Scientific) according to the manufacturer’s instructions. The following primers were used to detect the Ano6 transcript through exon 3 to exon 4: 5’-TGACCAGACAATTGTCTGCC-3’ and 5’-CACGAGGATGAAGTCGATTC-3’. The size of the expected product was 142 bp. The primers used for the detection of actin were: 5’-GTGGGCCGCTCTAGGCACCAA-3’ and 5’-CTCTTTGATGTCACGCACGATTTC-3’. The expected product size was 500 bp.

**Western Blotting**

Washed platelets were adjusted to $2 \times 10^6$ platelets/µL and lysed with RIPA buffer (150 mM sodium chloride, 0.1 % Triton-X 100, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris pH 8.0) containing protease inhibitors. Proteins were separated on an 8 % SDS-PAGE gel under reducing conditions and transferred onto a PVDF membrane by wet transfer. The membrane was blocked with non-fat milk for 1 hr at RT and subsequently incubated with rabbit anti-mouse Ano6 antibody (0.05 mg/ml) overnight at 4 °C. Proteins were visualized following incubation with HRP-conjugated secondary antibody for two hours at room temperature and using the ECL detection kit.

**Determination of platelet count and size**

Platelet count was determined as described previously. Briefly, 50 µL of heparinized blood was diluted 1:20 in PBS. 50 µL of this diluted sample was stained for 15 min at RT with phycoerythrin conjugated anti-integrin αIIbβ3 antibody (clone JON2) and anti-GPV fluorescein isothiocyanate conjugated antibody (clone DOM1) to identify the platelet population in whole blood. After 15 min, 500 µL of PBS was added, followed by addition of fluorescent beads (AccuCount
fluorescent particles, 5.2 µm; Spherotec). The samples were analyzed on a FACSCalibur (BD Biosciences). Platelet volume was determined by measuring the diluted heparinized blood in a hematology analyzer (Sysmex KX-21™), which gave MPV (mean platelet volume) values in fL.

**Measurement of phosphatidylserine (PS)-exposure in platelets**

Washed platelets were diluted 1:10 in modified Tyrodes-HEPES buffer containing 2 mM CaCl₂ to attain a final concentration of $5 \times 10^4$ platelets/µL. 50 µL of the diluted sample was stimulated with 10 µM ionomycin, 10 µM A23187 or co-stimulated with 10 µg/mL CRP and 0.1 U/mL thrombin for 5 min at RT. Unstimulated platelet samples were used as controls. The samples were stained with Annexin A5-DyLight-488 and JON/A-PE and analyzed on a FACSCalibur (BD Biosciences). JON/A-PE was used to identify the platelet population. The FL-1 histogram was gated into three regions, Annexin A5 Negative (99 % of events in wildtype resting condition), Annexin A5 High (90 % of the events in the wildtype ionomycin stimulated condition) and Annexin A5 Low (intermediate population between Annexin A5 Negative and Annexin A5 High). Simultaneously, 50 µL of the diluted sample was also stimulated with the same agonists under similar conditions, but stained with anti P-selectin FITC antibody as a measurement of α-granule release and thus platelet activation. Results representative of three individual experiments, n = 4 mice per group.

**Measurement of platelet membrane ballooning through changes in light transmission**

Increase in light transmission through a suspension of platelets (i.e. clearance of turbidity) served as a measure of transformation of platelets to a more rounded and translucent morphology (membrane ballooning)⁶,⁷ Changes in light transmission were recorded for 10 min on a Fibrintimer 4 channel aggregometer (APACT Laborgeräte und Analysensysteme). 50 µL of washed platelets were diluted into 110 µL Tyrodes-HEPES buffer containing 2 mM CaCl₂ and 100 µg/mL human fibrinogen. The platelet suspension was continuously stirred at 1,000 rpm. 30 s after starting the measurement, JON/A F(ab)’₂ fragments were added at a concentration of 20 µg/mL to block αIIbβ3 integrins and thus prevent aggregation.⁸ The platelet suspension was stimulated with 20 µM ionomycin after 150 s.

**Platelet adhesion and fibrin formation on collagen/tissue factor under flow conditions**

*Ex vivo* assay for detection of fibrin clot formation under flow was performed as described previously.⁹ Rectangular coverslips (26 × 60 mm) were coated with microspots of 100 ng Horm
type I collagen and 10 pg recombinant human tissue factor, and were subsequently blocked with 1 % BSA. Citrate anti-coagulated blood (1 part Na-citrate, 9 parts blood) was pre-labeled with DiOC₆ to mark platelets, Alexa fluor (AF)647-fibrinogen to detect fibrin(ogen) and AF568-Annexin A5 to visualize PS exposure and platelet membrane ballooning. Blood was taken in a 1 mL syringe and mixed with coagulation medium (Hepes buffer pH 7.45 supplemented with 32 mM MgCl₂ and 63 mM CaCl₂) taken in another 1 mL syringe and, with the aid of a Y-shaped flattened mixing tube and two pulse-free micro-pumps, perfused as a mixture (ratio 10:1) over the coated coverslip placed in a parallel plate flow chamber at a wall shear rate of 1000 s⁻¹. Brightfield and fluorescence images were recorded using an EVOS AMF 4300 digital inverted microscope at 60x magnification every 1.5 min until fibrin formation was observed, or for an observation period of 8 minutes.

**Thrombin generation assay (Thrombinscope)**

Thrombin generation was quantified by the calibrated thrombogram method adapted for mouse plasma as described previously.¹⁰,¹¹ Briefly, PRP was fractionated from citrate-anticoagulated whole blood by two cycles of centrifugation at 300 g for 6 min. Platelets were pelleted from PRP by another centrifugation step at 800 g for 5 min and the supernatant (PPP, platelet poor plasma) was pooled from the samples originating from mice of the same genotype. The platelets were adjusted to a concentration of 1.5 × 10⁵ platelets/µL in the respective pooled PPP to form the PRP. The PRP was stimulated with the indicated agonists and immediately pipetted into a 96 well plate (all samples in duplicate). Each well contained recombinant tissue factor (3 pmol/L). The machine was preheated to 37 °C and thrombin generation was monitored by addition of a fluorescent thrombin substrate (Z-Gly-Gly-Arg aminomethyl coumarin, 2.5 mmol/L) to each well. First-derivative curves of the accumulation of fluorescence intensity over time were converted into curves of nanomolar thrombin, using a thrombin calibrator. Results presented as mean ± SD; for PRP and 10 µM A23187 stimulation, data pooled from 3 independent experiments each with n = 4; for 20 µg/ml CRP/ 5 mM PAR-4 peptide, data pooled from 2 independent experiments, n = 4 animals per group. The Ca²⁺ ionophore A23187 was used in this assay and not ionomycin, as plasma proteins quench the latter.

**Tail bleeding assay**

Tail bleeding assay was performed as previously described.¹² Briefly, mice were anesthetized and 2 mm of the tail tip was cut off with a scalpel. Tail bleeding time was assessed by gently
absorbing the formed blood drop onto a filter paper every 20 s without making direct contact to the wound site. Bleeding was considered to have ceased when no blood was observed to blot onto the filter paper. The experiment was performed either until cessation of bleeding, or until the end of a 20 min observation period. Results from 14 WT vs 13 cKO mice tested in the assay.

**Model of FeCl$_3$ induced thrombosis in the carotid artery**

The right carotid artery of anesthetized mice was exteriorized through a midline incision in the neck. The artery was injured by topical application of a filter paper soaked with 6 % FeCl$_3$ for 100 s to induce thrombosis. Blood flow was measured with an ultrasonic flow probe placed around the vessel just above the injury (0.5PSB699, Transonic Systems) until full occlusion of the vessel (cessation of blood flow for at least 3 min) or until the end of an observation period of 30 min. Results from 8 WT vs 10 cKO mice.

**Triple Anesthesia**

Mice were anesthetized by intraperitoneal injection of a combination of midazolam/medetomidine/fentanyl (5/0.5/0.05 mg/kg body weight).

**Transient middle cerebral artery occlusion (tMCAO) model of stroke**

The tMCAO model of focal cerebral ischemia was performed as previously described. Briefly, mice were anesthetized with 2 % isoflurane, and the carotid artery was exteriorized. A silicon rubber coated nylon monofilament was advanced through the carotid artery to occlude the middle cerebral artery for 60 min, after which it was removed to allow reperfusion. Following 23 h of reperfusion, the mice were tested for global neurological defects by the Bederson score (0 best functional outcome, 5 worst deficit), as well as for motor functional and coordination deficits by the Grip test (5 best, 0 worst). The brains of the mice were also quantified for edema corrected infarct volumes by using of 2,3,5-triphenyltetrazolium chloride (TTC) staining on 2 mm thick coronal brain sections. Results from 15 WT vs 13 cKO mice.

**Data analysis**

Results are reported as mean ± SD. The unpaired Student’s $t$ test was used to determine the statistical significance between the experimental groups, except in the case of tail bleeding assay and FeCl$_3$ induced thrombosis in the carotid artery, where Fisher’s exact test was used.
The Bederson score and the grip test were analyzed by the Mann-Whitney U test. P<0.05 was considered to be statistically significant (*P<0.05; **P<0.01; ***P<0.001).

REFERENCES


SUPPLEMENTARY MATERIAL

Table I. TMEM16F cKO platelets have unaltered levels of surface glycoproteins. Heparinized whole blood was diluted in PBS and 50 µL stained for 15 min at RT with saturating amounts of fluorophore-labeled antibodies for determination of platelet surface glycoprotein expression. Samples were analyzed by flow cytometry on a FACSCalibur (BD Biosciences). Results are expressed as MFI ± SD (n = 4 mice per group; representative data of 3 individual experiments). No significant differences were observed between WT and cKO platelets. n.s = not significant; unpaired Students t test.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>cKO</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>αIIbβ3</td>
<td>460 +/- 27</td>
<td>426 +/- 5</td>
<td>n.s.</td>
</tr>
<tr>
<td>α2</td>
<td>112 +/- 15</td>
<td>117 +/- 4</td>
<td>n.s.</td>
</tr>
<tr>
<td>β1</td>
<td>152 +/- 2</td>
<td>140 +/- 24</td>
<td>n.s.</td>
</tr>
<tr>
<td>CD9</td>
<td>1309 +/- 34</td>
<td>1272 +/- 29</td>
<td>n.s.</td>
</tr>
<tr>
<td>CLEC-2</td>
<td>120 +/- 10</td>
<td>128 +/- 5</td>
<td>n.s.</td>
</tr>
<tr>
<td>GPIb</td>
<td>359 +/- 24</td>
<td>370 +/- 9</td>
<td>n.s.</td>
</tr>
<tr>
<td>GPIX</td>
<td>390 +/- 13</td>
<td>394 +/- 14</td>
<td>n.s.</td>
</tr>
<tr>
<td>GPV</td>
<td>220 +/- 7</td>
<td>221 +/- 5</td>
<td>n.s.</td>
</tr>
<tr>
<td>GPVI</td>
<td>37 +/- 1</td>
<td>38 +/- 2</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Table II. TMEM16F cKO mice display normal blood counts and unaltered global coagulation times. For determination of whole blood counts, diluted heparinized blood was analyzed in a hematology analyzer (Sysmex KX-21™). PT and aPTT were determined at the Zentrallabor, University Hospital Würzburg using standard methods. HGB, hemoglobin; HCT, hematocrit; PT, prothrombin time; aPTT, activated partial thromboplastin time. No significant differences were observed between WT and cKO platelets. n.s = not significant; unpaired Students t test.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>cKO</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC×10^3/µL</td>
<td>8.29 ± 2.31</td>
<td>9.29 ± 2.44</td>
<td>n.s.</td>
</tr>
<tr>
<td>RBC×10^6/µL</td>
<td>6.50 ± 0.87</td>
<td>6.40 ± 0.68</td>
<td>n.s.</td>
</tr>
<tr>
<td>HGB (g/dL)</td>
<td>9.62 ± 1.75</td>
<td>9.76 ± 1.20</td>
<td>n.s.</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>34.10 ± 4.67</td>
<td>33.65 ± 4.08</td>
<td>n.s.</td>
</tr>
<tr>
<td>PT (s)</td>
<td>9.3 ± 0.5</td>
<td>9.6 ± 0.2</td>
<td>n.s.</td>
</tr>
<tr>
<td>PTT (s)</td>
<td>25.4 ± 1.1</td>
<td>27.3 ± 3.4</td>
<td>n.s.</td>
</tr>
</tbody>
</table>
**Supplementary Figure I. Generation of TMEM16F conditional knockout mice.**

(A) Targeting strategy; *Anoctamin6 (Ano6)* gene has 20 exons spanning 184 kb on chromosome 15 in mice. The targeting strategy was designed to conditionally knockout exon 3 by Cre-loxP system, therefore exon 3 was flanked with loxP sites. Floxed mice were crossed with mice expressing Cre under the Pf4 promoter. This resulted in the generation of a frameshift mutation and the introduction of a premature stop codon. The resulting truncated protein was predicted to be non-functional.

(B) Genotyping strategy; the primers used for genotyping were For: 5’ ATGTCTGTGCAGATCTGCTCTTCA 3’ and Rev: 5’ CAGCAACTAGTT CATGCATGTTG 3’. The WT allele yielded a PCR product of 211 bp and the floxed allele yielded a PCR product of 267 bp. PCR was also used to determine successful Cre-recombination. Primers used were For: 5’ CTCTGACAGATGCCAGGACA 3’ and Rev: 5’ TCTCTGCCCAGAGTCATCCT 3’.

(C) Mendelian distribution of progeny of flox/flox Pf4 Cre +ve and flox/flox Pf4 Cre -ve mating. Knockout mice were born in accordance with the expected Mendelian distribution (50% WT and 50% cKO).
Supplementary Figure II. Platelets from TMEM16F cKO mice have grossly normal function. (A) cKO platelets have normal αIIbβ3 integrin activation in response to stimulation with indicated agonists and (B) unaltered α-granule release (as measured by P-selectin

Light transmission Time (min)
For determination of integrin activation and P-selectin exposure, heparinized whole blood was washed twice in Tyrode-HEPES buffer and then diluted in Tyrode-HEPES buffer containing 2 mM CaCl$_2$. 50 µL of diluted blood was activated with the indicated concentration of agonists and stained with antibodies (JON/A-PE and anti P-selectin-FITC) at saturating concentrations for 10 min at 37 °C. The samples were analyzed by flow cytometry (FACSCalibur, BD Biosciences). Results are presented as mean MFI ± SD (n = 4 mice per group; representative data of 3 individual experiments). (C) cKO platelets have normal aggregation responses after stimulation with the indicated agonists. These studies were performed on a Fibrin timer 4 channel aggregometer (APACT Laborgeräte und Analysensysteme). 50 µL of washed platelets were diluted into 110 µL Tyrodes-HEPES buffer containing 2 mM CaCl$_2$ and 100 µg/mL human fibrinogen (except in the case of thrombin stimulation where fibrinogen was not used). The agonists were added to the platelet suspension stirring at 1000 rpm. Changes in light transmission were recorded for 10 min. CRP, collagen related peptide; RC, rhodocytin

**Supplementary Figure III. TMEM16F knockout platelets have reduced pro-coagulant characteristics.** (A) Measurement of PS exposure (as indicated by Annexin A5 binding) and (B) Degranulation dependent P-selectin exposure by WT and cKO platelets measured by flow cytometry (MFI ± SD; n = 4 mice per group). (C) Measurement of PS exposure on stimulation of WT or cKO platelets with calcium ionophore A23187 by flow cytometry. The platelet population was gated into three groups, Annexin A5 Negative (White bars; 99 % of events in WT resting condition), Annexin A5 High (Black bars; 90 % of events in the WT ionomycin treated condition) and an intermediate population (Grey bars), Annexin A5 Low (percentage positive cells ± SD, significance shown for Annexin A5 High population). ***P<.001; unpaired Student $t$ test.
Supplementary Figure IV. Total amount of thrombin production supported by TMEM16F cKO platelets is comparable to WT platelets. Quantification of tissue factor initiated thrombin generation measurements at 37 °C. cKO samples show comparable Endogenous Thrombin Potential (ETP, integrated amount of thrombin produced over time) to WT samples.

Supplementary Figure V. TMEM16F cKO mice have frequent incidences of embolization in a model of arterial thrombosis. Representative blood flow curves during thrombus development after FeCl₃ injury of the carotid artery. WT and cKO mice were subjected to a chemical injury of the carotid artery and blood flow was monitored in the injured vessel using an ultrasonic flow probe. Blood flow readings from a representative injury in a WT (black) and cKO (grey) mouse are presented over time. cKO mice had higher incidence of embolization events and showed defects in formation of occlusive thrombi in the injured vessel.