

## ORIGINAL ARTICLE

# A *TMEM16F* point mutation causes an absence of canine platelet *TMEM16F* and ineffective activation and death-induced phospholipid scrambling

M. B. BROOKS,\* J. L. CATALFAMO,\* R. MACNGUYEN,\* D. TIM,\* S. FANCHER\* and J. A. MCCARDLE†

\*Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University; and †Institute of Biotechnology, Cornell University, Ithaca, NY, USA

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**Summary.** *Background:* *TMEM16F* is an ion channel and calcium-dependent lipid scramblase that mediates phosphatidylserine (PS) exposure on the plasma membrane. Two disparate disease phenotypes are associated with *TMEM16F* loss-of-function mutations: a rare bleeding disorder (Scott syndrome) and skeletal malformations due to aberrant bone mineralization in a *TMEM16F* knock-out mouse. We therefore undertook comparative studies of *TMEM16F* expression in canine Scott syndrome (CSS), an autosomal recessive platelet defect. *Objectives:* To define anoctamin proteins and scramblase response of CSS platelets and to determine whether *TMEM16F* is the CSS disease gene. *Methods:* CSS *TMEM16F* cDNA and gene were sequenced and mutation detection was performed in CSS pedigrees. Platelet fractions from CSS dogs were isolated for proteomic and immunologic characterization of *TMEM16F*. Annexin V was used as a flow cytometric marker of induced platelet PS externalization. *Results:* A *TMEM16F* splice site mutation segregated with the CSS trait and *TMEM16F* protein was undetectable in CSS platelet membranes; however, a second anoctamin, *TMEM16K*, was found. Proteomic analyses revealed a network of 32 proteins that differentially cosegregated with platelet plasma membrane *TMEM16F*. CSS platelets had profoundly impaired scramblase response to pharmacologic and physiologic agents that increase intraplatelet calcium and conditions that induce apoptotic and necrotic cell death. *Conclusions:* CSS plate-

lets represent a *TMEM16F*-null mutant model that demonstrates a central role for *TMEM16F* in mediating platelet PS externalization in response to activating and death signals. Platelet *TMEM16F* may prove a novel drug target for modulating platelet procoagulant activity and extending platelet life span.

**Keywords:** animal models; blood coagulation disorders, inherited; blood platelet disorders; phospholipid scramblase; platelet activation.

## Introduction

Procoagulant platelets, present at sites of thrombus formation, are characteristically non-aggregated, rounded cells that externalize phosphatidylserine (PS) and shed membrane microparticles (PMPs) that express PS [1–4]. Outer membrane PS supports assembly of highly active tenase and prothrombinase complexes that drive platelet-mediated thrombin generation [5–7]. *In vitro* studies of procoagulant platelets reveal that sustained high intraplatelet calcium, triggered by signaling through the thrombin and collagen receptors or by ionophore treatment, induces transbilayer lipid movement and PS-positive microvesiculation within 5 min of stimulation [1,4,8–10]. In addition to this rapid activation response, platelets also demonstrate a slow loss of membrane lipid asymmetry, PS exposure, and membrane blebbing as they undergo cell death from injury or senescence [11,12]. The differential regulation and mechanisms of platelet PS exposure under these varying pathophysiologic conditions are not yet fully defined. The circulatory life span of platelets is delimited by the intrinsic apoptotic pathway. The disinhibition of the BCL2 family proteins Bax and Bak initiates this pathway, leading to mitochondrial release of cytochrome C, and subsequent activation of effector caspases and cleavage of cytoskeletal proteins [13–15]. Caspase cascade activation in apoptotic

Correspondence: Marjory B. Brooks, Comparative Coagulation Laboratory-Cornell University, 240 Farrier Road, Ithaca, NY 14853, USA.

Tel.: +1 607 253 3648; fax: +1 607 253 3471.

E-mail: mbb9@cornell.edu

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PS externalization differs from the calcium and agonist-triggered procoagulant PS exposure that appears caspase independent and involves downstream response to cyclophilin D-mediated collapse of mitochondrial membrane potential [4,16,17]. The transmembrane protein, *TMEM16F*, has recently been identified as an important mediator of rapid, calcium-activated aminophospholipid transbilayer transport or 'scrambling' [18–21]. The participation of this protein in PS exposure in platelets and other cell types, and in response to varying stimuli, is the subject of active investigation [19,22–24].

*TMEM16F* is an anoctamin family protein. Anoctamins were first described as having eight transmembrane domains; however, crystallography supports a dimeric structure composed of subunits with 10 transmembrane domains and a calcium-binding site within a hydrophobic core [25–28]. Functionally diverse and with wide tissue distribution, individual family members demonstrate calcium-activated chloride, cation, and non-selective ion channel activities and/or lipid scrambling. Distinct loss of function mutations in *TMEM16F* have been described in two unrelated patients with Scott syndrome (OMIM: 262890), the rare bleeding disorder defined by a specific lack of platelet scramblase activity and defective procoagulant platelet formation [21,29]. The phenotype of a *TMEM16F*-null mouse, lacking exon 2, was characterized by prolonged tail bleeding times and reduced platelet scrambling in response to calcium ionophore treatment [30]. Surprisingly, a second knockout mouse, generated by excision/replacement of *TMEM16F* exon 1 and adjacent sequence, had a unique phenotype of gross skeletal deformities and high neonatal mortality due to a developmental defect of osteoblast matrix mineralization [31]. In addition, this murine model demonstrated an immunologic defect of macrophage phagocytosis [32].

Canine Scott syndrome (CSS) represents a naturally occurring, autosomal recessive bleeding disorder discovered in German shepherd dogs (GSDs) whose trait locus contains the *TMEM16F* homolog [33,34]. Platelets from CSS dogs demonstrate a similar lack of procoagulant activity as described for the human disease counterpart, with no evidence of skeletal malformation [34–36]. We therefore undertook molecular analyses and expression studies of platelet *TMEM16F* in the CSS model. In comparative proteomic analyses and functional studies, we determined the effects of *TMEM16F*'s absence on platelet membrane composition and the platelets' ability to externalize PS in response to varying stimuli that induce platelet activation or death.

## Materials and methods

### Materials

Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Fresh, ultrapure water (18-m $\Omega$  EMD, Millipore, Billerica, MA, USA) was used. Polyethylene glycol (PEG) 3350 (JT Baker™) and

dextran (DEX) T500 (Spectrum) were from VWR (Philadelphia, PA, USA). Precast TBX™ 10% polyacrylamide gels, polyvinylidene fluoride membranes, chemiluminescent molecular weight markers, buffers, and reagents for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were from Bio-Rad (Hercules, CA, USA). Halt™ protease and phosphatase inhibitor cocktail (No. 78442, 100 $\times$ ), BCA™ and 660™ protein assay kits were from Thermo Scientific (Rockford, IL, USA). Colloidal Coomassie blue stain kit was from Life Technologies (Grand Island, NY, USA), and anti-rabbit IgG-horseradish peroxidase conjugate and ECL™ select chemiluminescent detection kits were from GE Healthcare Bio-Sciences (Piscataway, NJ, USA).

### Animals, nucleic acid isolation and amplification, and platelet preparations

The phenotypic characterization of CSS in GSDs, the genetic trait locus, and description of its clinical expression have been previously reported [33–37]. Blood samples from a total of 114 GSDs (36 affected, 78 unaffected) were used for *TMEM16F* genotype analyses. The GSDs were classified as CSS-affected based on high residual serum prothrombin and flow cytometric detection of decreased stimulated platelet PS externalization and microvesiculation. The affected GSDs included 30 related dogs from the index CSS colony, five GSDs from three distinct pedigrees with no common ancestors for at least six generations, and one GSD with no pedigree information. For more detailed analyses of platelet function and *TMEM16F* expression studies, blood samples were collected on-site from four affected GSDs (two male, two female) and four control dogs (one each GSD, beagle, Labrador retriever, hound) housed at an American Association for Accreditation of Laboratory Animal Care-approved facility. All procedures followed protocols approved by an institutional animal care and use committee.

Platelet pellets prepared from acid citrate dextrose–A anticoagulated blood were used for Western blotting, proteomics, and RNA isolation. Whole blood was sequentially centrifuged three times at 650  $\times g$  for 3 min, and the recovered platelet-rich plasma (PRP) was pooled. After the addition of 1  $\mu\text{mol L}^{-1}$  PGE<sub>1</sub>, the PRP was centrifuged at 14 000  $\times g$  for 40 s, washed twice in a HEPES/EDTA buffer (10 mmol L<sup>-1</sup> HEPES, 54 mmol L<sup>-1</sup> EDTA, 150 mmol L<sup>-1</sup> NaCl, pH 7.5), incubated at 37 °C in a red cell lysis buffer, and washed in HEPES buffer. Immediately after removal of the final supernatant, the platelet pellet was combined with a lysis buffer and processed for subsequent experiments. Platelet preparations contained 0 to 1 leukocytes/10<sup>6</sup> platelets.

Genomic DNA and leukocyte RNA were isolated from blood by using Gentra Puregene Blood Kits (Qiagen,

Valencia, CA, USA) and Tempus Spin RNA Isolation Kits (Life Technologies), and RNA from platelet pellets by using RNAqueous-Micro Total RNA Isolation Kits (Life Technologies). Reverse transcription (RT) and polymerase chain reaction (PCR) were performed using One-Taq RT-PCR and Hot Start Polymerase (New England Biolabs, Beverly, MA, USA). First-strand cDNA synthesis was primed with random hexamers, and the entire *TMEM16F* cDNA and 3' UTR were amplified in a series of overlapping fragments. Preliminary experiments revealed alternate splicing in controls at the junction of exons 1 and 2; therefore, the *TMEM16F* 5' flanking region with exon 1 was amplified from genomic DNA. To characterize the CSS mutation, primers were designed for genomic amplification of exons 16 and 17 with adjacent splice junctions and a series of fragments spanning intron 16. Primer sequences with expected product sizes are listed in Table S1. Amplification products were sequenced in both directions at a core facility (Biotechnology Resource Center, Cornell University, Ithaca, NY, USA).

PRP for stimulation experiments was isolated from citrate anticoagulated blood combined with an equal volume of buffer (129 mmol L<sup>-1</sup> NaCl, 14 mmol L<sup>-1</sup> Na<sub>3</sub>citrate, 11 mmol L<sup>-1</sup> glucose, 10 mmol L<sup>-1</sup> NaHPO<sub>4</sub>, pH 7.3). For *ex vivo* aging experiments, whole blood samples were collected in EDTA by referral veterinarians from 20 CSS-affected dogs and paired non-GSD healthy dogs, and 20 GSDs with normal platelet function. These samples were shipped to the laboratory and held at room temperature until cytometric analyses 2 days after collection.

#### Plasma membrane enrichment

Frozen, washed canine platelets (150 mg wet weight) were suspended in 300 mL equilibrated PEG/DEX, then 19.7 mL equilibrated PEG/DEX was added. The suspension was subjected to repetitive mechanical and ultrasonic bursts (6 × 15 s, 30 s rest between bursts) using a Kintematica-Polytron (Kinematica, Inc., Bohemia, NY, USA) homogenizer at setting 7 with chilling of homogenate in the ice water bath and processed as detailed for human platelets [38].

#### One-dimensional SDS-PAGE and Western blot analyses

SDS-PAGE and Western blot analyses was performed as previously described [39] with modification. Briefly, washed platelet pellets or plasma membranes were thawed and lysed by resuspension in hot (75 °C) 1× Laemmli sample buffer containing 1× Halt™, 1 mg mL<sup>-1</sup> pepstatin A, and 5 mmol L<sup>-1</sup> EDTA; vortexed for 30 s; and then heated for 15 min at 75 °C to facilitate solubilization of transmembrane proteins. Proteins were separated for 30 min at 200 V on precast TBX gels, electroblotted to polyvinylidene fluoride membranes, blocked with 5% dry

milk in PBS-Tween (0.2%), washed 4× with PBS-Tween, and probed using antibodies to TMEM16F. Immune-reactive bands were visualized by enhanced chemiluminescence and then captured and analyzed using a ChemiDoc XRS system (Bio-Rad) and manufacturer-supplied analysis software.

For proteomic analyses, gels were fixed for 1 h in 40% methanol:50% water:10% acetic acid then stained using colloidal Coomassie blue. SDS-PAGE gel lanes were cut into segments corresponding to molecular weight regions, 80–120, 120–160, 160–250, and > 250 kDa (stacking gel). In additional experiments, regions between 50 and 80 kDa were excised and analyzed. Gel regions < 50 kDa were excluded to avoid actin.

#### Nano-liquid chromatography, tandem mass spectroscopy, and data analysis

Excised gel segments were subjected to in-gel trypsin digestion and peptides were separated by nano liquid chromatography (LC) and detected by tandem mass spectroscopy (MS) at a core facility (Biotechnology Resource Center). MS and MS/MS raw spectra were processed using Proteome Discoverer 1.2-1.3 (Thermo Scientific), and the spectra from each DDA file were output as an MGF file for database searches using Mascot Daemon version 2.3.02 (Matrix Science, Boston, MA, USA). Raw data were searched against the NCBI Canis reference sequence database, with allowance for one missed trypsin cleavage site. Peptide mass tolerance was set to 20 ppm and MS/MS tolerance was set to 0.8 Da. Carbamidomethylation of cysteine was selected as a fixed modification, and oxidation of methionine and deamidation of asparagine and glutamine were set as variable modifications. The final results were filtered at the 99% confidence threshold, and an ion score cut-off of 20 was applied. Peptides with scores greater than the identity threshold were used for protein identifications.

#### Flow cytometric analyses of platelet PS externalization

Treatments to induce *ex vivo* platelet PS exposure were performed as previously described [35,40]. In brief, 100 µL (total) reaction mixtures contained 1 µL PRP and 0.4 mmol L<sup>-1</sup> gly-pro-arg-pro-NH<sub>2</sub> in a 2.0 mmol L<sup>-1</sup> CaCl<sub>2</sub> buffer (Annexin V Binding Buffer; R & D Systems, Minneapolis, MN, USA) with vehicle control or one of the following: ionophore (A23187, 3 µmol L<sup>-1</sup>), thrombin (0.5 U mL<sup>-1</sup>), thrombin (0.5 U mL<sup>-1</sup>) plus convulxin (500 ng mL<sup>-1</sup>; Pentapharm, Basel, CH, Switzerland), ABT-737 (2 µmol L<sup>-1</sup>; Abbott Laboratories, Abbott Park, IL, USA), and 0.5% paraformaldehyde (BD Cytofix; BD Biosciences, San Jose, CA, USA). After reaction for 10 min (ionophore, thrombin, thrombin/convulxin) or 1 h (ABT737, paraformaldehyde), the PRP was dual-la-

beled by the addition of a platelet marker (CD61-PE [clone SZ21; Beckman Coulter, CA, USA]) and Annexin V-FITC (R& D Systems). EDTA blood samples were combined in equal volume HEPES/EDTA buffer before dual-labeling. Samples were analyzed with a flow cytometer (BD FacsCalibur; BD Biosciences) set with a forward scatter threshold of 20 to collect 7500 platelet (CD61-positive) events. CD61-positive events that fell below the first decade of the log forward scatter scale were considered small platelets and PMPs.

## Results

### Identification of a *TMEM16F* splicing defect in CSS-affected dogs

Sequence analyses of the control dog amplicons confirmed their identity with canine *TMEM16F* cDNA including the entire 3' UTR, and exon 1 with ~ 500-bp flanking region. The CSS dogs' RNA templates generated the same-sized amplicons and sequence as controls, with the exception of slightly smaller fragments span-

ning exons 14–17. Sequencing electropherograms of these products revealed aberrant and overlapping peaks at the junction of exons 16 and 17 (Fig. 1). Repeated RT-PCR of CSS leukocyte and platelet RNA spanning this junction consistently generated high-quality forward direction sequence through exon 16 to ~15 nucleotides from the 3' splice junction and no defined exon 17, whereas sequencing the same amplicons in the reverse direction yielded distinct peaks only to the first nucleotide of exon 17. Due to the erratic splicing and resultant frame shifts and premature stop codons, the predicted CSS translation products are variably truncated at exon 16. The corresponding region of *TMEM16F* protein spans amino acids 613–928 at the C-terminus and includes a conserved calcium-binding region at amino acids 635–645.

Genomic DNA from CSS dogs was amplified to span *TMEM16F* from exons 16–17. The CSS template generated appropriate-sized fragments for all reactions; however, sequence analyses revealed a unique G → A transition at the exon 16 splice donor site (G → A IVS16 + 1) at nucleotide 8912219 of *TMEM16F*.

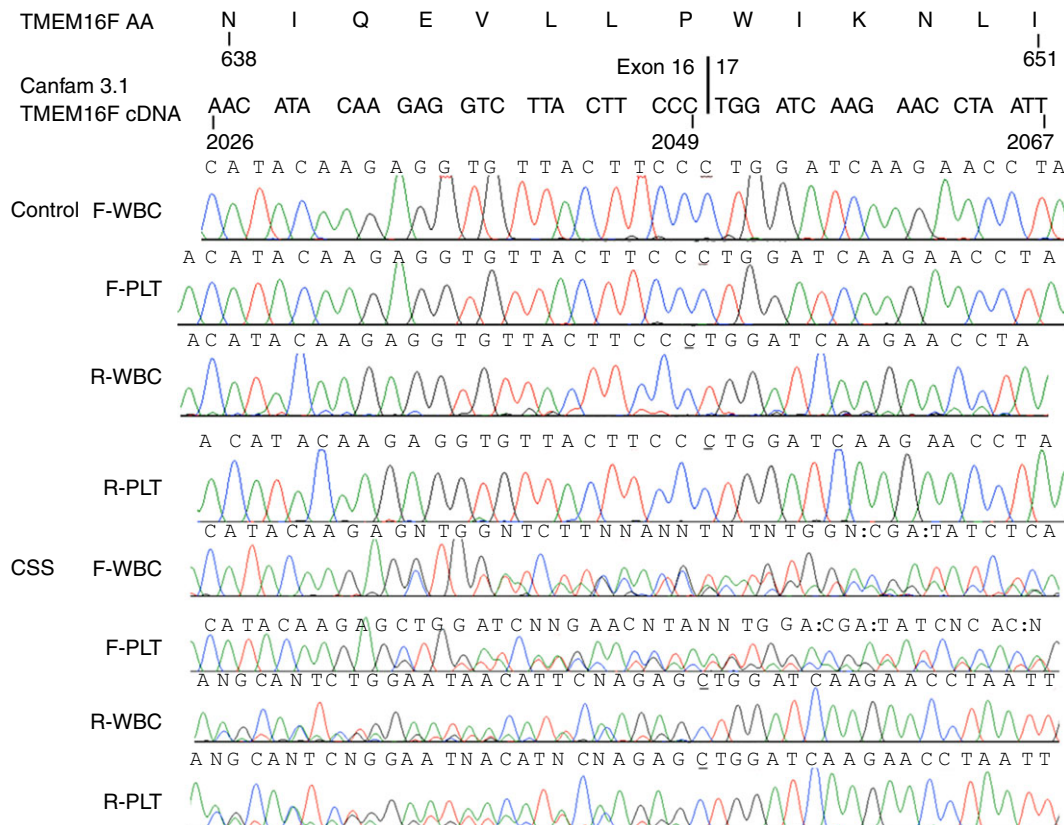


**Fig. 1.** Aligned sequence at the *TMEM16F* exons 16 and 17 splice junction from control and canine Scott syndrome (CSS) dogs. Chromatograms depict the forward (F) and reverse (R) sequencing reactions from cDNA amplicons generated from leukocytes (WBC) and platelets (PLT) with assigned nucleotides (Sequencher, Gene Code). The CSS reactions reveal aberrant sequence and ambiguous nucleotide calls (N) due to small overlapping peaks and deterioration of sequence from either side of the normal join. In contrast, control dog amplicons match the dog genome sequence (CanFam3.1, XM\_005636953). For reference, the last nucleotide in exon 16, 'C' is underlined above each chromatogram and the amino acid sequence of the predicted translation product (XP\_005637010.1) is displayed above the corresponding *TMEM16F* codons.

### A TMEM16F transition mutation segregates with the CSS phenotype

A total of 36 CSS-affected GSDs and 13 obligate carrier parents were screened for the *TMEM16F* g. 8912219 G>A mutation by genomic DNA amplification and sequencing (Fig. 2). All affected GSDs were homozygous (AA), and all carriers were heterozygous (AG). Pedigree studies revealed perfect segregation of the mutation with the CSS phenotype through six generations in the index colony and in an unrelated pedigree of white GSD (Fig. 3). A non-random sample of 81 additional GSDs from the index colony whose normal platelet function was confirmed by flow cytometry had genotype GG ( $n = 35$ ) or AG ( $n = 47$ ). Genotyping of 30 healthy dogs representing five different breeds (Beagle, Doberman pinscher, Foxhound, German wirehaired pointer, Labrador retriever, Portuguese water dog, Shetland sheepdog) revealed only the G allele at the 3' exon 16 splice junction.

### CSS platelets contain TMEM16K but lack TMEM16F

A shot-gun proteomic approach identified > 400 proteins > 80 kDa in control and CSS-enriched platelet plasma membranes (Tables S2 and S3); however, TMEM16F protein was uniquely absent from CSS. Table 1 displays the relative abundance of selected platelet proteins based on estimated emPAI scores. TMEM16K, present in CSS

and controls, was the only additional anoctamin in platelets. We found the large multipass transmembrane proteins ATP-binding cassette subfamily D, IP3 receptor type 1, and sarcoplasmic/endoplasmic reticulum calcium ATPase 3, in CSS platelets and controls, and glycoproteins IIb and IIIa were the most abundant transmembrane proteins in both. Examination of the data for other protein mediators of PS transport revealed that control and CSS platelets contained the P4-ATPase flippase, ATP8A1. Other P4-ATPases, including ATP11, were not detected.

TMEM16 protein expression was independently evaluated by Western blot analyses of whole platelet lysates and of enriched platelet plasma membrane fractions. Immunoblots from control dog platelet lysates probed with an anti-human TMEM16F antibody generated four bands (Fig. 4A, lane 1). Broad dominant bands (centered at 104 kDa) were seen in the expected region for monomeric TMEM16F. Additional bands at 134 kDa were consistent with dimeric TMEM16K, and at 73 kDa and 53 kDa with isoforms of monomeric TMEM16K. The CSS platelet lysates had reactive bands only at 73 and 53 kDa, representing TMEM16K (Fig. 4A, lane 2). Enriched platelet plasma membranes from controls (Fig. 4B, lane 1) had a broad band > 250 kDa in the region predicted for oligomeric TMEM16F, a strong band compatible with monomeric TMEM16F between 92 and 107 kDa, and TMEM16K at 137, 73, and 53 kDa. Reactions carried out with a 5-fold excess of a

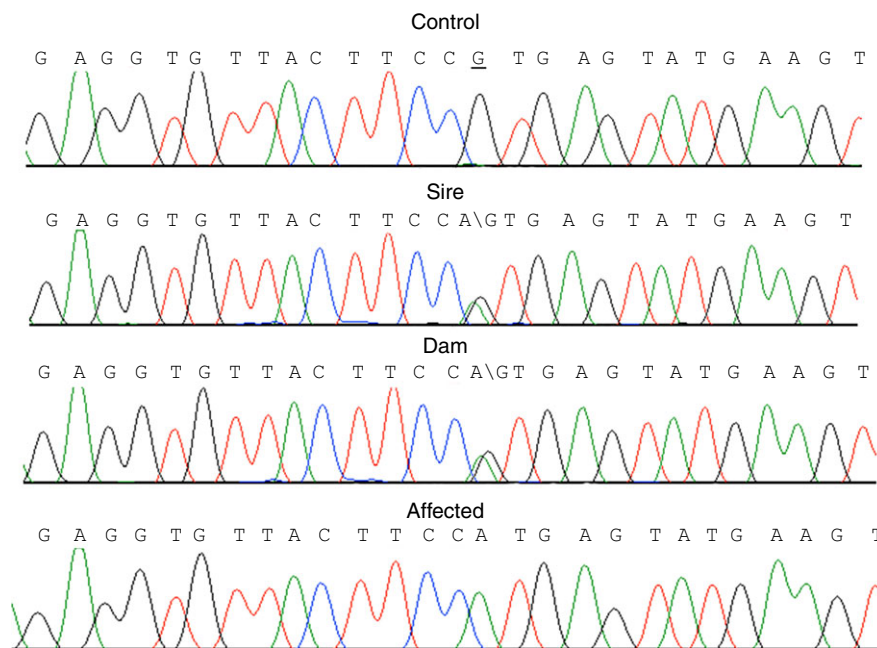


**Fig. 2.** Genomic DNA sequence at the *TMEM16F* intron 16 splice donor site from a control dog (top panel), canine Scott syndrome (CSS) obligate carrier sire and dam (center panels) and a CSS affected dog (bottom panel). Above the control chromatogram the position of the canonical splice donor 'G' is underlined at the first nucleotide of *TMEM16F* intron 16 (g.8912219). The control dog is homozygous for this wild type sequence, whereas the carrier sire and dam are heterozygous (AG) and the affected dog is homozygous (AA) for a transition mutation.

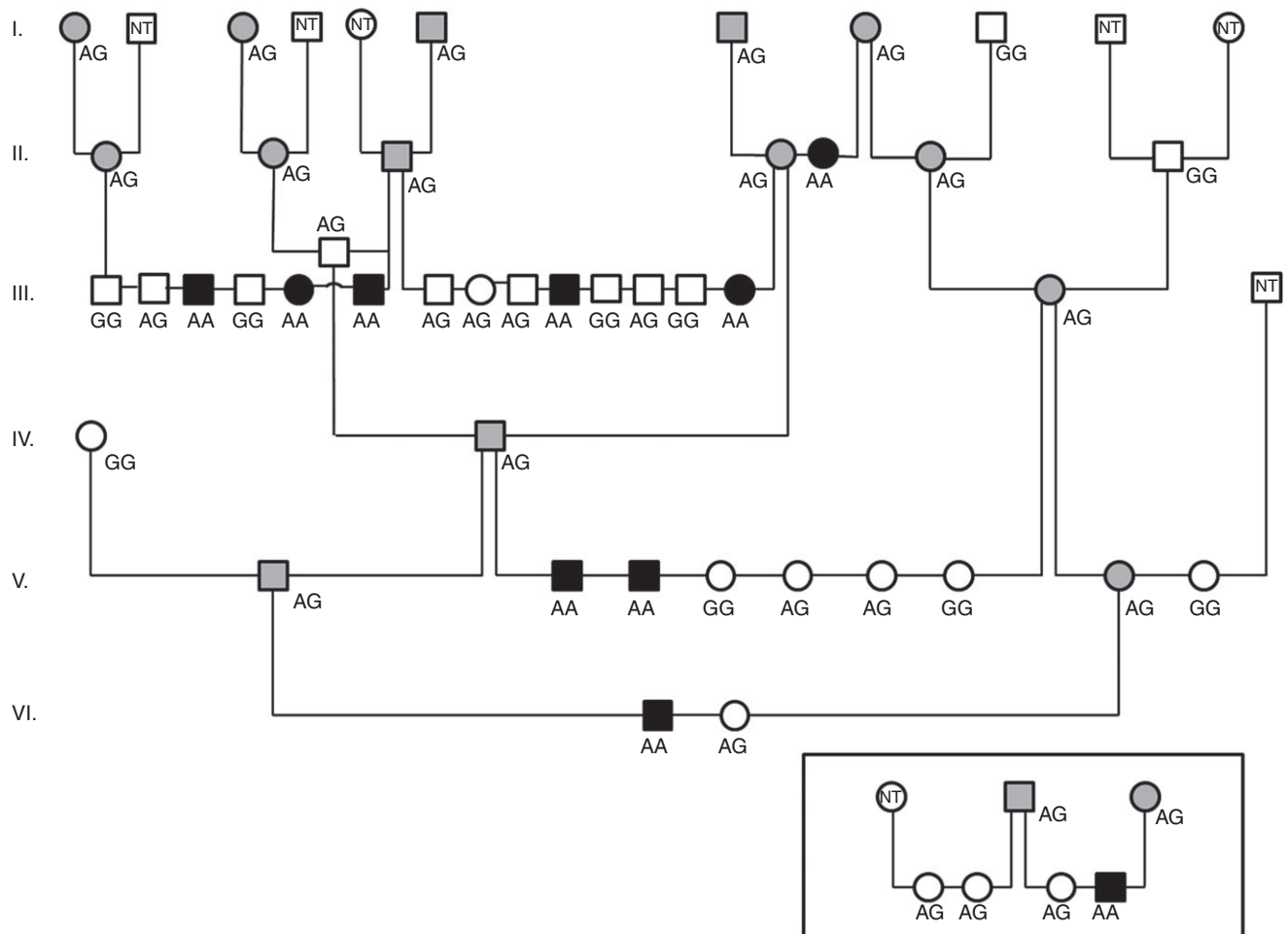


**Fig. 3.** Segregation of the TMEM16F splice site mutation in pedigrees of canine Scott syndrome (CSS) dogs. Solid black symbols represent clinically affected CSS dogs, gray filled symbols represent obligate carrier parents that produced at least one affected offspring. Genotypes for the TMEM16F g. 8912219 G>A mutation are indicated for each symbol as follows: heterozygous (AG), homozygous affected (AA), homozygous wild type (GG). The roman numerals at the left of the pedigree indicate six generations in the index colony and the small inset at lower right displays an unrelated pedigree of white German shepherd dogs. NT, dogs unavailable for molecular analyses.

TMEM16F blocking peptide (Fig. 4B, lane 3) supported the specificity of antibody binding to canine TMEM16 proteins. In contrast, enriched CSS platelet plasma membranes (Fig. 4B, lane 2), showed no reactivity in the molecular weight region for monomeric TMEM16F. A faint band detected at > 250 kDa was considered unlikely to represent TMEM16F given its presence in reactions that contained excess blocking peptide (Fig. 4B, lane 4) and the absence of TMEM16F in the CSS platelet proteomic analyses.

Immunoblots of platelet lysates were also probed with a polyclonal anti-murine TMEM16F antibody. This antibody did not react with canine TMEM16K. The control dogs' lysates demonstrated broad bands in the region of monomeric TMEM16F between 90 and 109 kDa (Fig. 4C, lane 1); however, TMEM16F was not detected in platelet lysates from the four CSS dogs (Fig. 4C, lanes 2–5).

#### TMEM16F cosegregating proteins

A differential proteomics strategy was used to examine proteins segregating in gel fractions with membrane localized TMEM16F. The putative interactome included 32 of 490 proteins whose relative concentration (emPAI) was increased in control vs. CSS-enriched platelet membrane fractions by  $\geq 3$ -fold (Table 2). The list is restricted to proteins migrating > 80 kDa. Seventeen proteins are cytosolic, including Munc 18-1, profilin-1, ADA-1, PKC- $\beta$ , PKA-C  $\beta$ , Bif-1, and 14-3-3 proteins  $\eta$  and  $\epsilon$ . Cytoskeletal, actin-interacting, and lipid raft proteins included  $\alpha$ - and  $\beta$ -actins, F-actin capping protein, flotillin-1, coronin-1, cofilin-1, and B-2 integrin, and Rab GTPases included Rab 13, 14, 29 (7L1), and 32. Additional proteins enriched in the control membranes included CLIC1, an intracellular chloride ion channel, the

**Table 1** Protein abundance (emPAI) estimates for TMEM16 family members and selected membrane proteins in enriched platelet plasma membranes

| Gene    | Protein name                                                      | TM domains | Mass kDa | emPAI <sup>a</sup> |       |
|---------|-------------------------------------------------------------------|------------|----------|--------------------|-------|
|         |                                                                   |            |          | Control            | CSS   |
| ANO6    | Anoctamin-6 (TMEM16F)                                             | 8          | 106      | 3.01               | ND    |
| ANO10   | Anoctamin-10 (TMEM16K)                                            | 8          | 76       | 0.09               | 0.04  |
| ITGA2B  | Platelet glycoprotein IIb                                         | 1          | 114      | 19.92              | 24.47 |
| ITGB3   | Platelet glycoprotein IIIa                                        | 1          | 89       | 14.34              | 16.08 |
| ITGA6   | Integrin $\alpha$ -6                                              | 1          | 123      | 8.53               | 9.31  |
| ITGB1   | Integrin $\beta$ -1                                               | 1          | 91       | 6.19               | 6.19  |
| ITGA5   | Integrin $\alpha$ -V                                              | 1          | 109      | 2.64               | 2.33  |
| ITGA2   | Integrin $\alpha$ -2                                              | 1          | 136      | 2.2                | 2.12  |
| SELP    | P-selectin                                                        | 1          | 94       | 3.32               | 3.95  |
| PECAM1  | Platelet endothelial cell adhesion molecule (PECAM-1)             | 1          | 83       | 2.7                | 2.84  |
| TSPAN29 | CD9 antigen                                                       | 4          | 23       | 0.69               | 0.69  |
| TSPAN14 | Tetraspanin-14                                                    | 4          | 31       | 0.11               | 0.11  |
| ABCD1   | ATP-binding cassette sub-family D member 1                        | 5          | 90       | 0.04               | 0.04  |
| ITPR1   | Inositol 1,4,5-trisphosphate receptor type 1                      | 6          | 309      | 0.04               | 0.16  |
| ATP8A1  | Probable phospholipid-transporting ATPase 1A (P4-ATPase flippase) | 10         | 132      | 0.34               | 0.64  |
| ATP2A3  | Sarcoplasmic/endoplasmic reticulum calcium ATPase 3               | 10         | 115      | 5.68               | 8.35  |

emPAI<sup>a</sup> = Modified protein abundance index (emPAI) values obtained by spectral counting for the mixture of carbonate treated membrane associated proteins extracted from excised gel segments above 80 kDa, including the stacking gel. All proteins identified by proteomic analysis in trypsin digests of gel segments above 80 kDa are listed in Tables S2 and S3, for the control and CSS dog, respectively. TM, transmembrane domains from Uniprot GO annotation; ND, not detected.

calcium-dependent PS binding protein, copine-1, and several proteins implicated in apoptotic signaling.

#### *Defective CSS platelet PS externalization on activation, apoptosis, chemical injury, and ex vivo aging*

Control dog platelets treated with activating stimuli demonstrated rapid scrambling, with PS exposure and release of PS-positive PMP after a 10-min reaction time. In dogs, as in people [3,41], thrombin alone was a weak

stimulus of scrambling that was enhanced by convulxin, while calcium ionophore induced up to 90% PS-positive small platelets and PMP (Fig. 5, top row). The CSS platelets, however, had a greatly impaired response to these activating stimuli (Fig. 5, bottom row). In addition, treatment with a proapoptotic small molecule the BH3 mimetic ABT-737 [42], failed to evoke normal PS externalization in CSS platelets. While ABT737 treatment for 1 h generated up to 95% PS-positive platelets in controls, CSS platelets had maximal values of only 10% PS externalization. Similarly, the CSS platelets had relatively little scrambling response to paraformaldehyde treatment. Paraformaldehyde induces ATP depletion and loss of intracellular calcium regulation in platelets, features that mimic necrotic cell death [43]. Finally, the exposure of platelet PS in stored EDTA blood samples collected remotely from CSS dogs was significantly diminished compared with paired samples from healthy non-GSD or GSD controls. The group mean (SD) percentage of PS positive platelets after 48 h of storage for CSS dogs was 4.9% (2.8) vs. non-GSD control dog mean of 26.7% (10.5) and GSD control mean of 28.9% (12.8) (ANOVA,  $P < 0.001$ ).

## Discussion

This study defines the CSS model as an autosomal recessive trait caused by a highly penetrant splice-site mutation in *TMEM16F*. The disease phenotype resulting from *TMEM16F* inactivation in this naturally occurring genetic disease is limited to signs of platelet dysfunction [34,37,39]. As null mutants, the CSS platelets demonstrate that the absence of platelet membrane TMEM16F results in markedly impaired PS externalization and microvesiculation in response not only to activating stimuli but also to agents that trigger cell death through apoptosis or necrosis and during *ex vivo* storage. Our data indicate a common convergence for the signaling pathways of these diverse processes on TMEM16F's action as a lipid scramblase. We also identified a second platelet anoctamin, TMEM16K, that, unlike TMEM16F, is localized intracellularly rather than within the plasma membrane. The presence of TMEM16K within platelets, however, does not compensate for the lack of TMEM16F-mediated scrambling under any of the stimuli or reaction conditions we tested.

TMEM16F has been previously characterized as the scramblase required for rapid, calcium-activated PS externalization in platelets but with a limited role in apoptosis-induced PS movement [24,28,44]. The CSS platelets' markedly impaired PS externalization in response to cell death triggers and delayed *ex vivo* aging was therefore unexpected, and contrasts with that of a human Scott patient (Scott<sub>UK</sub>) [24]. In that report, similar proportions of Scott<sub>UK</sub> and control platelets labeled with annexin V after treatment with ABT-737 and during storage. Platelet

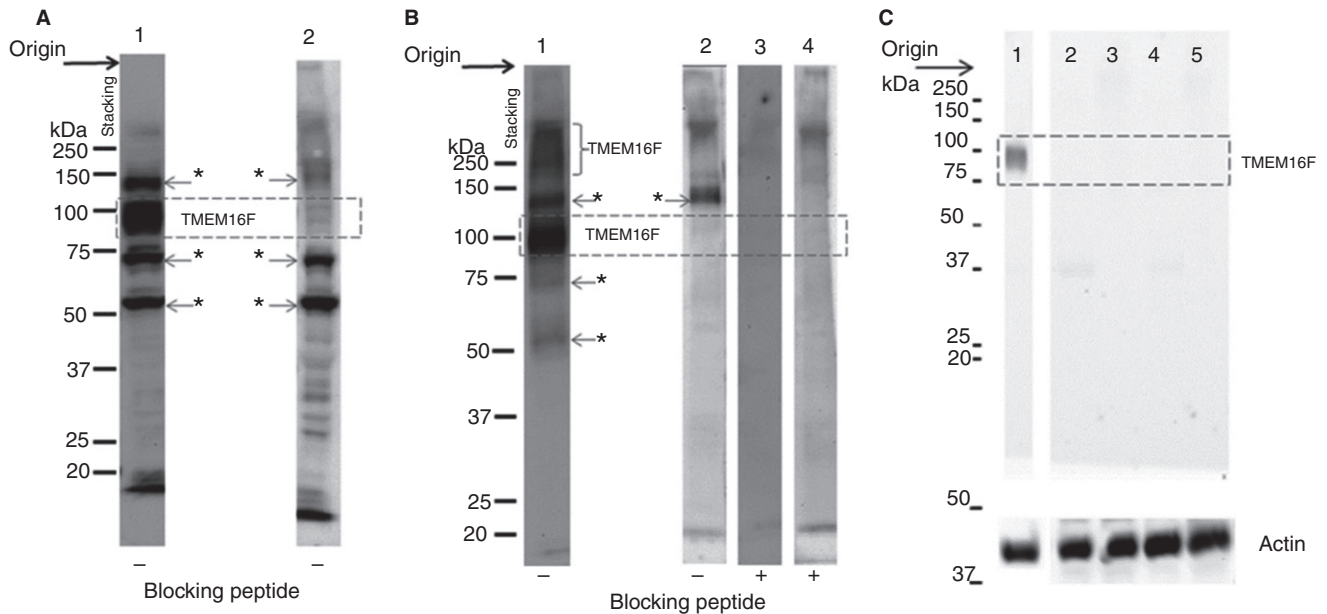


**Fig. 4.** Western blot analyses of TMEM16F protein expression. Washed canine platelets (panel A; control, lane 1, canine Scott syndrome [CSS], lane 2) or enriched canine platelet plasma membranes (panel B; control, lanes 1, 3; CSS, lanes 2, 4) were lysed and solubilized using hot SDS electrophoresis buffer. Equivalent concentrations of cell lysate or membrane proteins were loaded in each lane and separated on 10% gels by 1-D SDS-PAGE, under non-reducing conditions. Proteins were electrotransferred to polyvinylidene fluoride membranes and detected by enhanced chemiluminescence using an antibody (Santa Cruz G14) to a peptide that maps to a region within the third extracellular loop between the fifth and sixth transmembrane domains of human TMEM16F. This region shares 92% homology with canine TMEM16F and 62% homology with canine TMEM16K (isoforms 1–4), with no homology to other TMEM16 family members. Symbols + or – refer to the presence or absence, respectively, of 5-fold excess blocking peptide. Blots of platelet lysates from four CSS dogs (panel C) were probed using a rabbit polyclonal antibody to a peptide corresponding to the N terminus of mouse TMEM16F (generous gift, Dr. Andrew Kim, University of California, San Francisco); control, lane 1; CSS, lanes 2–5. Platelet actin served as loading control. Dashed boxes are in molecular weight region expected for monomeric TMEM16F. In addition to reacting with canine TMEM16F, the Santa Cruz G14 antibody to human TMEM16F reacted with protein bands having molecular weights reported for TMEM16K at approximately 53, 73 and 134 kDa. \*Proteomic analysis of excised gel segments in these regions confirmed the presence of canine TMEM16K; no other TMEM16 family members were detected.

senescence and cell death induced by ABT-737 involve the intrinsic apoptotic pathway, with activation of caspase 9 and downstream effectors such as caspase 3, 6, and 7 [14,42,45]. While caspase-mediated proteolysis of cytoskeletal proteins is not calcium dependent, the rate and extent of platelet PS externalization under apoptotic conditions are influenced by intracellular calcium and enhanced by store-operated calcium entry [24,46]. Apoptotic and necrotic stimuli that readily induced PS externalization in our control dog experiments had minimal effect on CSS platelets in the 1-h reaction time in the absence of TMEM16F-mediated lipid scrambling. Although TMEM16F protein expression has not been reported for human Scott platelets, semiquantitative RT-PCR and Western blot analyses of lymphocytes from the Scott<sub>UK</sub> patient reveal the presence of TMEM16F mRNA and protein [44]. This patient is compound heterozygous for two distinct TMEM16F mutations [29]. It is possible that some TMEM16F is also present in platelets, albeit at very low levels. In this case, the differential apoptotic response may reflect the virtual absence of membrane TMEM16F in CSS platelets vs. a quantitative deficiency in Scott<sub>UK</sub> platelets. Direct comparisons of response are complicated, however, by differences in reaction

conditions and species differences between dogs and people in platelet physiology.

The clinical manifestation of the *TMEM16F* splicing defect in CSS also contrasts with the fetal and neonatal deaths and gross skeletal deformities described in a murine *TMEM16F* knockout generated through excision/replacement of *TMEM16F* exon 1 and adjacent sequence [31]. Detailed longitudinal health surveillance of the index GSD colony has revealed no abnormalities in body conformation or radiographic signs of aberrant bone mineralization and no reduction in fertility or life span [47–49]. Similarly, the few human Scott cases have had no apparent skeletal malformations [50–52], and neonatal mortalities were not reported in a second mouse model generated through deletion of *TMEM16F* exon 2 [30]. Manipulation of the 5' promoter region of *TMEM16F* may have induced tissue-specific and temporal alterations in gene expression or dominant negative effects unique to an engineered model, rather than features of *TMEM16F* mutation per se.

Proteomic analyses of platelet membrane fractions from CSS and control dogs provide additional information on TMEM16F function and regulation. The limited disease phenotype of platelet dysfunction in CSS dogs implies a

**Table 2** Candidate members of a platelet TMEM16F interactome

| Gene            | Protein name                                                   | emPAI   |       | Fold-change | Location             | Function                                                                                                                                              |
|-----------------|----------------------------------------------------------------|---------|-------|-------------|----------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------|
|                 |                                                                | Control | CSS   |             |                      |                                                                                                                                                       |
| <i>PRKCB</i>    | Protein kinase C $\beta$                                       | 0.4     | 0     | ND          | Cytosol, PM          | Ca <sup>2+</sup> DAG activated in the presence of phosphatidylserine; Ca <sup>2+</sup> transport; apoptosis; signal transduction; platelet activation |
| <i>STXBP1</i>   | Syntaxin-binding protein 3 isoform 1 (MUNC 18-1)               | 0.1     | 0     | ND          | Cytosol, PM, Mito    | SNARE and syntaxin binding; Ca <sup>2+</sup> -dependent platelet exocytosis; protein localization to PM                                               |
| <i>PKM</i>      | Pyruvate kinase isozymes M1/M2 isoform 1                       | 1.68    | 0.25  | 6.7         | Cytosol              | ATP binding; carbohydrate metabolism; kinase; transferase; general role in caspase-independent cell death                                             |
| <i>PFN1</i>     | Profilin-1                                                     | 3.79    | 0.19  | 19.9        | Cytosol, CS, MP      | Binds to actin; affects the structure of CS when bound to PIP2; inhibits the formation of IP3 and DAG                                                 |
| <i>ADA</i>      | Adenosine deaminase isoform 1 (ADA-1)                          | 1.16    | 0.08  | 14.5        | Cytosol, PM          | Key enzyme in purine metabolism; necessary to the coupling of A1 adenosine receptors and heterotrimeric G proteins                                    |
| <i>PRKACB</i>   | PKA C $\beta$                                                  | 0.32    | 0.07  | 4.6         | Cytosol, PM          | Mediates cAMP-dependent signaling via GPCRs; activation regulates diverse cellular processes; microtubule dynamics; transport mechanisms and ion flux |
| <i>ACTC1</i>    | Actin, $\alpha$ cardiac muscle 1 isoform 3                     | 45.4    | 10.11 | 4.5         | Cytosol, CS          | ATP binding; myosin binding; actin-myosin filament binding; negative regulation of apoptosis                                                          |
| <i>ACO3M</i>    | Aconitate hydratase, mitochondrial isoform 3                   | 0.51    | 0.12  | 4.25        | Cytosol, Mito        | TCA cycle; DNA binding; iron-sulfur cluster binding                                                                                                   |
| <i>HSP90AA1</i> | Heat shock protein HSP 90- $\alpha$ isoform 1                  | 0.51    | 0.13  | 3.9         | Cytosol, CM          | Chaperone protein; ATPase; protein homodimerization; NO metabolism; protein refolding; protein import to outer Mito membrane; signal transduction     |
| <i>LIMS3</i>    | LIM and senescent cell antigen-like domains 3-like isoform 2   | 0.28    | 0.08  | 3.5         | Cytosol              | Zn and metal ion binding                                                                                                                              |
| <i>PLEK</i>     | Pleckstrin                                                     | 4.17    | 1.19  | 3.5         | Cytosol, PM          | PKC and PtdIns(3,4)P2 binding; CS reorganization; platelet activation and degranulation; PAR and integrin signaling; actin filament bundle assembly   |
| <i>BIN2</i>     | Bridging integrator 2                                          | 0.37    | 0.11  | 3.3         | Cytosol, PM          | Membrane tubulation; phospholipid and protein binding                                                                                                 |
| <i>SHLB1</i>    | Endophilin-B1 (BIF-1), BAX-interacting factor-1                | 0.32    | 0.1   | 3.2         | Cytosol, PM, G, Mito | Apoptosis; binds BAX; protein homodimerization, oligomerization; recruits proteins to membranes with high curvature; promotes membrane fusion         |
| <i>YWHAE</i>    | 14-3-3 protein $\epsilon$ isoform 2                            | 2.27    | 0.71  | 3.19        | Cytosol              | Phosphoserine, ion channel and enzyme binding; intrinsic apoptotic signaling pathway; membrane repolarization; intracellular signal transduction      |
| <i>YWHAH</i>    | 14-3-3 protein $\eta$                                          | 3.72    | 1.17  | 3.18        | Cytosol, Mito, VM    | Enzyme; ion channel and phosphoserine binding; intrinsic apoptotic signaling; K <sup>+</sup> ion transport; intracellular signal transduction         |
| <i>LTA4H</i>    | Leukotriene A-4 hydrolase                                      | 0.15    | 0.05  | 3           | Cytosol              | Lipid metabolism; aminopeptidase activity; Zn ion binding                                                                                             |
| <i>BIN1</i>     | Myc box-dependent interacting protein 1, bridging integrator 1 | 0.15    | 0.05  | 3           | Cytosol, CS, PM      | Endocytosis; apoptosis; protein heterodimerization activity                                                                                           |
| <i>RAB14</i>    | Ras-related protein Rab-14 isoform 1                           | 0.3     | 0     | ND          | VM, G                | Protein transport; nucleotide binding—GDP, GTP                                                                                                        |
| <i>CAPZB</i>    | F-actin capping protein subunit $\beta$                        | 0.49    | 0     | ND          | CS                   | Binds actin filaments (barbed end); regulation of cell morphology and CS organization                                                                 |
| <i>ACTB</i>     | Actin, cytoplasmic-1, $\beta$ -actin                           | 456.62  | 73.51 | 6.2         | CS, PM, MP           | Protein complexes; structural constituent of cytoskeleton; ATP binding; membrane organization; platelet aggregation; protein folding                  |
| <i>RAB13</i>    | Ras-related protein Rab-13                                     | 0.22    | 0     | ND          | PM                   | Transports transmembrane proteins to PM                                                                                                               |
| <i>CLIC1</i>    | Chloride intracellular channel protein 1                       | 0.41    | 0     | ND          | PM,                  | Chloride ion channel; stabilizes cell membrane potential; regulation cell volume and pH                                                               |
| <i>FLOT1</i>    | Flotillin-1 isoform 2                                          | 0.31    | 0     | ND          | PM, caveolae         | Flotillin complex; protein localization to plasma membrane; cell adhesion                                                                             |
| <i>RAB29</i>    | Ras-related protein Rab-7L1 isoform 1                          | 0.14    | 0     | ND          | PM                   | Lipid anchor- cytoplasmic side; Rab protein signal transduction; protein transport—PM to Golgi                                                        |

Table 2 (Continued)

| Gene           | Protein name                                              | emPAI   |      | Fold-change | Location        | Function                                                                                                                                                      |
|----------------|-----------------------------------------------------------|---------|------|-------------|-----------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                |                                                           | Control | CSS  |             |                 |                                                                                                                                                               |
| <i>CORO1C</i>  | Coronin-1C isoform 2                                      | 0.61    | 0.06 | 10.1        | Cytosol, PM, CS | Cell projections; lamellipodium; motility and signal transduction                                                                                             |
| <i>CFL1</i>    | Cofilin-1 isoform 1                                       | 2.75    | 0.39 | 7           | PM, CS          | Cytoskeleton organization; platelet activation and degranulation; actin filament depolarization; Rho signal transduction                                      |
| <i>ITGB2</i>   | B-2 integrin                                              | 0.29    | 0.08 | 3.6         | PM              | ICAM receptor activity; cell adhesion molecule and protein kinase binding; apoptosis; extracellular matrix organization; integrin and Toll receptor signaling |
| <i>CPNE1</i>   | Copine-1                                                  | 0.31    | 0.05 | 6.2         | PM              | Transporter activity; Ca <sup>2+</sup> -dependent phosphatidylserine binding                                                                                  |
| <i>RAB32</i>   | Ras-related protein Rab-32                                | 0.31    | 0    | ND          | ER, Mito        | Functions as a PKA-anchoring protein; regulator of mitochondria-associated membrane properties; speed of apoptosis onset                                      |
| <i>ACSL1</i>   | Long-chain fatty acid CoA ligase 1 isoform 5              | 0.13    | 0.04 | 3.25        | ER, Mito        | ATP binding and long-chain fatty acid CoA ligase activity; regulation of extrinsic apoptotic signaling                                                        |
| <i>ABAT</i>    | 4-aminobutyrate aminotransferase, mitochondrial isoform 2 | 0.48    | 0.06 | 8           | Mito            | Aminotransferase and protein homodimerization activity                                                                                                        |
| <i>TOMM70A</i> | Mitochondrial import receptor subunit TOM70               | 0.15    | 0.05 | 3           | Mito            | Protein targeting to mitochondria                                                                                                                             |

Location and function assigned using UniProt GO annotation. ND, not detected in membrane fraction; present in whole platelet lysate; PM, plasma membrane; CS, cytoskeleton; MP, microparticles; Mito, mitochondria; CM, cell membrane; G, golgi; VM, cytoplasmic vesicle membranes; ER, endoplasmic reticulum. DAG, diacylglycerol; SNARE, soluble NSF (N-ethylmaleimide sensitive fusion proteins) attachment protein receptor; IP3, inositol trisphosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; GPCR, G protein-coupled receptor; TCA, tricarboxylic acid NO = nitric oxide; HSP, heat shock protein protein; LIM, LIM domain; PKC, protein kinase C; PtdIns(3,4)P2, Phosphatidylinositol (3,4)-bisphosphate; PAR, protease-activated receptor; BAX, Bcl-2-associated X protein; GDP, guanosine diphosphate; GTP, guanosine triphosphate; ICAM, intracellular adhesion molecule; ER, endoplasmic reticulum; CoA, coenzyme A.

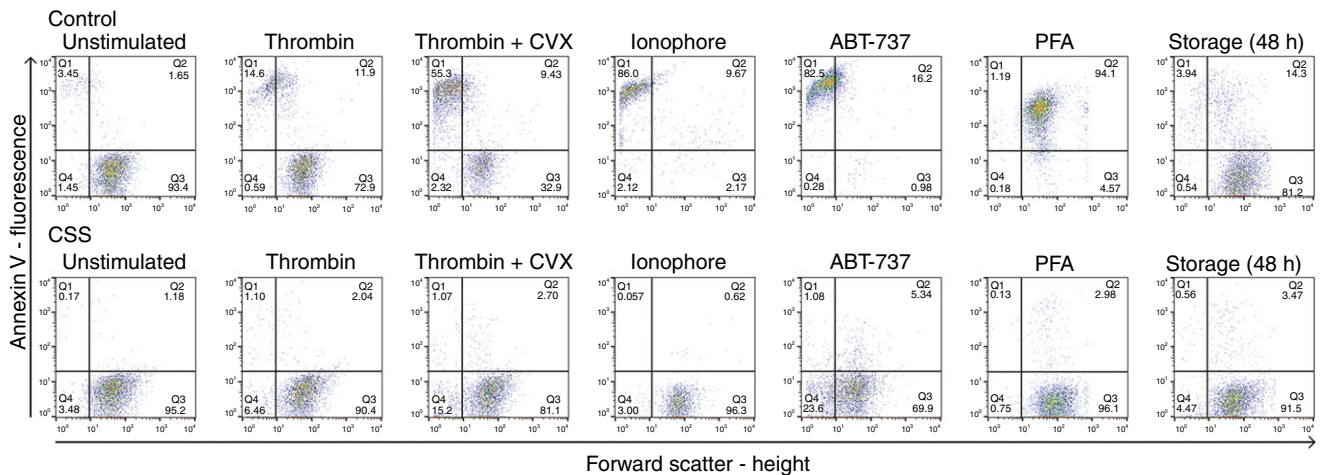


**Fig. 5.** Flow cytometric detection of phosphatidylserine externalization in control dog and canine Scott syndrome (CSS) platelets in response to activating agents, proapoptotic ABT737, paraformaldehyde, and *ex vivo* aging. Density plots depict fluorescence intensity (*y* axis) and forward scatter (*x* axis) for platelet rich plasma from a control dog (top row) and a CSS dog (bottom row). The samples were labeled with Annexin V-FITC after the following treatments: Incubation in a calcium buffer at 37 °C for 15 min with no agonist (unstimulated), thrombin (0.5 U mL<sup>-1</sup>), combined thrombin (0.5 U mL<sup>-1</sup>) with convulxin (CVX, 500 ng mL<sup>-1</sup>), ionophore (A23187, 3 μmol L<sup>-1</sup>), or 1 h with ABT737 (2 μmol L<sup>-1</sup>), or 0.5% paraformaldehyde (PFA), or held at room temperature in EDTA whole blood for 48 h (storage). Representative plots from a single dog are shown. The median (range) of percent annexin V-labeled platelet events for CSS dogs (*n* = 4) was: unstimulated, 4% (1–9); thrombin, 10% (3–13); thrombin + CVX, 12% (2–20); ionophore, 12% (1–22); ABT737 8% (2–10); and PFA, 3% (2–18). Mean percent annexin V labeled for EDTA blood samples (*n* = 20), 4.9%.

greater reliance for TMEM16F-mediated scrambling in platelets compared with other cell types. For example, immortalized lymphocytes from the Scott<sub>UK</sub> patient and

the index Scott patient, Scott<sub>USA</sub>, demonstrate normal PS externalization in response to ABT737 [44], a response markedly reduced in CSS platelets lacking TMEM16F. In

contrast to platelets, immortalized lymphocytes and leukemia/lymphoma cell lines express a membrane flippase, ATP11C, and a membrane scramblase, XKR8, that are activated by caspases resulting in calcium-independent PS exposure [53,54]. These lipid transporters are not detected in human platelets [38,55] and were not found in our proteomic analyses of canine platelets.

Differential proteomic analyses revealed a subset of proteins markedly reduced in the CSS platelet plasma membranes compared with controls. Considering endogenous TMEM16F as 'bait' for potential interacting partners, we found that control platelet membranes contained actin-binding and regulatory proteins, small GTPases, calcium-binding proteins, kinases, and lipid-reactive proteins lacking in the CSS membrane fractions. This putative TMEM16F interactome overlaps the categories of binding partners identified for TMEM16A, a related anoctamin, overexpressed in HEK cells [56]. Unlike TMEM16A, however, we did not find an apparent association of TMEM16F with the ezrin–radixin–moesin network of signaling and scaffold proteins. The calcium transporter, NCX1, has previously been described as a binding partner of TMEM16F in osteoblasts [57]. We did not identify NCX1 in the canine platelet proteome, but CLIC1, a chloride channel with a regulatory role in osteoblast differentiation [58], was detected. Relevant to platelet TMEM16F's role as a phospholipid scramblase mediating procoagulant activity and apoptosis, cosegregating proteins included the calcium-dependent PS binding protein, copine-1, and proteins such as SHLB1 (Bax interacting factor -1), BIN1, and RAB32, known as regulators of the intrinsic apoptotic pathway [59–61]. The semiquantitative nature of the CSS differential proteome did not provide any information on stoichiometry and temporal or spatial relationships among proteins. The composition of the putative interactome was also biased by 'basal' intraplatelet  $\text{Ca}^{2+}$ , size selection, method of platelet disruption, extent of cytosolic and organelle contamination, and carbonate extractability of membrane-associated proteins. Studies using protein crosslinking agents will be required to provide direct evidence of protein interaction with TMEM16F.

TMEM16F represents a potential target for pharmacologic modulation of platelet function. The CSS model demonstrates that a specific lack of TMEM16F-mediated scramblase activity does not alter platelet aggregation or degranulation or cause side effects such as thrombocytopenia or thrombocytosis. TMEM16F inhibition may also enhance viability and prolong storage times of blood-banked platelets. However, the variable disease phenotypes associated with naturally occurring TMEM16F mutations and the engineered mouse model demonstrate gaps in the understanding of TMEM16F's expression and regulation across species and among cell types.

## Addendum

M. B. Brooks and J. L. Catalfamo designed the study, conceived experiments, analyzed data, and wrote the manuscript. R. MacNguyen, D. Tim, and S. Fancher designed and performed molecular genetic experiments and analyzed data. J. A. McCardle performed proteomics, analyzed data, and wrote the manuscript.

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## Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Primer pairs used for genomic DNA and cDNA amplification of canine TMEM16F.

**Table S2.** A list of all proteins confidently identified in the enriched platelet plasma membrane sub proteome of the control dog. The sub proteome represents proteins migrating above 80 kDa including those within the stacking gel above 250 kDa. The NCBI canis (8/2013) database was not manually curated to remove duplicate protein entries. Anoctamin family members are highlighted in green (TMEM16F, Anoctamin 6, rank protein hit rank 49 and TMEM16K, Anoctamin 10, protein hit rank 303). Membrane associated integrins are highlighted in blue. Platelet membrane glycoproteins IIb and IIIa are highly enriched.

**Table S3.** A list of all proteins confidently identified in the enriched platelet plasma membrane sub proteome of the CSS dog. The sub proteome represents proteins migrating above 80 kDa including those within the stacking gel above 250 kDa. The NCBI canis (8/2013) database was not manually curated to remove duplicate protein entries. Anoctamin family members are highlighted in green. Membrane associated integrins are highlighted in blue. Platelet membrane glycoproteins IIb and IIIa are highly enriched. In contrast to TMEM16F, anoctamin 10 which was readily identified as a low abundance protein in enriched membranes from the control and CSS dog, TMEM16F, Anoctamin 6 was not detected in enriched platelet plasma membranes from the CSS dog.

## References

- 1 Dale GL. Coated-platelets: an emerging component of the procoagulant response. *J Thromb Haemost* 2005; 3: 2185–92.

- 2 de Witt SM, Verdoold R, Cosemans JM, Heemskerk JW. Insights into platelet-based control of coagulation. *Thromb Res* 2014; **133**(Suppl. 2): S139–48.
- 3 Freyssinet JM, Toti F. Formation of procoagulant microparticles and properties. *Thromb Res* 2010; **125**(Suppl. 1): S46–8.
- 4 Heemskerk JW, Siljander PR, Bevers EM, Farndale RW, Lindhout T. Receptors and signalling mechanisms in the procoagulant response of platelets. *Platelets* 2000; **11**: 301–6.
- 5 Heemskerk JW, Mattheij NJ, Cosemans JM. Platelet-based coagulation: different populations, different functions. *J Thromb Haemost* 2013; **11**: 2–16.
- 6 Mazepa M, Hoffman M, Monroe D. Superactivated platelets: thrombus regulators, thrombin generators, and potential clinical targets. *Arterioscler Thromb Vasc Biol* 2013; **33**: 1747–52.
- 7 Zwaal RF, Comfurius P, Bevers EM. Surface exposure of phosphatidylserine in pathological cells. *Cell Mol Life Sci* 2005; **62**: 971–88.
- 8 Bevers EM, Comfurius P, Dekkers DW, Harmsma M, Zwaal RF. Regulatory mechanisms of transmembrane phospholipid distributions and pathophysiological implications of transbilayer lipid scrambling. *Lupus* 1998; **7**: S126–31.
- 9 Dachary-Prigent J, Toti F, Satta N, Pasquet JM, Uzan A, Freyssinet JM. Physiopathological significance of catalytic phospholipids in the generation of thrombin. *Semin Thromb Hemost* 1996; **22**: 157–64.
- 10 Zwaal RF, Comfurius P, Bevers EM. Platelet procoagulant activity and microvesicle formation. Its putative role in hemostasis and thrombosis. *Biochim Biophys Acta* 1992; **1180**: 1–8.
- 11 Dasgupta SK, Argaz ER, Mercado JE, Maul HO, Garza J, Enriquez AB, Abdel-Monem H, Prakasam A, Andreff M, Thiarajan P. Platelet senescence and phosphatidylserine exposure. *Transfusion* 2010; **50**: 2167–75.
- 12 Leytin V, Allen DJ, Mutlu A, Mykhaylov S, Lyubimov E, Freedman J. Platelet activation and apoptosis are different phenomena: evidence from the sequential dynamics and the magnitude of responses during platelet storage. *Br J Haematol* 2008; **142**: 494–7.
- 13 Gyulkhandanyan AV, Mutlu A, Freedman J, Leytin V. Markers of platelet apoptosis: methodology and applications. *J Thromb Thrombolysis* 2012; **33**: 397–411.
- 14 Kile BT. The role of apoptosis in megakaryocytes and platelets. *Br J Haematol* 2014; **165**: 217–26.
- 15 Mason KD, Carpinelli MR, Fletcher JI, Collinge JE, Hilton AA, Ellis S, Kelly PN, Ekert PG, Metcalf D, Roberts AW, Huang DC, Kile BT. Programmed anuclear cell death delimits platelet life span. *Cell* 2007; **128**: 1173–86.
- 16 Bevers EM, Williamson PL. Phospholipid scramblase: an update. *FEBS Lett* 2010; **584**: 2724–30.
- 17 Jobe SM, Wilson KM, Leo L, Raimondi A, Molkentin JD, Lentz SR, Di Paola J. Critical role for the mitochondrial permeability transition pore and cyclophilin D in platelet activation and thrombosis. *Blood* 2008; **111**: 1257–65.
- 18 Kunzelmann K, Nilius B, Owsianik G, Schreiber R, Ousingsawat J, Sirianant L, Wanitchakool P, Bevers EM, Heemskerk JW. Molecular functions of anoctamin 6 (TMEM16F): a chloride channel, cation channel, or phospholipid scramblase? *Pflügers Arch* 2014; **466**: 407–14.
- 19 Lhermusier T, Chap H, Payrastré B. Platelet membrane phospholipid asymmetry: from the characterization of a scramblase activity to the identification of an essential protein mutated in Scott syndrome. *J Thromb Haemost* 2011; **9**: 1883–91.
- 20 Suzuki J, Fujii T, Imao T, Ishihara K, Kuba H, Nagata S. Calcium-dependent phospholipid scramblase activity of TMEM16 protein family members. *J Biol Chem* 2013; **288**: 13305–16.
- 21 Suzuki J, Umeda M, Sims PJ, Nagata S. Calcium-dependent phospholipid scrambling by TMEM16F. *Nature* 2010; **468**: 834–8.
- 22 Jackson SP, Schoenwaelder SM. Procoagulant platelets: are they necrotic? *Blood* 2010; **116**: 2011–8.
- 23 Juul CA, Grubb S, Poulsen KA, Kyed T, Hashem N, Lambert IH, Larsen EH, Hoffmann EK. Anoctamin 6 differs from VRAC and VSOAC but is involved in apoptosis and supports volume regulation in the presence of Ca(2+). *Pflügers Arch* 2014; **466**: 1899–910.
- 24 van Kruchten R, Mattheij NJ, Saunders C, Feijge MA, Swieringa F, Wolfs JL, Collins PW, Heemskerk JW, Bevers EM. Both TMEM16F-dependent and TMEM16F-independent pathways contribute to phosphatidylserine exposure in platelet apoptosis and platelet activation. *Blood* 2013; **121**: 1850–7.
- 25 Brunner JD, Lim NK, Schenck S, Duerst A, Dutzler R. X-ray structure of a calcium-activated TMEM16 lipid scramblase. *Nature* 2014; **516**: 207–12.
- 26 Kunzelmann K, Tian Y, Martins JR, Faria D, Kongsuphol P, Ousingsawat J, Thevenod F, Roussa E, Rock J, Schreiber R. Anoctamins. *Pflügers Arch* 2011; **462**: 195–208.
- 27 Pedemonte N, Galletta LJ. Structure and function of TMEM16 proteins (anoctamins). *Physiol Rev* 2014; **94**: 419–59.
- 28 Piccolo A, Malvezzi M, Accardi A. TMEM16 proteins: unknown structure and confusing functions. *J Mol Biol* 2015; **427**: 94–105.
- 29 Castoldi E, Collins PW, Williamson PL, Bevers EM. Compound heterozygosity for 2 novel TMEM16F mutations in a patient with Scott syndrome. *Blood* 2011; **117**: 4399–400.
- 30 Yang H, Kim A, David T, Palmer D, Jin T, Tien J, Huang F, Cheng T, Coughlin SR, Jan YN, Jan LY. TMEM16F forms a Ca<sup>2+</sup>-activated cation channel required for lipid scrambling in platelets during blood coagulation. *Cell* 2012; **151**: 111–22.
- 31 Ehlen HW, Chinenkova M, Moser M, Munter HM, Krause Y, Gross S, Brachvogel B, Wuelling M, Kornak U, Vortkamp A. Inactivation of anoctamin-6/Tmem16f, a regulator of phosphatidylserine scrambling in osteoblasts, leads to decreased mineral deposition in skeletal tissues. *J Bone Miner Res* 2013; **28**: 246–59.
- 32 Ousingsawat J, Wanitchakool P, Kmit A, Romao AM, Jantarajit W, Schreiber R, Kunzelmann K. Anoctamin 6 mediates effects essential for innate immunity downstream of P2X7 receptors in macrophages. *Nat Commun* 2015; **6**: 6245.
- 33 Brooks M, Etter K, Catalfamo J, Brisbin A, Bustamante C, Mezey J. A genome-wide linkage scan in German shepherd dogs localizes canine platelet procoagulant deficiency (Scott syndrome) to canine chromosome 2L. *Gene* 2010; **450**: 70–5.
- 34 Brooks MB, Catalfamo JL, Brown HA, Ivanova P, Lovaglio J. A hereditary bleeding disorder of dogs caused by a lack of platelet procoagulant activity. *Blood* 2002; **99**: 2434–41.
- 35 Brooks MB, Catalfamo JL, Friesse P, Dale GL. Scott syndrome dogs have impaired coated-platelet formation and calcein-release but normal mitochondrial depolarization. *J Thromb Haemost* 2007; **5**: 1972–4.
- 36 Brooks MB, Randolph J, Warner K, Center S. Evaluation of platelet function screening tests to detect platelet procoagulant deficiency in dogs with Scott syndrome. *Vet Clin Pathol* 2009; **38**: 306–15.
- 37 Jandrey KE, Norris JW, Tucker M, Brooks MB. Clinical characterization of canine platelet procoagulant deficiency (Scott syndrome). *J Vet Intern Med* 2012; **26**: 1402–7.
- 38 Lewandrowski U, Wortelkamp S, Lohrig K, Zahedi RP, Wolters DA, Walter U, Sickmann A. Platelet membrane proteomics: a novel repository for functional research. *Blood* 2009; **114**: e10–9.
- 39 Brooks MB, Catalfamo JL, Etter K, Brisbin A, Bustamante CD. Exclusion of ABCA-1 as a candidate gene for canine Scott syndrome. *J Thromb Haemost* 2008; **6**: 1608–10.
- 40 Remenyi G, Szasz R, Friesse P, Dale GL. Role of mitochondrial permeability transition pore in coated-platelet formation. *Arterioscler Thromb Vasc Biol* 2005; **25**: 467–71.

- 41 Dale GL, Friese P. Bax activators potentiate coated-platelet formation. *J Thromb Haemost* 2006; **4**: 2664–9.
- 42 Vogler M, Weber K, Dinsdale D, Schmitz I, Schulze-Osthoff K, Dyer MJ, Cohen GM. Different forms of cell death induced by putative BCL2 inhibitors. *Cell Death Differ* 2009; **16**: 1030–9.
- 43 Wong K, Li X, Ma Y. Paraformaldehyde induces elevation of intracellular calcium and phosphatidylserine externalization in platelets. *Thromb Res* 2006; **117**: 537–42.
- 44 Kmit A, van Kruchten R, Ousingsawat J, Mattheij NJ, Senden-Gijsbers B, Heemskerk JW, Schreiber R, Bevers EM, Kunzelmann K. Calcium-activated and apoptotic phospholipid scrambling induced by Ano6 can occur independently of Ano6 ion currents. *Cell Death Dis* 2013; **4**: e611.
- 45 Schoenwaelder SM, Yuan Y, Josefsson EC, White MJ, Yao Y, Mason KD, O'Reilly LA, Henley KJ, Ono A, Hsiao S, Willcox A, Roberts AW, Huang DC, Salem HH, Kile BT, Jackson SP. Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function. *Blood* 2009; **114**: 663–6.
- 46 Clark SR, Thomas CP, Hammond VJ, Aldrovandi M, Wilkinson GW, Hart KW, Murphy RC, Collins PW, O'Donnell VB. Characterization of platelet aminophospholipid externalization reveals fatty acids as molecular determinants that regulate coagulation. *Proc Natl Acad Sci USA* 2013; **110**: 5875–80.
- 47 Cole JB, Franke DE, Leighton EA. Population structure of a colony of dog guides. *J Anim Sci* 2004; **82**: 2906–12.
- 48 Helmink SK, Rodriguez-Zas SL, Shanks RD, Leighton EA. Estimated genetic parameters for growth traits of German shepherd dog and Labrador retriever dog guides. *J Anim Sci* 2001; **79**: 1450–6.
- 49 Leighton EA. Genetics of canine hip dysplasia. *J Am Vet Med Assoc* 1997; **210**: 1474–9.
- 50 Munnix IC, Harmsma M, Giddings JC, Collins PW, Feijge MA, Comfurius P, Heemskerk JW, Bevers EM. Store-mediated calcium entry in the regulation of phosphatidylserine exposure in blood cells from Scott patients. *Thromb Haemost* 2003; **89**: 687–95.
- 51 Toti F, Satta N, Fressinaud E, Meyer D, Freyssinet JM. Scott syndrome, characterized by impaired transmembrane migration of procoagulant phosphatidylserine and hemorrhagic complications, is an inherited disorder. *Blood* 1996; **87**: 1409–15.
- 52 Weiss HJ. Scott syndrome: a disorder of platelet coagulant activity. *Semin Hematol* 1994; **31**: 312–9.
- 53 Segawa K, Kurata S, Yanagihashi Y, Brummelkamp TR, Matsuda F, Nagata S. Caspase-mediated cleavage of phospholipid flippase for apoptotic phosphatidylserine exposure. *Science* 2014; **344**: 1164–8.
- 54 Suzuki J, Denning DP, Imanishi E, Horvitz HR, Nagata S. Xk-related protein 8 and CED-8 promote phosphatidylserine exposure in apoptotic cells. *Science* 2013; **341**: 403–6.
- 55 Burkhart JM, Vaudel M, Gambaryan S, Radau S, Walter U, Martens L, Geiger J, Sickmann A, Zahedi RP. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood* 2012; **120**: e73–82.
- 56 Perez-Cornejo P, Gokhale A, Duran C, Cui Y, Xiao Q, Hartzell HC, Faundez V. Anoctamin 1 (Tmem16A)  $\text{Ca}^{2+}$ -activated chloride channel stoichiometrically interacts with an ezrin-radixin-moesin network. *Proc Natl Acad Sci USA* 2012; **109**: 10376–81.
- 57 Ousingsawat J, Wanitchakool P, Schreiber R, Wuelling M, Vorkamp A, Kunzelmann K. Anoctamin 6 controls bone mineralization by activating the calcium transporter NCX1. *J Biol Chem* 2015; **290**: 6270–80.
- 58 Yang JY, Jung JY, Cho SW, Choi HJ, Kim SW, Kim SY, Kim HJ, Jang CH, Lee MG, Han J, Shin CS. Chloride intracellular channel 1 regulates osteoblast differentiation. *Bone* 2009; **45**: 1175–85.
- 59 Bui M, Gilady SY, Fitzsimmons RE, Benson MD, Lynes EM, Gesson K, Alto NM, Strack S, Scott JD, Simmen T. Rab32 modulates apoptosis onset and mitochondria-associated membrane (MAM) properties. *J Biol Chem* 2010; **285**: 31590–602.
- 60 Etxebarria A, Terrones O, Yamaguchi H, Landajuela A, Landeta O, Antonsson B, Wang HG, Basanez G. Endophilin B1/Bif-1 stimulates BAX activation independently from its capacity to produce large scale membrane morphological rearrangements. *J Biol Chem* 2009; **284**: 4200–12.
- 61 Prokic I, Cowling BS, Laporte J. Amphiphysin 2 (BIN1) in physiology and diseases. *J Mol Med* 2014; **92**: 453–63.