

Calpain cleaves phospholipid flippase ATP8A1 during apoptosis in platelets

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Key Points

- ATP8A1 is highly expressed in platelets, but is not present in the plasma membrane.
- ATP8A1 is cleaved by calpain during platelet apoptosis in a caspase-dependent manner.

The asymmetric distribution of phospholipids in the plasma/organelle membranes is generated and maintained through phospholipid flippases in resting cells, but becomes disrupted in apoptotic cells and activated platelets, resulting in phosphatidylserine (PS) exposure on the cell surface. Stable PS exposure during apoptosis requires inactivation of flippases to prevent PS from being reinternalized. Here we show that flippase ATP8A1 is highly expressed in both murine and human platelets, but is not present in the plasma membrane. ATP8A1 is cleaved by the cysteine protease calpain during apoptosis, and the cleavage is prevented indirectly by caspase inhibition, involving blockage of calcium influx into platelets and subsequent calpain activation. In contrast, in platelets activated with thrombin and collagen and exposing PS, ATP8A1 remains intact. These data reveal a novel mechanism of flippase cleavage and suggest that flippase activity in intracellular membranes differs between platelets undergoing apoptosis and activation.

Introduction

Mammalian platelets are enucleated specialized blood cells essential for hemostasis and thrombosis.¹ Similar to all mammalian cells, the plasma membrane of platelets consists of an asymmetrical phospholipid bilayer with phosphatidylcholine and sphingomyelin concentrated mainly in the outer leaflet, and phosphatidylserine (PS) and phosphatidylethanolamine confined predominantly to the cytoplasmic leaflet.² In general, the asymmetric distribution of phospholipids is generated and maintained by 2 groups of adenosine triphosphate (ATP)-dependent transporters; floppases, belonging to ATP-binding cassette transporters, are responsible for the transport of phosphatidylcholine and sphingomyelin to the exoplasmic leaflet, whereas flippases, belonging to the P4-type ATPase family, mediate the transport of PS and phosphatidylethanolamine to the inner plasma leaflet.^{3,4} The asymmetric distribution of phospholipids is disrupted by a third group of transporters, known as scramblases, that function in an ATP-independent manner, resulting in PS exposure on the cell surface.^{5,6} In platelets, PS exposure occurs as a result of the 2 distinct pathways, apoptosis and activation, contributing to the clearance of apoptotic platelets and blood coagulation, respectively.^{1,6}

In addition to the plasma membrane, flippases also function in generating phospholipid asymmetry in intracellular membranes, including endoplasmic reticulum, *trans*-Golgi network, and endosomes,^{5,7-9} and play a critical role in vesicle-mediated protein trafficking.^{10,11} Flippases are made up of 10 transmembrane helices and 3 cytosolic domains involved in the ATPase catalytic cycle. Most flippases function in a complex with the ancillary β -subunit, cell cycle control protein 50 (CDC50).^{4,12} There are 14 members of the P4-type ATPase family in human and 15 members in mice,¹³ some of which have been shown to play important roles in many physiological and pathological processes. For example, previous studies from our group and others showed that ATP11C mediates significant PS flipping in murine leukocytes¹⁴ and human erythrocytes.¹⁵ The loss of ATP11C activity causes B-cell deficiency,^{16,17} cholestasis,¹⁸ and

anemia¹⁹ in mice, and congenital hemolytic anemia in humans.¹⁵ In platelets, however, transcriptome²⁰ and proteome²¹⁻²⁴ studies suggest that ATP11C expression is low, with expression of the related flippase, ATP8A1, being much higher.²⁰⁻²⁴ Previous evidence, gathered in immune cells and related cell lines, suggested that inactivation of flippases by caspases was required to support PS exposure in apoptotic cells.²⁵⁻²⁷ In addition, a recent study revealed a mechanism of flippase downregulation via clathrin-mediated endocytosis in nonapoptotic cells.²⁸ Despite many studies being undertaken on flippases, it remains unclear, however, which flippase is active in platelets, and how flippase activity is regulated during platelet apoptosis and activation.²⁹

We report here that ATP8A1 is the most abundant flippase in mouse and human platelets, but is not located at the plasma membrane. During apoptosis, it is cleaved by a previously unrecognized pathway involving the calcium-dependent cysteine protease calpain, and not by caspases. Consistently, prevention of calcium influx into platelets through inhibition of caspases indirectly protects ATP8A1 from cleavage because of a lack of calpain activation. In contrast to apoptosis, ATP8A1 remains intact during the activation of platelets induced by physiological agonists. Our results provide evidence of a novel pathway of flippase cleavage that happens in apoptotic platelets, but not in activated platelets.

Methods

Blood collection and platelet preparation

Mouse. All animal procedures were approved by the Australian National University's Animal Experimentation Ethics Committee (Protocol A2014/62 and A2017/54). C57BL/6J mice and ambrosius mouse strain with an X-linked *N*-ethyl-*N*-nitrosourea-induced point mutation in the gene encoding ATP11C has been described previously.¹⁶ Blood was collected by cardiac puncture from mice (male, 8-20 weeks of age) that had been euthanized by CO₂, and anticoagulated with acid-citrate-dextrose (69 mM citric acid, 85 mM sodium citrate, 20 mg/mL D-glucose at pH 4.6). Washed platelets were prepared as described previously³⁰ and resuspended in modified Tyrode's buffer (138 mM NaCl, 3 mM KCl, 0.5 mM MgCl₂, 5.5 mM glucose, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid at pH 7.4) at a density of 5 × 10⁸ cells/mL. For reverse transcription polymerase chain reaction, washed platelets were purified further through depletion of residual leukocytes and erythrocytes, using biotin-labeled anti-mouse CD45 and TER-119 antibodies (clone 30-F11 and TER-119, 1:200; Biolegend), followed by separation using Streptavidin MicroBeads, LD Columns, and a MidiMAC Separator following manufacturer's instructions (Miltenyi Biotec).

Human. Human blood was obtained with consent from healthy donors in accordance with the Declaration of Helsinki, under protocols reviewed by the Australian National University's Human Research Ethics Committee (Protocol 2016/317). Human platelets were isolated from acid-citrate-dextrose-anticoagulated blood, as previously described,³¹ and resuspended in modified Tyrode's buffer at 5 × 10⁸ cells/mL.

Reverse transcription polymerase chain reaction

Total RNA isolation, reverse transcription, and cDNA amplification were performed as previously described.³² The purity of platelet cDNA was assessed by polymerase chain reaction, using primers for CD41 (platelet-specific marker, positive control) and CD45

(leukocyte-specific marker, negative control). Primer sequences are available on request.

Surface biotinylation

Surface biotinylation of cells was performed as described previously.³² In brief, platelets were washed with prelysis phosphate-buffered saline (PBS) buffer (PBS supplemented with 1 mM CaCl₂, 0.5 mM MgCl₂ at pH 8.0) followed by biotinylation in 0.5 mg/mL EZ-Link Sulfo-NHS-Biotin (Thermo Fisher Scientific). After washing with 100 mM glycine to quench unbound reagent, cells were lysed by lysis buffer (150 mM NaCl, 1% Triton X-100, 20 mM Tris-HCl at pH 7.6). After centrifugation, the supernatant of cell lysate was incubated with high-capacity streptavidin-agarose beads (Thermo Fisher Scientific). After washing with lysis buffer, the streptavidin beads were applied to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis.

Plasma membrane extraction

The plasma membrane of platelets was extracted and enriched using a Plasma Membrane Protein Extraction Kit (Abcam) according to manufacturer's instructions. The kit uses an aqueous polymer 2-phase system to separate plasma membranes from organelle membranes.

Platelet apoptosis and activation

Washed platelets (5 × 10⁷) remained untreated or were preincubated with vehicle (dimethyl sulfoxide [DMSO]; Sigma), Calpeptin (CP; 50 μg/mL; Sigma), or Q-VD-OPh (QVD; 25 μM; Sigma) at room temperature for 15 minutes. To induce apoptosis, platelets were treated with ABT737 (1 μM, Selleckchem) at 37°C in the absence or presence of CaCl₂ (2 mM) for different periods of time or with vehicle (DMSO) in the controls. To induce activation, platelets were treated with A23187 (at different concentrations; Sigma), thrombin (1 U/mL; Sigma), collagen (50 μg/mL; CHRONO-LOG), or thrombin and collagen in the presence of CaCl₂ (2 mM) at room temperature for 20 minutes or with vehicle (DMSO) in the controls.

Calpain cleavage assay

Platelet membrane fractions (10 μg) were incubated with purified human calpain-1 (4U; Sigma) in the absence or presence of CaCl₂ (5 mM) or CP (50 μg/mL; Sigma) at room temperature for 30 minutes in PBS at pH 7.4 (Medicago). Controls remained untreated. Samples were then denatured for western blot analysis.

Western blot analysis and antibodies

Platelet total membrane and mouse liver homogenate were prepared as described previously.^{32,33} Protein samples were applied to 4% to 12% Bis-Tris Plus Gels running in 4-morpholinepropanesulfonic acid-SDS running buffer (Bolt, Thermo Fisher Scientific), as previously described.³² The separated proteins were transferred to nitrocellulose blotting membranes (GE Healthcare), followed by blocking with 5% skim milk in PBS-Tween 20 at pH 7.4 (Medicago). Membranes were probed with primary antibodies against ATP8A1 (1:1000; Proteintech), ATP11C (1:1000; customized, Pineda Antikörper-Service), caspase-3 (1:1000; Cell Signaling), calpain-1 large subunit (1:1000; Cell Signaling), gelsolin (1:1000; Abcam), β-actin (1:2000; Abcam), or Na⁺/K⁺-ATPase (Clone EP1845Y, 1:5000; Abcam) in PBS-Tween 20 with 2% membrane blocking reagent (GE Healthcare) at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated donkey anti-rabbit

immunoglobulin G (1:2000; GE Healthcare) at room temperature for 1 hour. Stained blots were visualized with Luminata Forte Western Horseradish Peroxidase Substrate (Millipore). For reprobing, the same blots were stripped for 30 minutes at 70°C in stripping buffer (62.5 mM Tris-HCl at pH 6.8, 2% SDS, and 100 mM 2-mercaptoethanol). Membranes were washed in PBS-Tween 20, blocked in 5% skim milk, and reprobed with different antibodies, as described earlier.

Measurement of intracellular calcium

Intracellular calcium levels were measured by using a Fluo-4 NW Calcium Assay Kit (Thermo Fisher Scientific) according to the manufacturer's manual. In brief, washed platelets (5×10^7) were preloaded in Fluo-4 NW dye loading solution in the presence of CaCl_2 (1.3 mM) and probenecid (5 mM) at 37°C for 30 minutes, and then at room temperature for an additional 30 minutes. Platelets were then treated with ABT737 (1 μM ; Selleckchem) at 37°C in the absence or presence of CP (50 $\mu\text{g}/\text{mL}$; Sigma) or QVD (25 μM ; Sigma), or with vehicle (DMSO) in the controls. Fluorescence was measured continuously for a total of 3 hours, using a Tecan Infinite M1000 PRO microplate reader (Tecan Group Ltd.) with excitation at 494 nm and emission at 516 nm.

Measurement of PS exposure

PS exposure on the cell surface was assessed by measuring the binding of Annexin-V to platelets by flow cytometry, as previously described.²⁹ Doublets and microparticles were excluded by forward scatter and side scatter gates. Platelets were defined by CD41^+ gates, followed by quantification of Annexin-V.

Statistical analysis

Statistical significance between 2 groups was analyzed using an unpaired Student *t* test with 2-tailed *P* values. All statistical analyses were performed using GraphPad Prism software. Data are presented as means \pm standard error of the mean (SEM), where *n* is the number of independent experiments performed. **P* < .05; ***P* < .01; ****P* < .001.

Results

ATP8A1 is highly expressed in mouse platelets, but not located at the cell surface

Earlier work indicated that expression of flippases is tissue-specific.^{26,34} To determine the major flippase or flippases in mouse platelets, we first tested the gene expression profile of 15 flippases and 3 CDC50 proteins in RNA isolated from mouse platelets.¹³ We found that ATP8A1 had the highest expression level compared with other flippases, and CDC50A was the only CDC50 protein expressed in mouse platelets (Figure 1A). These data are in agreement with analysis of the murine platelet transcriptome²⁰ (Figure 1B) and proteome²¹ (Figure 1C).

Further investigations showed that ATP8A1 was not present in the plasma membrane. As shown in Figure 1D, ATP8A1 was detected in total membranes of platelets, but not in the biotinylated cell surface protein fractions. The absence of ATP8A1 in the plasma membrane was further confirmed by the observation that ATP8A1 was depleted rather than enriched in the plasma membrane extracted from total membranes of platelets. As a marker of plasma membrane protein, Na^+/K^+ -ATPase was enriched in both biotinylated and

extracted plasma membrane protein fractions. Together, these data indicate that ATP8A1 is predominantly present in the intracellular membranes, rather than the plasma membrane of platelets.

In addition, using ATP11C-deficient (ambrosius) mice and a customized antibody against ATP11C, we demonstrated the absence of protein expression of ATP11C in mouse platelets (Figure 1E). Consistent with high expression of ATP11C in liver,^{16,17,25,26,34} the presence of a strong band of approximately 120 kDa in liver from wild-type mice was detected, and this band was absent in the liver of ATP11C-deficient mice (Figure 1E, right panel), confirming that the antibody was specific to ATP11C. As a result of the lack of high-quality antibodies against ATP11B, we were unable to test for ATP11B protein expression by western blot.

Calpain-mediated cleavage of ATP8A1 depends on caspase activation and calcium influx during platelet apoptosis

It has been reported that PS flippases, ATP11A and ATP11C, are inactivated through caspase-mediated cleavage enabling PS exposure in apoptotic leukocytes.^{35,36} To examine whether flippase ATP8A1 is similarly cleaved by caspases during apoptosis in platelets, we assessed levels of cleaved and intact ATP8A1 protein in apoptotic murine platelets. We used the BH3 mimetic compound ABT737 to induce intrinsic apoptosis in murine platelets.³⁵ After 30 minutes of treatment with ABT737 in the absence of extracellular calcium, we noted cleavage of procaspase-3 from a 32-kDa zymogen into an active form of approximately 17 kDa, which indicated the activation of caspases and onset of platelet apoptosis (Figure 2A, left panel). Under these conditions, no cleavage of ATP8A1 was observed, as shown by immunodetection of the intact protein at 120 kDa (Figure 2A, left panel). This suggests that ATP8A1 is not cleaved by caspases during platelet apoptosis.

Intriguingly, ATP8A1 cleavage was observed after 1 hour of treatment with ABT737 in the presence of extracellular calcium, resulting in a lower-molecular-weight product of around 100 kDa (Figure 2A, middle panel). This suggested that ATP8A1 cleavage was most likely mediated by a calcium-dependent protease activated through calcium influx. Calpain is a calcium-dependent cysteine proteinase and functions in apoptosis by cleaving apoptosis-regulating factors and cytoskeleton-associated proteins.³⁷ Thus, we next investigated whether calpain was involved in ATP8A1 cleavage during apoptosis in platelets. Activation of calpain can be demonstrated by the autolytic cleavage of a peptide from the 80-kDa large catalytic subunit, resulting in the generation of a fully active form at 76 kDa.³⁸ As shown in the middle and left panels of Figure 2A, fully active calpain-1 was only detected in apoptotic platelets treated with ABT737 + CaCl_2 , not ABT737 alone. This is consistent with the previous observation that calpain activation is calcium influx-dependent in platelets.³⁹ Moreover, pretreatment of platelets with a specific calpain inhibitor, CP, completely inhibited the activation of calpain and cleavage of ATP8A1 in apoptotic platelets despite the presence of active caspase-3 (Figure 2A, right panel). Thus, these data indicate that ATP8A1 was cleaved during platelet apoptosis in a calpain-mediated manner.

Interestingly, the addition of the broad spectrum caspase inhibitor, QVD, not only inhibited pro-caspase 3 conversion to active caspase-3 but also inhibited the cleavage of ATP8A1 (Figure 2A, last lane of right panel). This suggests that although ATP8A1 is not directly cleaved by caspases during platelet apoptosis, it is inactivated by a

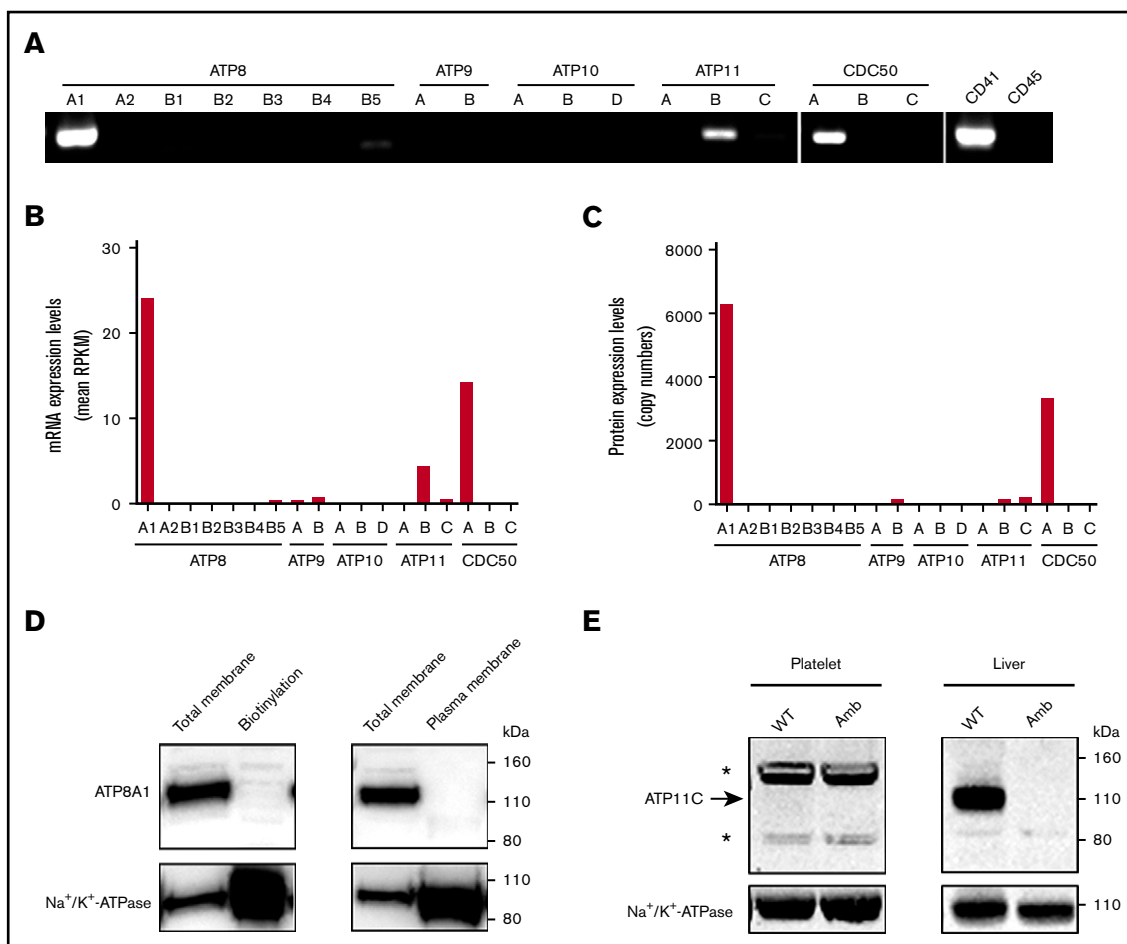


Figure 1. ATP8A1 is highly expressed in mouse platelets, but not located at the cell surface. (A) Gene expression of the P4-type ATPases and CDC50 proteins in mouse platelets. Reverse transcription polymerase chain reaction was performed using total RNA isolated from mouse platelets. CD41 and CD45 were used as positive control and negative control for the purity of platelet cDNA, respectively. (B) mRNA expression pattern of the P4-type ATPase and CDC50 genes derived from the genome-wide RNA-seq analysis of mouse platelet transcriptome.²⁰ RPKM, reads per kilobase of exon model per million mapped reads. Data were extracted from supplementary Table S4 in Rowley et al.²⁰ (C) Protein expression pattern of the P4-type ATPases and CDC50 proteins derived from the copy number analysis of murine platelet proteome.²¹ Data were extracted from supplementary Table S2 in Zeiler et al.²¹ (D) Total membrane, biotinylated surface protein, and extracted plasma membrane from mouse platelets were denatured for SDS-PAGE and then probed for ATP8A1 by western blotting. Na⁺/K⁺-ATPase was used as a marker of plasma membrane protein. Immunoblots are representative of 3 independent experiments. (E) Expression of ATP11C in platelets and liver from *Atp11c*^{+/-} (wild-type) and *Atp11c*^{amb/0} (ambrosius) mice. Mouse platelet cell lysates and liver tissue homogenates were denatured for SDS-PAGE and then probed for ATP11C through western blot. *Nonspecific bands. Immunoblots are representative of 3 independent experiments.

caspase-dependent mechanism. The lack of ATP8A1 cleavage after QVD treatment most likely resulted from a lack of calpain activation after caspase inhibition, as evident by the observation that autoprolysis of calpain-1 was absent (Figure 2A, last lane of right panel). To test whether the lack of calpain activation was a result of the absence of calcium influx when caspase activity was inhibited, we measured intracellular calcium ([Ca²⁺]_i) levels of platelets, using the cytosolic Ca²⁺ probe Fluo-4 (Figure 2B). The measurement of Fluo-4 revealed that ABT737-treated apoptotic platelets had elevated [Ca²⁺]_i, as detected by a significant 2-fold increase in mean fluorescence intensity of Fluo-4 compared with resting platelets (Figure 2B). The elevation of [Ca²⁺]_i in apoptotic platelets was prevented by caspase inhibition. Under this condition, [Ca²⁺]_i remained at the same level as in resting platelets (Figure 2B), indicating that influx of extracellular Ca²⁺ across the plasma membrane was blocked. Inhibition of calpain by CP did not affect elevation of [Ca²⁺]_i (Figure 2B)

or caspase activation (Figure 2A, right panel) during platelet apoptosis. Also, absence of extracellular calcium did not affect activation of caspases in platelets treated with ABT737 (Figure 2A, left panel). Taken together, these data demonstrate that calcium influx and subsequent calpain activation are downstream events of caspase activation; therefore, cleavage of ATP8A1 mediated by calpain is caspase-dependent during platelet apoptosis.

ATP8A1 is a direct substrate of calpain

To further confirm the molecular interactions leading to ATP8A1 cleavage by calpain, platelet membrane fractions were incubated with purified calpain-1, the isoform that accounts for 80% of the calpain activity in mouse platelets,⁴⁰ followed by western blotting analysis of ATP8A1. As shown in Figure 3A, ATP8A1 was cleaved into the same size protein fragment of approximately 100 kDa, by calpain-1 in a Ca²⁺-dependent fashion, as observed in apoptotic

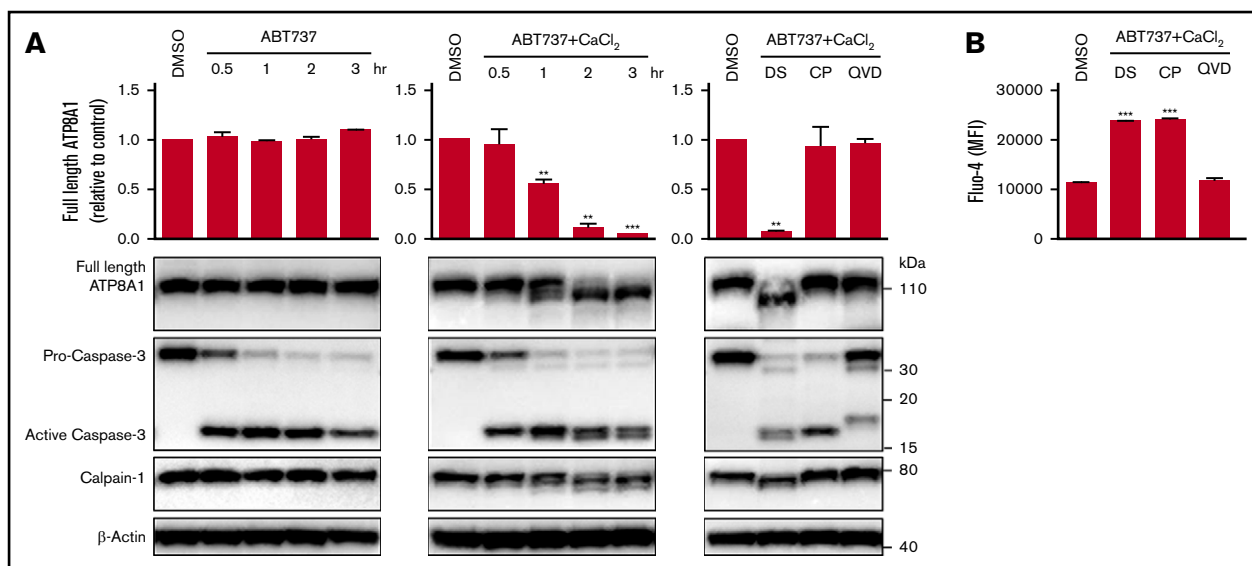


Figure 2. Calpain-mediated cleavage of ATP8A1 depends on caspase activation and calcium influx during platelet apoptosis. (A) Washed mouse platelets (5×10^7) were treated with vehicle (DMSO) or ABT737 (1 μ M, 37°C) in the absence or presence of CaCl₂ (2 mM) for the indicated time. In some experiments, platelets were preincubated with DMSO (DS), CP (50 μ g/mL), or QVD (25 μ M) at room temperature for 15 minutes before treatment with ABT737 (2 hours). Platelets were subsequently lysed for western blot analysis of ATP8A1, Caspase-3, Calpain-1, and β -actin. (B) Washed mouse platelets (5×10^7) were preloaded with Fluo-4 and treated with vehicle (DMSO) or ABT737 (1 μ M) and CaCl₂ (1.3 mM) at 37°C for 2 hours in the absence or presence of CP (50 μ g/mL) or QVD (25 μ M). Fluo-4 fluorescence was measured, and data are expressed as mean fluorescence intensity (MFI) \pm SEM (n = 3). Immunoblots are representative of 3 independent experiments. Bar graphs represent blot quantification of full-length ATP8A1 by densitometric analysis (means \pm SEM, n = 3) of stained bands using Image J, corrected for loading control (β -Actin). Statistical significance was calculated using Student 2-tailed *t* test. ***P* < .01; ****P* < .001.

platelets (Figure 2A, middle and right panel). The cleavage was fully inhibited by CP (Figure 3A). These data demonstrate that ATP8A1 is a direct substrate of calpain.

As a substrate of calpain, ATP8A1 should be cleaved by calcium-activated endogenous calpain even in the absence of apoptosis and caspase activity. To test this, viable resting platelets were treated with calcium ionophore, A23187, resulting in the activation of endogenous calpain (Figure 3B). As shown in Figure 3B, the activation of calpain coincided with cleavage of ATP8A1, and both events were completely inhibited by CP. In this model, activation of calpain was directly achieved by A23187-induced calcium flux, bypassing apoptosis and caspase pathways. Caspases remain inactive in this case, as shown by the absence of active caspase-3. Accordingly, caspase inhibitor QVD had no effect on calpain activation and ATP8A1 cleavage.

Immunoblots also revealed a lower-molecular-weight band below pro-caspase-3 (Figure 3B) that represents a nonactivated cleaved form of pro-caspase-3 generated by active calpain³⁶ (Figure 3C). Gelsolin, a known caspase substrate, was cleaved by the active form of caspase-3 in ABT737-treated apoptotic platelets (Figure 3C, left panel), but remained intact in A23187-treated platelets, indicating that the cleaved form of pro-caspase-3 generated by calpain does not activate pro-caspase-3 (Figure 3C, right panel).

Cleavage of human ATP8A1 and predicted calpain cleavage sites in mammalian ATP8A1 orthologs

As demonstrated in murine platelets, ATP8A1 and CDC50A were also found to be highly expressed in human platelets (Figure 4A-B) in platelet transcriptomic and proteomic studies.²²⁻²⁴ Human ATP8A1 protein was also cleaved by endogenous calpain, rather than caspases,

in human platelets treated with ABT737 to induce apoptosis in a caspase- and calcium influx-dependent manner (Figure 4C).

Searches for calpain cleavage sites using the prediction tool GPS-CCD1.0⁴¹ revealed several phylogenetically conserved predicted calpain cleavage sites in mammalian ATP8A1 orthologs. The predicted cleavage site at position R139 in the actuator (A) domain of ATP8A1 generates a fragment of around 100 kDa (Figure 4D-E), consistent with the cleaved form observed in the western blot analysis of murine and human platelets. These data indicate that calpain-mediated cleavage of ATP8A1 is highly likely to be a common event during apoptosis of mammalian platelets, and potentially in other ATP8A1-expressing cell types.

ATP8A1 is not cleaved in platelets activated by physiological agonists

We next investigated whether calpain-mediated cleavage of ATP8A1, observed in apoptotic platelets, was also seen in activated platelets. As an artificial agonist, calcium ionophore, A23187, induces strong and sustained calcium flux increasing intracellular Ca²⁺ levels in a dose-dependent manner.⁴²⁻⁴⁴ As shown in the upper panel of Figure 5A, 0.5 μ M A23187 and 1 μ M A23187 induced PS exposure on ~80% and 100% of platelets, respectively, as detected by the PS-specific probe, Annexin-V.⁴⁵ However, calpain activation and the ensuing cleavage of ATP8A1 and pro-caspase-3 were only detected in platelets treated with 1 μ M A23187 (Figure 5A, lower panel). These data indicate that ATP8A1 is cleaved by calpain only under conditions of high Ca²⁺ concentration in A23187-activated platelets.

To determine the effect of physiological agonists on ATP8A1 cleavage, platelets were treated with thrombin and collagen at

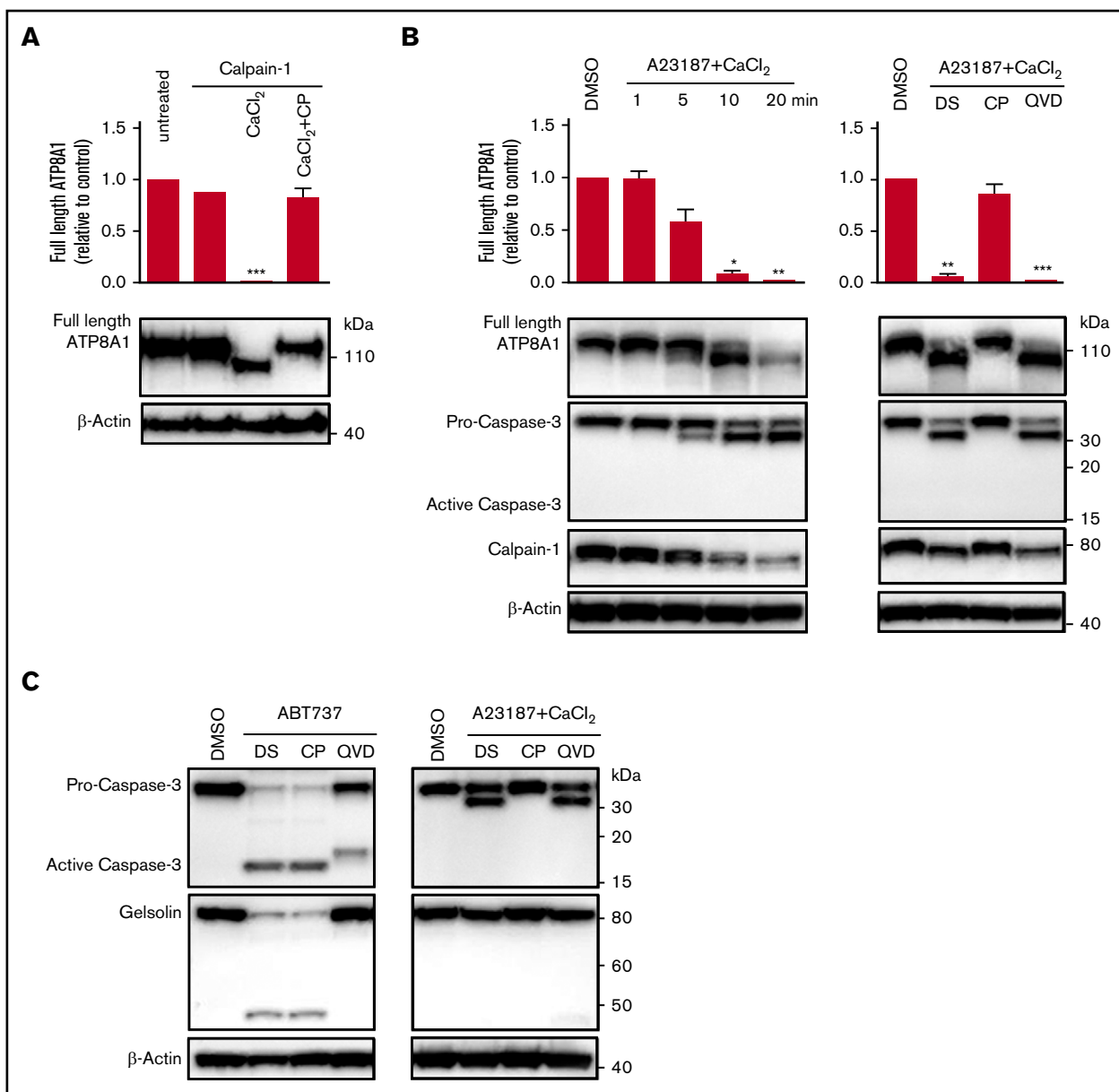


Figure 3. ATP8A1 is a direct substrate of calpain. (A) Mouse platelet membrane fractions (10 μ g) remained untreated or were incubated with purified human calpain-1 (4U) in the absence or presence of CaCl₂ (5 mM) or CP (50 μ g/mL) at room temperature for 30 minutes. Samples were then denatured for western blot analysis of ATP8A1 and β -actin. (B-C) Washed mouse platelets (5×10^7) were treated with vehicle (DMSO) or A23187 (1 μ M; room temperature) in the presence of CaCl₂ (2 mM) for the indicated time. In some experiments, platelets were preincubated with DMSO (DS), CP (50 μ g/mL) or QVD (25 μ M) at room temperature for 15 minutes before treatment with A23187 (20 minutes) or ABT737 (1 μ M, 37°C, 2 hours, in the absence of CaCl₂). Platelets were subsequently lysed for western blot analysis of ATP8A1, Caspase-3, Calpain-1, Gelsolin, and β -actin. Immunoblots are representative of $n = 3$ independent experiments. Bar graphs represent blot quantification of full-length ATP8A1 by densitometric analysis (means \pm SEM, $n = 3$) of stained bands using Image J, corrected for loading control (β -Actin). Statistical significance was calculated using Student 2-tailed *t* test. * $P < .05$; ** $P < .01$; *** $P < .001$.

concentrations known to induce platelet activation.^{46,47} In contrast to A23187, Figure 5B (upper panel) shows that each agonist alone was a weak trigger of PS exposure, resulting in approximately 20% of Annexin-V-positive platelets.^{46,47} Costimulation of platelets with thrombin and collagen, however, led to a substantial fraction of platelets (approximately 80%) that exposed PS on their surface,^{29,48,49} similar to that observed on platelets treated with 0.5 μ M A23187. Despite this level of PS exposure, no calpain activation, ATP8A1 cleavage, or pro-caspase-3 cleavage was detected (Figure 5B,

lower panel). Together, these data indicate that ATP8A1 remains intact in PS-exposed platelets under conditions of physiological activation.

Discussion

In the present study, we show that ATP8A1 is highly expressed in both murine and human platelets, but is not present in the plasma membrane, and calpain, rather than caspase, catalyzes

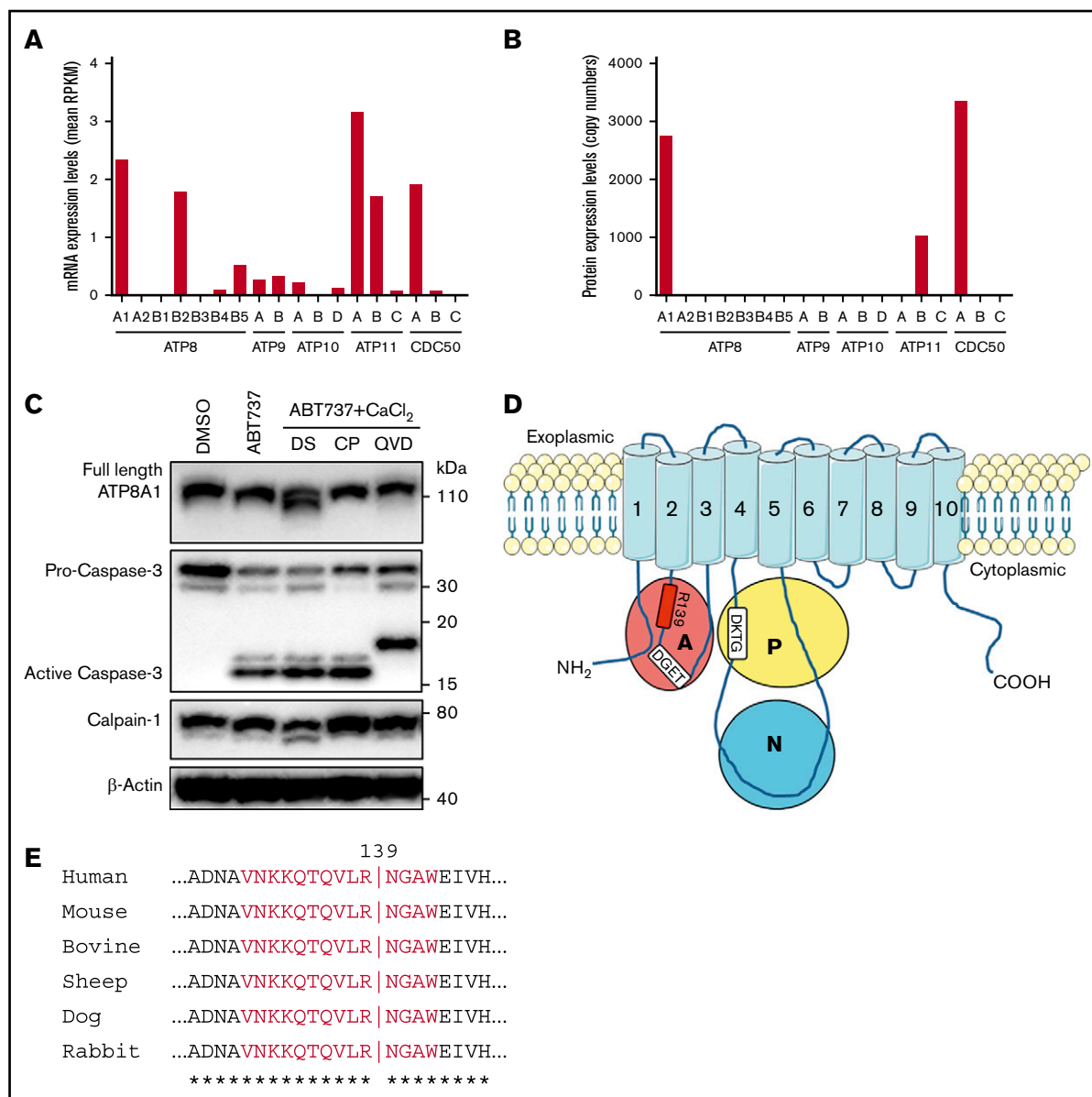


Figure 4. Cleavage of human ATP8A1 and predicted calpain cleavage sites in mammalian ATP8A1 orthologs. (A) mRNA expression pattern of the P4-type ATPase and CDC50 genes derived from the genome-wide RNA-seq analysis of human platelet transcriptome.²⁰ Data were extracted from supplementary Table S4 in Rowley et al.²⁰ (B) Protein expression pattern of the P4-type ATPases and CDC50 proteins derived from the quantitative analysis of human platelet protein composition.²² Data were extracted from supplemental Table 3 in Solari et al.²² (C) Washed human platelets (5×10^7) were preincubated with DMSO (DS), CP (50 μ g/mL), or QVD (25 μ M) at room temperature for 15 minutes, and then treated with vehicle (DMSO) or ABT737 (1 μ M; 37°C; 2 hours) in the absence or presence of CaCl₂ (2 mM). Platelets were subsequently lysed for western blot analysis. Immunoblots are representative of $n = 3$ independent experiments. (D) Topology of ATP8A1 and its predicted calpain cleavage site. Transmembrane helices are numbered. Three cytosolic domains involved in the ATPase catalytic cycle are shown as colored circles: nucleotide-binding (N) domain, which binds ATP; phosphorylation (P) domain, which contains the conserved phosphorylation site in the DKTG (Asp-Lys-Thr-Gly) motif; and actuator (A) domain, which has the DGET motif that facilitates the dephosphorylation of the phosphorylated aspartate intermediate.^{4,66,67} Calpain cleavage sites were predicted using GPS-CCD 1.0 (<http://ccd.biocuckoo.org>).⁴¹ Although several cleavage sites are predicted, the site at position R139 in the A-domain (indicated by a red box) generates a fragment of around 100 kDa, which is consistent with the western blot fragment size in apoptotic platelets. (E) Amino acid sequence alignment around the calpain cleavage site in 6 mammalian ATP8A1 orthologs: human (UniProt, Q9Y2Q0), mouse (P70704), bovine (Q29449), sheep (W5PYS1), dog (F1PHG9), and rabbit (G1TF29). Sequences are aligned. *Fully conserved residues. The calpain recognition sequence is shown in red. A vertical bar (|) indicates the cleavage site.

the cleavage of ATP8A1 during platelet apoptosis. To our knowledge, this is the first evidence for calpain-mediated cleavage of flippases. We also ruled out ATP11C as a candidate of flippase activity in murine platelets. Interestingly, given the

critical role for ATP11C in leukocytes and erythrocytes, and its inactivation by caspases,^{14-17,19,25,26} these results suggest that expression and regulation of flippases in platelets is distinct from these other cell types.

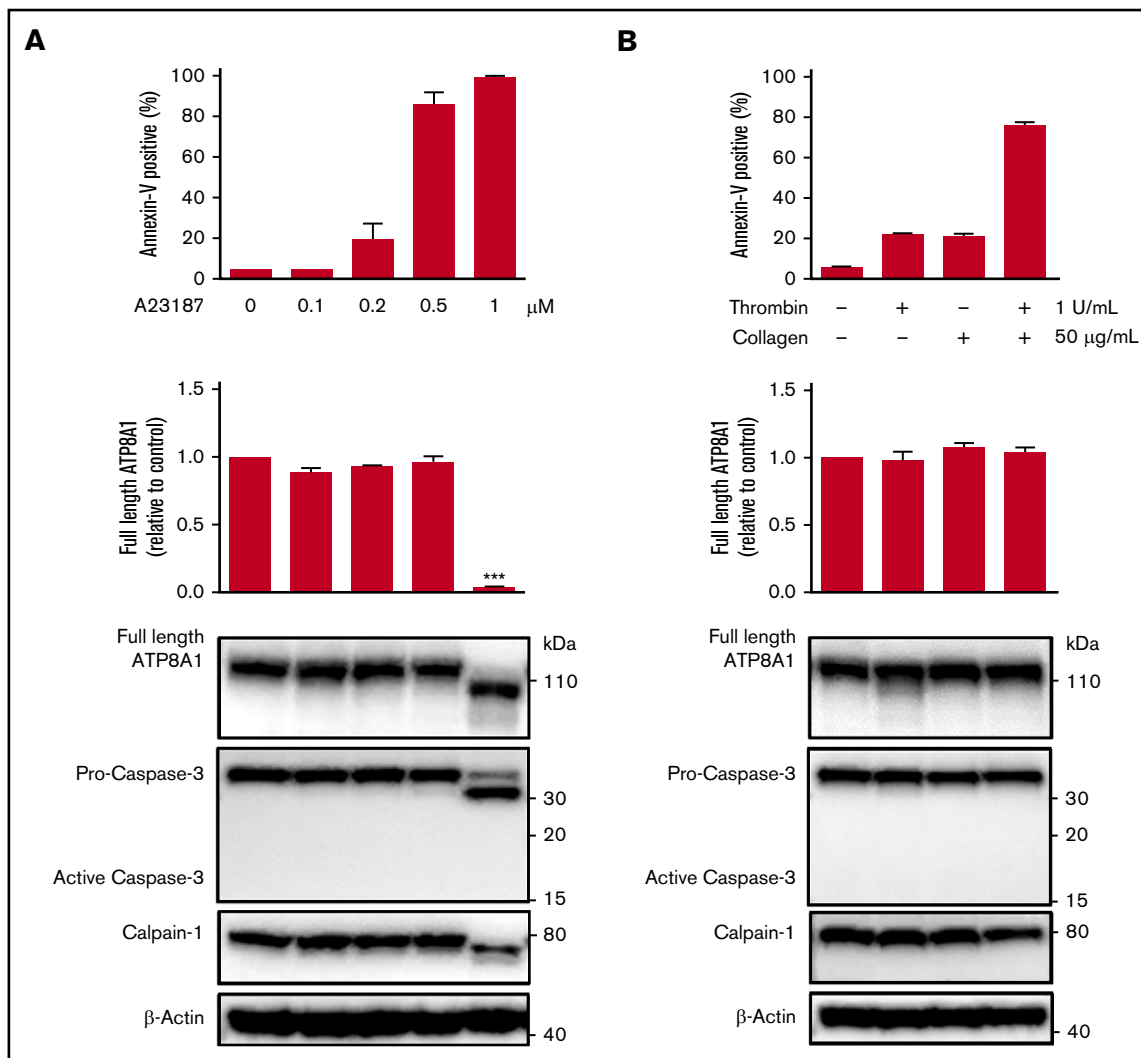


Figure 5. ATP8A1 is not cleaved in platelets activated by physiological agonists. (A-B, upper panels) Bar graphs represent means \pm SEM ($n = 3$) of the percentage of Annexin-V-positive cells in platelets stimulated by A23187, thrombin, collagen, or thrombin and collagen in the presence of CaCl_2 (2 mM) at room temperature for 20 minutes. (A-B, lower panels) Washed mouse platelets (5×10^7) were treated with vehicle, A23187, thrombin, collagen, or thrombin and collagen in the presence of CaCl_2 (2 mM) at room temperature for 20 minutes. Platelets were subsequently lysed for western blot analysis of ATP8A1, Caspase-3, Calpain-1, and β -actin. Immunoblots are representative of 3 independent experiments. Bar graphs represent blot quantification of full-length ATP8A1 by densitometric analysis (means \pm SEM, $n = 3$) of stained bands using Image J, corrected for loading control (β -Actin). Statistical significance was calculated using Student 2-tailed t test. *** $P < .001$.

Both calpain and caspase play important roles in cell apoptosis. Studies have shown that calpain possesses both prosurvival and pro-apoptotic functions,^{37,38} and the biological outcome of calpain activity is dependent on cell types and stimulants.^{50,51} In this study, we provide further evidence for the pro-apoptotic role of calpain in platelets treated with ABT737. In this setting, calpain facilitates apoptosis by cleaving flippase ATP8A1 (Figure 2A). In contrast, caspase proteinases are centrally involved in pro-apoptotic signaling and execution. Although caspases do not directly cleave ATP8A1 in apoptotic platelets, they contribute to calpain-mediated cleavage in an indirect manner, through their actions on regulating pathways of calcium signaling (Figure 2B). In platelets, Ca^{2+} elevation in the cytosol occurs via both the release of stored Ca^{2+} from the dense tubular system and the influx

of extracellular Ca^{2+} across the plasma membrane.⁵²⁻⁵⁵ Various components of the Ca^{2+} signaling machinery have been described to be cleaved by caspases⁵⁶; in addition, previous reports have shown that calpain activity may play a role downstream of caspases in the degradation phase of apoptosis.⁵⁷ The specific signaling mechanisms that link caspase activity, calcium signaling, and calpain activation in platelets are beyond the scope of this study.

It is well documented that ATP8A1 associates with CDC50A to exit the endoplasmic reticulum, but mainly remains in intracellular membranes and to some extent at the plasma membrane.^{26,58-60} ATP8A1 has been shown to localize to recycling endosomes, and PS flipping by ATP8A1 is required for endosome-mediated protein trafficking.^{61,62} Given the absence of ATP8A1 in the

plasma membrane of platelets (Figure 1D), we reason that ATP8A1 is predominantly located in intracellular membranes and is involved in vesicle-mediated trafficking in platelets. Platelets have complete intracellular membrane systems⁶³ and possess fundamental membrane trafficking processes such as endocytosis.⁶⁴ Moreover, upon activation, platelets undergo dramatic shape change¹ and carry out exocytosis such as secretion of procoagulant components and release of granules (dense-/ α -granule).⁶⁵ These processes require a large number of membrane structures; therefore, it is tempting to speculate that flippase-mediated asymmetric distribution of phospholipids in organelles enables the normal operation of these activities. As opposed to activation, ATP8A1 was found to be cleaved during platelet apoptosis (Figure 2). The cleavage is likely to inactivate flippase activity of ATP8A1 and disrupt lipid asymmetry of intracellular membranes and trafficking, which may contribute to the process of apoptosis. In summary, cleavage of ATP8A1 was observed in apoptotic platelets (Figure 2), but not in activated platelets (Figure 5), indicating that inactivation of flippases is required for apoptosis; however, maintenance of flippase activity is required for normal function of platelets during activation.

In conclusion, our findings support a model of calpain-mediated cleavage of phospholipid flippases in apoptotic platelets. In contrast, flippases remain intact in activated platelets despite the presence of PS on the cell surface. Hence, we propose that the modulation of flippase activity in the intracellular membranes of platelets plays a distinct role in apoptosis and activation.

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Authorship

Contribution: W.J., A.E., and S.B. conceived and designed the study; W.J. planned and performed the experiments; W.J., M.Y., and A.B. analyzed the data; L.C. and E.E.G. contributed to experiments on human platelets; W.J., A.E., and S.B. wrote the manuscript; and M.Y., A.B., L.C. and E.E.G. edited the manuscript.

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