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ORIGINAL ARTICLE

The critical role of platelet adenylyl cyclase 6 in hemostasis and thrombosis

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Abstract

Background: Platelet activation is constrained by endothelial-derived prostacyclin (PGI_2) through cyclic adenosine-3′,5′-monophosphate (cAMP) signaling involving multiple isoforms of adenylyl cyclase (AC). The roles of specific AC isoforms in controlling hemostasis remain unclear and require clarification.

Objectives: To understand the specific contribution of AC6 in platelet hemostatic and thrombotic function.

Methods: A platelet-specific AC6 knockout mouse was generated. Biochemical approaches were used to determine intracellular signaling, with flow cytometry, tail bleeding time assays, and *in vivo* thrombosis by ferric chloride were used to measure the hemostatic and thrombotic importance of platelet AC6.

Results: Loss of AC6 resulted in diminished accumulation of platelet cAMP in response to PGI_2, while basal cAMP was unaffected. We found no differences in phosphodiesterase 3A activity, suggesting the defect was in generation rather than hydrolysis of cAMP. Consistent with this, phosphorylation of protein kinase A substrates, vasodilator-stimulated phosphoprotein, and glycogen synthase kinase were diminished but not ablated. Functional studies demonstrated that the inhibition of thrombin-induced fibrinogen binding and P-selectin expression by PGI_2 was severely compromised, while inhibition of glycoprotein VI-mediated platelet activation was largely unaffected. Under conditions of flow formed stable thrombi, but in the absence of AC6, thrombi were insensitive to PGI_2. Diminished *in vivo* sensitivity to PGI_2 manifested as significantly reduced tail bleeding and accelerated occlusive arterial thrombus formation in response to vascular injury that were highly unstable and prone to embolization in AC6 knockout mice.

Conclusion: These data demonstrate that AC6 is linked directly to PGI_2-mediated platelet inhibition and regulation of hemostasis and thrombosis *in vivo*.

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KEYWORDS
adenylyl cyclase, cAMP, hemostasis, platelets, thrombosis

1 | INTRODUCTION

Cross talk between endothelial-derived mediators, such as prostacyclin (PGI_2) and nitric oxide, and platelets is critical to the control of hemostasis [1–7]. PGI_2 is the more potent of these 2 inhibitors based on the much higher expression of the downstream signaling apparatus and effectors [8]. In circulation, the marginalization of platelets during blood flow facilitates their continual exposure to PGI_2, ensuring they remain inactive. Ligation of the prostaglandin I_2 (IP) receptor by PGI_2 is coupled to a complex system involving enzymes and adaptors that facilitate the generation, propagation, and termination of cyclic adenosine-3′,5′-monophosphate (cAMP) signaling. The binding of PGI_2 to the IP receptor activates membrane adenylyl cyclase (AC) through $G\alpha_s$ and G-protein coupled receptors, resulting in elevations in cAMP and activation of protein kinase A (PKA) isoforms. The subsequent phosphorylation of a plethora of key protein substrates results in the inhibition of multiple aspects of platelet function [9]. Increased platelet cAMP is associated with reduced Ca^{2+} mobilization, dense granule secretion, integrin $\alpha_{IIb}\ \beta_3$ activation and aggregation *in vitro* [10], and reduced platelet accrual at sites of vascular injury *in vivo* [11]. However, much of the data regarding the role of cAMP in platelet function have been gained from *in vitro* studies using cAMP mimetics that act as global cAMP modulators or bypass AC as well as pharmacologic inhibitors that have consistently been shown to have off-target effects [12]. Consequently, a precise understanding of the relationship between specific AC and PKA isoforms, individual substrates/signaling targets, and the distinct platelet functions highlighted above is still lacking.

Seminal gene deletion studies demonstrated that loss of the IP receptor led to a prothrombotic phenotype confirming PGI_2 as a key regulator of thrombosis *in vivo* and are supported by clinical studies suggesting impaired cAMP signaling in subjects with acute coronary syndromes [13–15]. However, our understanding of how cAMP signaling controls platelet function through a myriad of signaling enzymes and where the systems fail in atherothrombotic disease is unclear. Dissecting the functionality of the pathway downstream of the IP receptor has been hampered by a lack of pharmacologic tools and genetic models [16]. The precise role of PKA isoforms has proved difficult to decipher because the deletion of PKA regulatory subunits is either embryonic lethal [17,18] or results in compensation through upregulation of other subunits [17,19]. Given this complexity, and as a first step to understanding the role of different components of cAMP systems, we focused our attention on the roles of AC. This first step in the pathway has recently been shown to be critical in acute coronary syndromes where dysfunctional AC activity is linked to reduced efficacy of $P2Y_{12}$ receptor antagonists [20].

The soluble AC isoform, AC9, was shown to be the most dominant isoform in mice with a copy number of ~2493 [21,22]; however, because this isoform is not expressed in human platelets, we decided to focus our efforts on isoforms expressed in both human and murine platelets. Further studies indicate that AC6 is highly expressed in both human and murine platelets with a copy number of ~2166 in mice [21] and ~2500 in humans [23], with significantly lower expression of AC3 (human) and AC5 (murine), respectively [21–24]. The reason for multiple AC isozymes in platelets is unclear, and to explore the possibility that AC in platelets play functionally distinct roles, we generated a platelet-specific deletion of AC6. Our data demonstrate that AC6 is responsible for selective inhibition of platelet activation and plays a central role in controlling hemostasis and thrombosis in mice.

2 | MATERIALS AND METHODS

2.1 | Reagents

Protease-activated receptor 4 (PAR4) peptide (Anaspec); Cross-linked collagen-related peptide (CRP-XL) (CambCol Laboratories); PGI_2 and Forskolin (Cayman Chemical); Amersham cAMP Biotrak Enzymeimmunoassay system (Cytiva); S-nitrosoglutathione (GSNO), 8–(4-chlorophenyl)thio-cAMP (8-CPT-cAMP), and mouse anti-adenylate cyclase (sc-377243) (Santa Cruz Biotechnology); SQ22537 (Calbiochem); Annexin V allophycocyanin ready flow conjugate and Fast RNA extraction with PureLink RNA mini kit (Invitrogen); anti-phospho–vasodilator-stimulated phosphoprotein $(VASP)^{Ser239}$ (3114), phospho-$VASP^{Ser157}$ (3111), anti-phospho-PKA substrate (RRXS*/T*) recognizing RRXpSerine/threonine PKA consensus (9624), anti-phospho-glycogen synthase kinase 3 beta $(GSK3\beta)^{Ser9}$, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (2118) (Cell Signaling Technologies); BD Phosflow Lyse/Fix Buffer 5X; fluorescein isothiocyanate (FITC) rat immunoglobulin (Ig) G1 λ isotype control (553995); FITC rat anti-mouse CD62P (553744); BB700 rat anti-mouse CD41 (742148); FITC hamster anti-rat CD49b (554999); FITC hamster IgG1 κ isotype control (553971); FITC rat anti-mouse CD41 (553848); FITC rat IgG1 κ isotype control (553924); FITC hamster anti-mouse CD61 (553346); and FITC hamster IgG1 κ isotype control (553971) (BD Biosciences); Phycoerythrin (PE) rat anti-mouse integrin $\alpha_{IIb}\ \beta_3$ (M023-2); FITC rat anti-mouse glycoprotein (GP) 1b α (M040-1), FITC rat anti-mouse integrin α2 (M071-1), FITC rat anti-mouse GPVI (M011-1), FITC rat IgG polyclonal (P190-1), and PE rat IgG polyclonal (P190-2) (Emfret); Anti–phospho-phosphodiesterase 3A $(PDE3A)^{Ser312}$ (MRC-PPU, University of Dundee); Collagen Reagens HORM suspension (Takeda); Restore Western Blot Stripping buffer; SuperSignal West Pico

Plus Pierce ECL; TaqMan probes (Adcy6: Mm00475773_g1, Adcy5: Mm00674122_m1, Adcy3: Mm00460371_m1, and GAPDH: Mm99999915_g1) and TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific); and Reverse Transcription System (Promega). All other reagents were from Sigma–Aldrich.

2.2 | Experimental animals

All animal husbandry, housing, and procedures were carried out in line with the regulations and guidelines of the University of Leeds Central Biological Services facility under the Animals (Scientific Procedures) Act 1986 and United Kingdom Home Office approved project licenses (PP0499799 and PP9539458). Animals received standard rat and mouse no.1 maintenance diet (RM1, Special Diet Services) and water by Hydropac pouches. All mice were housed in individually ventilated cages (GM500, Techniplast), with 12 hours of light/dark cycles, at 21 °C and 50% to 70% humidity.

Platelet factor 4 (*Pf4*)-*Cre*$^{+}$/adenylyl cyclase 6 (*Adcy6*)$^{fl/fl}$ experimental mice were generated via a *Cre*-loxP approach by cross-breeding *Adcy6*$^{fl/fl}$ (RRID:IMSR_JAX:022503) and *Pf4-Cre*$^{+}$ (RRID:IMSR_JAX:008535) mice purchased from the Jackson Laboratory. Homozygous female *Adcy6* floxed mice were bred with hemizygous male mice expressing Cre recombinase under the control of the *Pf4* promoter, generating heterozygous floxed *Adcy6* and hemizygous *Pf4-Cre*. Male mice hemizygous for *Pf4-Cre* and heterozygous for floxed *Adcy6* were bred with females heterozygous for floxed *Adcy6*. This yielded animals homozygous for floxed *Adcy6* with and without *Pf4-Cre*. Mice with *Pf4-Cre* expression have platelet-specific knockout (KO) of *Adcy6*, while mice without *Pf4-Cre* function as littermate controls. Throughout this study, *Pf4-Cre*$^{+}$/*Adcy6*$^{fl/fl}$ are denoted as AC6-KO, and *Adcy*$^{fl/fl}$ littermate controls are denoted as wild type (WT). All mice generated were in a C57BL/6 background, and both male and female mice were used throughout this study. Prior to use, all pups were ear notched at weaning (~3 weeks of age) to allow for identification and isolation of genomic DNA for automated genotyping by Transnetyx.

2.3 | mRNA expression, flow cytometry, immunoblotting, cAMP measurement, *in vitro* flow, and *in vivo* thrombosis

Detailed methods are described in the Supplementary Methods.

2.4 | Statistics

Results are expressed as means ± SD unless otherwise stated, and statistical analyses were performed using GraphPad Prism 9.0. Comparisons between WT and AC6-KO mice were performed by 2-way analysis of variance with Šídák's multiple comparisons post hoc test unless otherwise stated, and statistical significance was accepted at $P < .05$.

3 | RESULTS

3.1 | Establishment of the platelet-specific AC6-KO mouse

The novel platelet-specific AC6-KO mouse was generated using the *Pf4* promotor to ensure the AC6 gene was deleted in cells of the megakaryocyte lineage (Figure 1A). To confirm the platelet specificity of the AC6-KO mouse, we tested the mRNA levels of the AC6 gene (*Adcy6*) in WT and AC6-KO platelets using real-time quantitative polymerase chain reaction. AC6 was successfully deleted in platelets (Figure 1B) but not in other tissues (eg, heart and kidney) (Supplementary Figure S1), confirming that the deletion of AC6 was restricted to the platelet–megakaryocyte lineage (Figure 1B). This was confirmed via immunoblotting using a pan-AC antibody, whereby total AC protein expression was unchanged in WT vs AC6-KO in heart and kidney (Supplementary Figure S2). Furthermore, we observed no change in the expression of AC9 and AC5, and consistent with other studies, we found no expression of AC3 in murine platelets [21,24] (Figure 1B). Immunoblotting for total AC showed significantly reduced protein expression in platelets, with residual protein likely to be the remaining AC5 and AC9 (Figure 1C). Importantly, we observed no change in PDE3A and PDE2A mRNA expression in WT and AC6-KO platelets (Supplementary Figure S3). In addition, we found no changes in protein expression of key components of the cAMP signaling system, including PDE3A (Figure 2C), VASP (Supplementary Figure S4), and key PKA subunits (Supplementary Figure S5). Importantly, we observed no difference in hematologic blood parameters between WT and AC6-KO (Table). Analysis of the platelets from AC6-KO mice and littermate controls showed no major differences in the expression of key platelet receptors (Supplementary Figure S5A). Furthermore, platelet numbers drawn from CD42b-positive cells and young reticulated platelets from thiazole orange-positive events showed no difference between WT and AC6-KO platelets (Supplementary Figure S5B, C), suggesting that platelet counts and turnover were similar in both groups of mice.

3.2 | Contribution of AC6 to platelet cAMP production and PKA-mediated phosphorylation events

We first investigated the role of AC6 in platelet cAMP synthesis using a cAMP assay. Basal cAMP generation was unchanged between WT (303 ± 161 fmol/10^7 platelets) and AC6-KO (224 ± 65 fmol/10^7 platelets) mice. Treatment of platelets with PGI_2 (0-100 nM, 30 seconds) led to a concentration-dependent increase in cAMP in WT platelets, while in AC6-KO platelets, cAMP synthesis was severely compromised (Figure 2A). In response to PGI_2 (100 nM), there was a

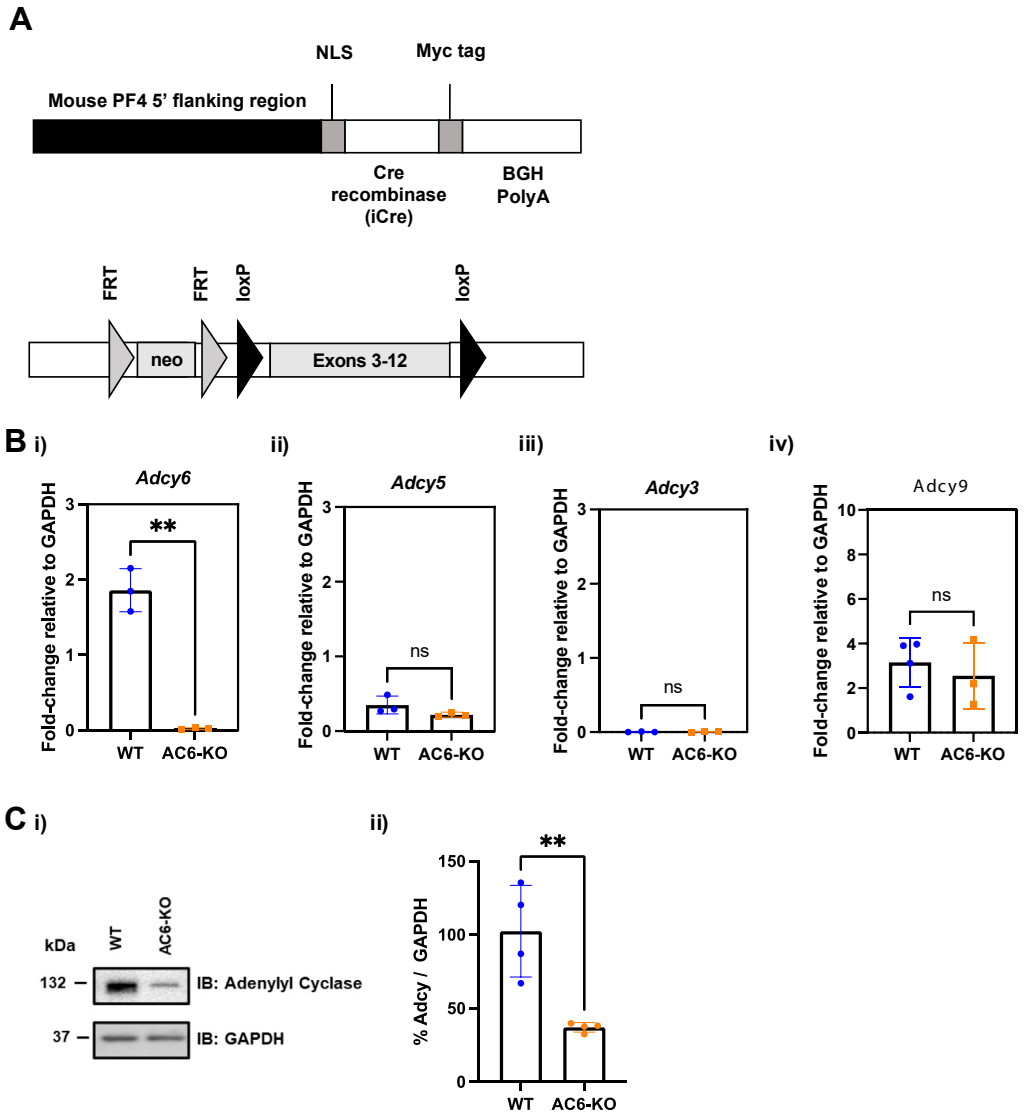

FIGURE 1 Confirmation of platelet-specific AC6-KO mouse. (A) Schematic representation of *Adcy6-lox* and *Pf4-Cre* constructs used in generating platelet-specific KO of the *Adcy6* gene. The top construct, *Pf4-Cre*, contains a mouse *Pf4* promoter, an amino-terminal NLS, a carboxy-terminal Myc epitope tag, iCre recombinase, and a BGH polyA sequence. The bottom construct is the floxed *Adcy6* gene with exons 3 to 12 (of 21 exons) flanked by *loxP* sites and an FRT-flanked neomycin resistance gene. (B) mRNA expression of individual AC isoforms was analyzed in washed platelets by real-time quantitative polymerase chain reaction. Comparisons were made between WT and AC6-KO using an unpaired Student's *t*-test with Welch's correction (means ± SD, n = 3-4; ns = not significant, $^{**}P < .01$). (C) Total AC expression in washed platelets via immunoblot analysis with data presented as fold of gene of interest over GAPDH control. Representative blot image and densitometry relative to GAPDH control. Comparisons were made between WT and AC6-KO using an unpaired Student's *t*-test with Welch's correction (means ± SD, n = 4, $^{**}P < .01$). AC, *Adcy*, adenylyl cyclase; BGH, bovine growth hormone; FRT, flippase recognition target; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IB, immunoblot; KO, knockout; mRNA, messenger RNA; NLS, nuclear localization sequence; *Pf4*, platelet factor 4; WT, wild type.

77.6% reduction in cAMP formation in AC6-KO compared with WT platelets (8860 ± 2699-1984 ± 417 fmol/10^7 platelets; $P < .0001$). When cAMP synthesis was assessed as a function of time, we found that its generation was severely delayed in AC6-KO platelets; at 1 minute, the levels were 8682 ± 1896 fmol/10^7 platelets (WT) and 4385 ± 1175 fmol/10^7 platelets (AC6-KO) (Figure 2B). Importantly, we found that in the absence of AC6, platelets retained some capacity to produce cAMP. Since cAMP concentrations are synergistically linked to synthesis and hydrolysis, we examined the potential role of PDE3A, the enzyme responsible for breaking down cAMP in platelets [25]. We observed no changes in the expression or activity of PDE3A between WT and AC6-KO platelets (Figure 2C, D).

Given the reduced cAMP generation, we next examined the importance of AC6 in cAMP-mediated PKA phosphorylation events using established platelet PKA substrates. In both groups, PGI_2 (1-100 nM) caused a concentration-dependent increase in phospho-VASP^{Ser157}, phospho-VASP^{Ser239}, phospho-GSK3β^{Ser9} (Figure 3), and phospho-PDE3A^{Ser312} (Supplementary Figure S7). The

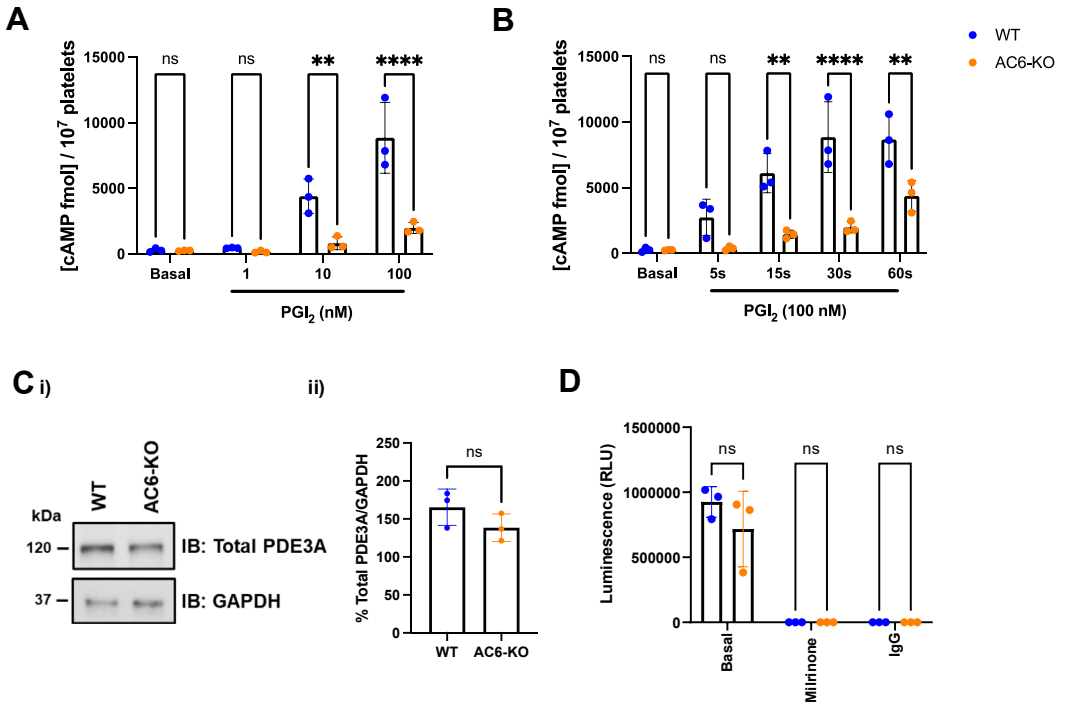

FIGURE 2 Contribution of AC6 to platelet cAMP production. (A) Washed platelets (2×10^8 platelets/mL) were treated with PGI_2 in increasing concentrations or (B) over time at fixed (100 nM) concentration. Reactions were terminated with 2.5% dodecyltrimeylammonium bromide, and intracellular cAMP generation was monitored. Data expressed as the concentration of cAMP (fmol) per 1×10^7 platelets. Comparisons were made between WT and AC6-KO using 2-way ANOVA with Šídák's multiple comparisons test (means ± SD, $n = 3$; ns = not significant, $^{**}P < .01$, $^{****}P < .0001$). (C) Total PDE3A expression in washed platelets via immunoblot analysis relative to GAPDH control presented as representative blot image and densitometry. (D) Washed platelets were treated with and without milrinone (10 μM) and assessed for PDE3A activity using a luciferase-based kinetic assay, and comparisons were made between WT and AC6-KO using 2-way ANOVA with Šídák's multiple comparisons test (means ± SD, $n = 3$; ns = not significant). AC6-KO, adenylyl cyclase 6 knockout; ANOVA, analysis of variance; cAMP, cyclic adenosine-3′,5′-monophosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IgG, immunoglobulin G; PDE3A, phosphodiesterase 3A; PGI_2, prostacyclin; WT, wild type.

phosphorylation of $VASP^{Ser157}$, $VASP^{Ser239}$, and $GSK3\beta^{Ser9}$ was reduced but not abolished in AC6-KO platelets at all concentrations of PGI_2 (Figure 3A). While phosphorylation of $PDE3A^{Ser312}$ appeared to be slightly reduced in the AC6-KO, this was not significant (Supplementary Figure S7A, B). We then applied forskolin (0.1-10 μM), which directly activates AC1 to AC8 (excluding AC9) to produce cAMP in a noncompartmentalized manner, independent of the IP receptor. Consistent with reduced capacity for cAMP generation, we found compromised phosphorylation of $VASP^{Ser157}$, $VASP^{Ser239}$, and $GSK3\beta^{Ser9}$ but little effect on $PDE3A^{Ser312}$ phosphorylation (Figure 3B and Supplementary Figure S7C, D). To assess cAMP production via receptors independent of the IP receptor, we measured $VASP^{Ser157}$ phosphorylation in response to adenosine (100 μM). Consistent with our findings in response to PGI_2, we found that $VASP^{Ser157}$ phosphorylation was impaired to basal levels in the AC6-KO compared with WT platelets ($P < .01$) (Supplementary Figure S8). To confirm that the cAMP signaling apparatus downstream of AC6 was unaffected, we treated platelets with the nonhydrolyzable analog of cAMP, 8–CPT-cAMP (1-100 μM), and we observed no differences in the phosphorylation of $VASP^{Ser157}$ between WT and AC6-KO (Figure 3C). Using phosphoflow cytometry [26,27], we found that platelets from AC6-KO mice produced significantly less phosphorylation of $VASP^{Ser157}$ and $VASP^{Ser239}$ than WT mice when stimulated with PGI_2 (100 nM) (16.9 ± 5.1 WT vs 11.9 ± 3.7 AC6-KO fold increase over basal phospho-$VASP^{Ser239}$; $P < .05$) (Figure 3D), confirming that cAMP signaling is also compromised in whole blood.

3.3 | Loss of AC6 leads to selective platelet hyposensitivity to PGI_2

We next assessed whether AC6 was linked to multiple platelet functions in the physiologic conditions of whole blood. We investigated the functional consequences using flow cytometric analysis of the key surface markers of platelet activation, active integrin $\alpha_{IIb}\ \beta_3$ (JON/A) and P-selectin (CD62P). We observed no difference in the ability of CRP-XL (1 μg/mL; Figure 4C) or PAR4 peptide (100 μM) to increase the surface expression of CD62P or active $\alpha_{IIb}\beta_3$ (data not shown). When whole blood was treated with PGI_2 (1-100 nM) before the addition of PAR4 peptide (100 μM) or CRP-XL (1 μg/mL), we observed a concentration-dependent inhibition of both markers in WT mice (Figure 4A, B). At 5 nM of PGI_2, we observed almost complete

TABLE Hematologic parameters in wild type and adenylyl cyclase 6 knockout mice.

Parameter	WT	AC6-KO	*P* value
WBC (10^9/L)	4.36 ± 1.29	4.74 ± 1.24	.66
RBC (10^{12}/L)	6.36 ± 0.32	6.35 ± 0.44	.90
HGB (g/L)	91.40 ± 2.95	91.56 ± 4.61	.65
HCT (%)	30.78 ± 1.27	30.84 ± 1.75	.96
PLT (10^9/L)	402.50 ± 40.64	466.22 ± 138.00	.50
MPV (fL)	6.59 ± 0.16	6.66 ± 0.10	.27
LYM (%)	79.90 ± 2.07	76.00 ± 10.79	.40
MXD (%)	8.09 ± 1.31	9.53 ± 4.13	.32
NEU (%)	12.01 ± 0.97	14.47 ± 6.95	.46

AC6-KO, adenylyl cyclase 6 knockout; HCT, hematocrit; HGB, hemoglobin; LYM, lymphocytes; MPV, mean platelet volume; MXD, mixed white blood cells; NEU, neutrophils; PLT, platelets; RBC, red blood cells; WBC, white blood cells; WT, wild type.

inhibition of both surface markers in WT platelets. However, in AC6-KO platelets, we found that the ability of PGI_2 to inhibit PAR4 peptide-stimulated P-selectin expression and activated α_{IIb} β_3 was significantly impaired (Figure 4A). For example, at PGI_2 (5 nM), we found 75% ± 14% inhibition of P-selectin expression in WT platelets compared with 16% ± 5% inhibition in AC6-KO platelets ($P < .0001$). To examine another pathway of platelet activation, we considered procoagulant activity; here, activated platelets facilitate coagulation by exposing procoagulant phosphatidylserine (PS) on their outer surface. In separate experiments, we assessed the inhibition of PS exposure by PGI_2 upon CRP-XL alone and dual agonist stimulation with PAR4 peptide and CRP-XL. Consistent with earlier experiments, PGI_2 inhibited CRP-XL-stimulated annexin V binding to procoagulant PS in both AC6-KO and WT mice (Figure 4C). Dual stimulation with CRP-XL/PAR4 peptide led to increased PS exposure compared with CRP-XL alone, but here, we observed a significant reduction in PS expression in response to PGI_2 in WT but not AC6-KO platelets (Figure 4C).

We next examined how the impaired cAMP signaling in AC6-KO platelets affected function. Measurement of static platelet adhesion showed that AC6-KO platelets adhered normally to both fibrinogen and collagen (Figure 4D). While it was not significantly different between WT and AC6-KO under direct comparison, the inhibitory actions of PGI_2 were compromised in the AC6-KO mice (Figure 4D). The number of WT platelets adhering to fibrinogen decreased from 45.7 ± 2.6 per field of view (FoV) to 14.8 ± 5.6 per FoV in the presence of PGI_2 (10 nM) ($P < .05$), whereas AC6-KO platelets only displayed a slight reduction from 38.8 ± 6.7 per FoV to 28.2 ± 5.7 per FoV in the presence of PGI_2 (10 nM) (Figure 4D). In contrast, platelet adhesion to collagen was significantly reduced in the presence of PGI_2 (10 nM) in both WT and AC6-KO platelets (Figure 4D). Taken together, these data suggest that AC6 may play a role in inhibiting platelet adhesion to fibrinogen but not to collagen. To examine the consequences of platelet AC6 deletion on thrombus formation under flow, we perfused (1000 s^{-1}) whole blood from AC6-KO and WT over immobilized fibrinogen (1 mg/mL). Under these conditions, platelets from both AC6-KO and WT mice adhered to the fibrinogen to form a series of microaggregates with no differences in overall surface coverage (Figure 4E). Preincubation of the blood from WT mice with PGI_2 (50 nM) led to a significant reduction in surface area coverage (7.1% ± 1.5% to 3.0% ± 1.5%; $P = .0005$). In contrast, PGI_2 did not affect platelet adherence and recruitment to fibrinogen in AC6-KO mice (Figure 4E and Supplementary Figure S9).

3.4 | AC6 controls hemostasis, thrombosis, and thrombus stability

Platelet aggregation and adhesion are critical to hemostasis *in vivo*, and our *in vitro* and *ex vivo* observations suggested that AC6-KO mice might be prone to accelerated thrombus formation due to PGI_2 hyposensitivity. To test this hypothesis, we first evaluated bleeding time using tail clip assays (Figure 5A). AC6-KO mice exhibited a significantly reduced bleeding time compared with WT mice, with bleeding time reduced from 228 ± 91 seconds for WT mice to 99 ± 62 seconds for AC6-KO mice ($P = .005$). Effective hemostasis is reliant upon the efficient formation of stable thrombi, and therefore we examined *in vivo* thrombus formation in response to ferric chloride ($FeCl_3$)-induced injury to mesenteric arterioles using fluorescence microscopy. Following $FeCl_3$ treatment, we found the rate of thrombosis was accelerated in the absence of AC6 (Figure 5B, C) as evidenced by a significantly reduced occlusion time from 25.33 ± 4.90 to 19.16 ± 4.30 minutes ($P = .007$). (Figure 5B). Further, we found that the area of the thrombus formed after injury was significantly larger in AC6-KO mice (Figure 5D). While observing thrombosis *in vivo*, we noticed that the thrombi formed were less stable. To examine this, we conducted individual kinetic analysis on thrombi by assessing sudden loss of fluorescence, a method we have used previously to measure embolization [28]. Embolic events were defined as a loss in thrombus area >15% between time points. We observed a 3.5-fold increase in the total number of embolic events in AC6-KO mice compared with WT mice (Figure 5E, F) (Welch's *t*-test, $P = .04$), suggesting a reduction in thrombus stability in the AC6-deficient mice.

4 | DISCUSSION

The tonic inhibition of platelets through endothelial-derived PGI_2 is critical to the effective management of systemic hemostasis. However, our understanding of the complex signaling events that allow cAMP to control platelets and the contribution of key signaling nodes has remained elusive, primarily due to a lack of precise pharmacologic and genetic tools. In this study, we generated a model to study AC6 mediated-cAMP signaling in platelets and show that AC6 is critical to hemostasis in mice while also highlighting potential redundancy in this signaling system. In our novel platelet-specific AC6-KO mouse, we found that the deletion of the *Adcy6* gene from the megakaryocyte

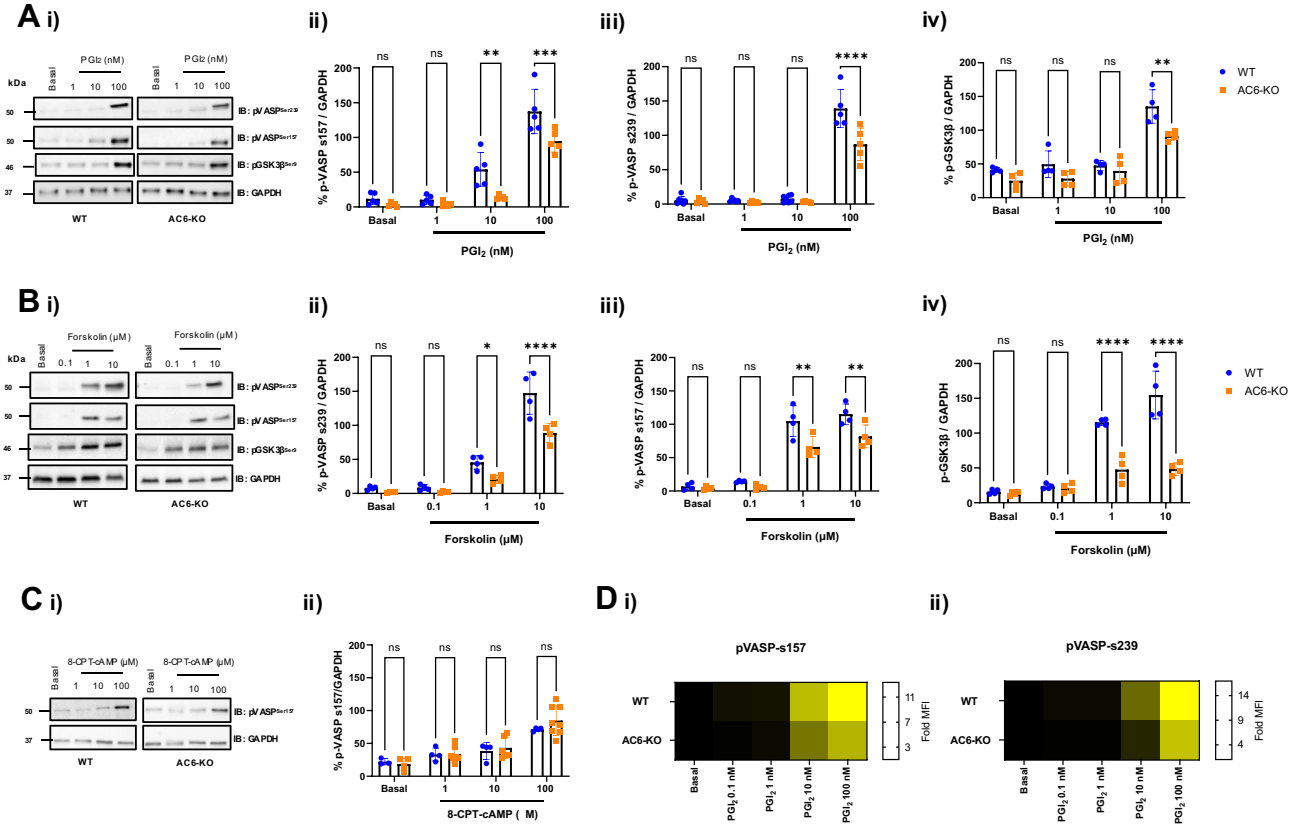

FIGURE 3 Contribution of AC6 to cAMP-mediated PKA phosphorylation events. (A–C) Washed platelets (5×10^8 platelets/mL) were treated with increasing concentrations of (A) PGI_2 (1-100 nM), (B) forskolin (0.1-10 μM), or (C) 8–CPT-cAMP (1-100 μM) before lysis with Laemmli buffer and separation of proteins via sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Phosphorylation of individual PKA substrates ($VASP^{Ser157}$, $pVASP^{Ser239}$, and GSK3β) was assessed via immunoblotting. Data presented as (i) representative images and (ii-iv) densitometry analysis relative to appropriate loading control. Comparisons were made between WT and AC6-KO using 2-way ANOVA with Šídák's multiple comparisons test (means ± SD, n = 3-5, ns = not significant, $^*P < .05$, $^{**}P < .01$, $^{****}P < .0001$). (D) Whole blood was treated with PGI_2 (0.1-100 nM), and $pVASP^{Ser157}$ and $pVASP^{Ser239}$ were measured by phosphoflow cytometry. The extent of phosphorylation is shown by representative heatmaps and data expressed as fold MFI over basal (n = 5). 8-CPT-cAMP, 8-(4-chlorophenylthio)adenosine-3′,5′-cyclic monophosphate; AC6, adenylyl cyclase 6; ANOVA, analysis of variance; cAMP, cyclic adenosine-3′,5′-monophosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KO, knockout; MFI, mean fluorescence intensity; p, phosphorylated; PKA, protein kinase A; PGI_2, prostacyclin; GSK3β, glycogen synthase kinase 3 beta; VASP, vasodilator-stimulated phosphoprotein; WT, wild type.

lineage had no effect on platelet numbers and morphology but did reveal that (1) AC6 is linked to PGI_2-induced cAMP generation and downstream PKA signaling, (2) AC6 generated cAMP is selectively coupled to the inhibition of PAR-mediated platelet activation, and (3) AC6-KO mice have an aggressive prothrombotic phenotype that is associated with reduced bleeding times despite the presence of other AC isoforms.

The first key observation, to our knowledge, was that the loss of AC6 does not affect "basal" cAMP generation in washed platelets *in vitro* in the absence of exogenous stimulation of the IP receptor/AC by PGI_2, Consistent with other studies [14,29], this suggests that *in vitro*, the residual AC activity is relatively small compared with *in vivo* studies and could suggest AC5 or soluble AC9 may control unstimulated cAMP production, as suggested for other cells [30,31]. In contrast, AC6-KO platelets exhibited impaired PGI_2-mediated cAMP production, with both amount and rate of cAMP accumulation diminished but not ablated. We confirmed that the reduced accumulation of cAMP was linked to synthesis rather than accelerated hydrolysis since there was no significant change in the expression of PDE3A and PDE2 or in PDE3A activity [15,32,33]. It has been well established that platelet PDE3A activity is essential to maintain low equilibrium levels of cAMP and to determine a threshold for platelet activation [33,34], which could account for the lack of PDE3A compensation in our AC6-KO mouse. These data suggest, to our knowledge, for the first time a potential redundancy between AC isoforms in platelets, and while the IP receptor is coupled to AC6, it must also be linked to other cAMP-generating enzymes. Mirroring the impaired cAMP production in the absence of AC6, there was diminished phosphorylation of established PKA substrates, VASP [35–39] and GSK [40]. The loss of signaling in the AC6-KO was not linked to changes in the expression of PKA regulatory or catalytic subunits. Furthermore, a loss of AC6 also significantly impaired adenosine-driven cAMP signaling as measured by $VASP^{Ser157}$

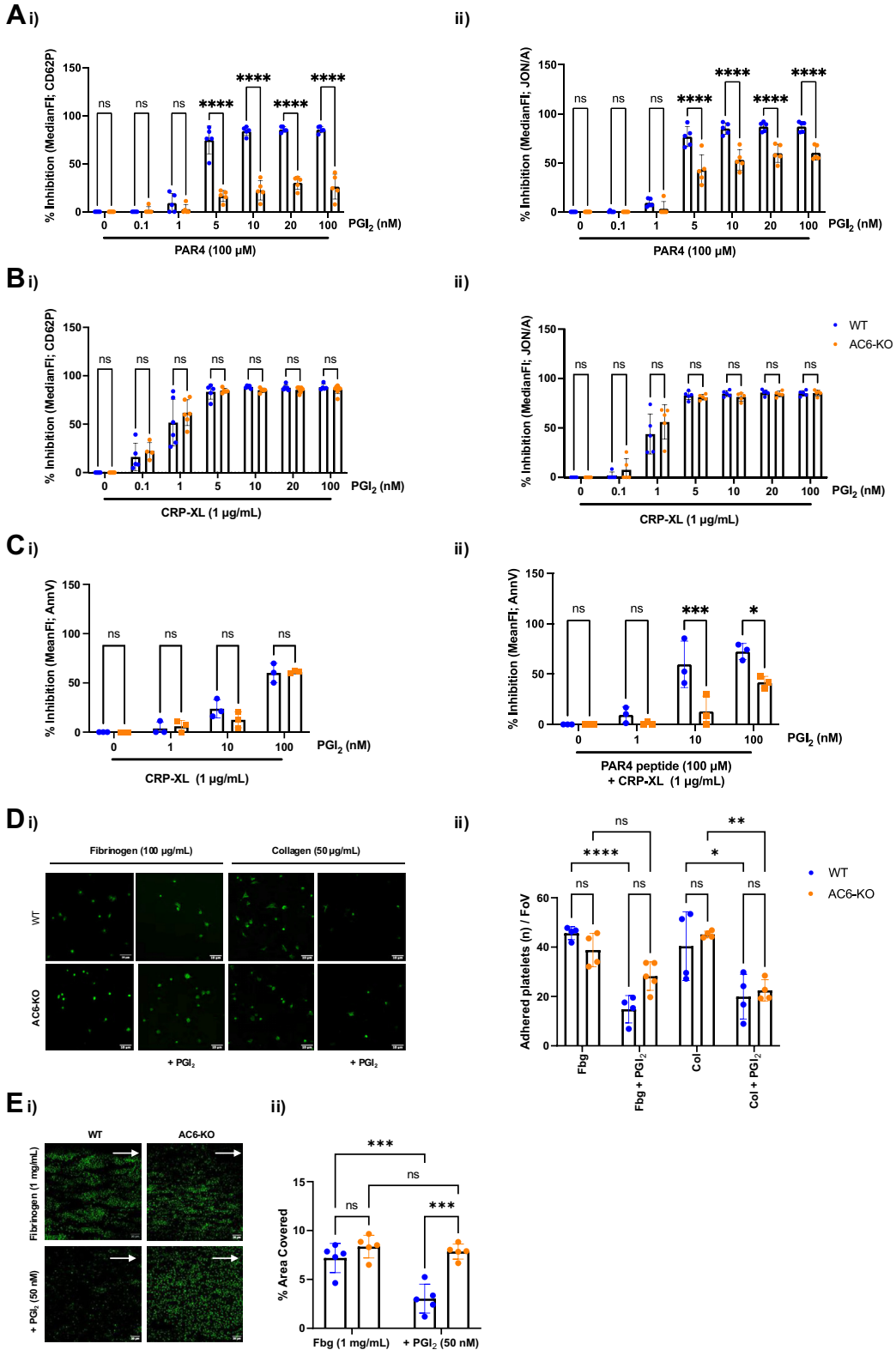

FIGURE 4 AC6 mediates inhibition of PAR4- but not CRP-XL-stimulated platelet activity. (A) Whole blood was treated with CRP-XL (1 µg/mL) or (B) PAR4 peptide (100 µM) in the absence or presence of PGI_2 (1-100 nM) and incubated with fluorescently conjugated markers for platelet activation (CD62P and JON/A) (n = 5). (C) Whole blood was treated with CRP-XL (1 µg/mL) alone or in combination with PAR4 (100 µM) and incubated with AnnV (platelet marker for phosphatidylserine exposure) (n = 3). Data expressed as percentage inhibition of median fluorescence intensity (MedianFI) and percentage inhibition of mean fluorescence intensity (MeanFI). Comparisons were made between WT and AC6-KO using 2-way ANOVA with with Šídák's multiple comparisons test (means ± SD, ns = not significant, $^*P < .05$, $^{***}P < .001$, $^{****}P < .0001$). (D) Washed platelets (1×10^7 platelets/mL) were spread on Fbg (100 µg/mL) or Col (50 µg/mL) for 30 minutes, washed twice with

phosphorylation, suggesting that AC6 activity may be localized to multiple cAMP-generating receptors.

The most relevant cell model to platelets are vascular smooth muscle cells (VSMCs), which share many features of cAMP signaling, including PKA isoforms and their known protein targets (VASP, IP3R1, and MYPT1). Among the AC isoforms expressed in VSMCs, AC3, AC5, and AC6 are the most prominent [41–45]. While studies have shown that AC6, in particular, is coupled to β-adrenergic receptors-mediated signaling, the precise coupling of IP to specific AC isoforms requires further investigation, although it is plausible given its regulatory role in cardiac muscle [45] and our own observations that AC6 may be involved in IP signaling in VSMCs [44,46]. In endothelial cells, ACs play a role in vascular permeability. PGI_2-mediated signaling via AC6 (not AC5) contributes to a feedback loop that increases barrier function. In human umbilical vein endothelial cells, adenoviral gene transfer of AC6, not AC5, led to an increase in IP receptor-stimulated cAMP, while thrombin-stimulated increases in endothelial cell barrier function were reduced [47]. Although beyond the scope of the current study, further investigation into the specific roles of platelet AC5, AC6, and soluble AC9 are warranted.

Interestingly, we found that cAMP-mediated $PDE3A^{Ser312}$ phosphorylation [33] and PDE3A activity were unaffected by loss of AC6. We believe that this is an important observation since it suggests to our knowledge for the first time that specific targets for PKA-mediated phosphorylation could be linked to distinct cAMP-generating systems. PDE3A can be phosphorylated and activated by protein kinase C (PKC) independently of PKA activity [48,49]. We have shown that in the absence of AC6, cAMP levels are reduced in response to PGI_2, with impaired PGI_2-mediated inhibition of PAR-stimulated platelet activity. It is well known that thrombin and protease-activated receptor peptides (SFLLRN and GYPGKF) activate PDE3A through PKC, thus increasing cAMP hydrolysis [33]. Our findings demonstrate that a loss of AC6 thereby tips the balance between cAMP generation and hydrolysis, resulting in impaired PGI_2-mediated inhibition of PAR-stimulated platelet activity. The lack of a phenotype in response to inhibition of GPVI-meditated platelet activity by PGI_2 indicates a potential role for novel PKC isoforms PKCδ or PKCθ, which have positive regulatory roles in PAR-stimulated responses but negative roles in GPVI signaling [49–52].

To understand the ability of AC6 to control platelet function, we focused on studies with whole blood. Using phosphoflow, we were able to establish that PGI_2 is active in whole blood and to induce cAMP signaling in platelets, which was diminished but not abolished by the absence of AC6. We next moved through a series of progressively more physiologic assays to pinpoint how diminished cAMP affects platelet function. Flow cytometry demonstrated that platelet activation was unaffected by the loss of the enzyme, which we believe is consistent with our observation that basal cAMP remains unaffected, as well as other studies in IP receptor-null mice in which thrombosis was accelerated while platelet aggregation remained normal [29]. However, we did find that the ability of PGI_2 to inhibit PAR-stimulated platelet activity was severely compromised, while GPVI-mediated activation was unaffected [53,54]. We know that our observations demonstrate that AC6-generated cAMP is not linked to all G-protein coupled receptors since inhibition of U46619- and ADP-mediated platelet aggregation were also unaffected by AC6 deficiency (data not shown). The reason for this is unclear but is consistent with earlier studies demonstrating that early GPVI signaling is unaffected by cAMP signaling [54]. Previously, we demonstrated that cAMP signaling prevents thrombin-induced membrane compartmentalization of RhoA [55]. This finding could explain why the PGI_2 signaling defect observed in AC6-KO platelets is observed more prominently in PAR- and GPVI-mediated pathways. Interestingly, when we examined static platelet adhesion, the inhibition of fibrinogen-mediated adhesion by PGI_2 was compromised in AC6-KO but not in WT platelets, while inhibition of collagen-mediated adhesion was unchanged in both genotypes. In many nucleated cells, cAMP signaling is partitioned to restrict signaling to intended targets and allows concurrent signals to drive specific functions [56]. We have shown previously that a similar system may exist in platelets, where anchored PKA-I is responsible for controlling, at least in part, signaling downstream of GPIb [57]. Therefore, the inhibition of GPVI-mediated activation may be linked specifically to a distinct isoform of AC, potentially AC5 or AC9, although this would require significant further investigation to establish. Nevertheless, we did find that the loss of AC6 severely compromised the ability of PGI_2 to inhibit *in vitro* thrombosis to immobilized fibrinogen, suggesting that AC6 may be important in thrombosis. Indeed, we show that despite the absence of AC6 not fully blocking cAMP synthesis and signaling, it has a profound effect on hemostasis and thrombosis *in vivo*. We found significantly reduced bleeding times in tail clip assays, which was coupled to a more aggressive and rapid thrombotic response to vascular injury. Interestingly, the thrombi formed *in vivo* in the AC6-deficient mice were inherently unstable, which may suggest potential problems with the

phosphate-buffered saline to remove nonadherent platelets, and then treated with PGI_2 (10 nM) for 2 minutes prior to fixation. Data presented as (i) representative images, (ii) average number of adhered platelets across 8 images (FoV = 74.97 × 74.97 μm). Comparisons were made between absence and presence of PGI_2 for each group using 2-way ANOVA with Tukey's multiple comparisons test (means ± SD, $n = 4$; ns = not significant, $^{*}P < .05$, $^{**}P < .01$, $^{****}P < .0001$). (E) Whole blood was perfused over Fbg (1 mg/mL) coated biochips at 1000 s^{-1} in the presence and absence of PGI_2 (50 nM) for 2 minutes. Data presented as (i) representative images and (ii) percentage area covered. Comparisons were made between WT and AC6-KO using 2-way ANOVA with Šídák's multiple comparisons test (means ± SD, $n = 5$; ns = not significant, $^{***}P < .001$). AC6, adenylyl cyclase 6; AnnV, annexin V; ANOVA, analysis of variance; CD62P, P-selectin; Col, collagen; CRP-XL, cross-linked collagen-related peptide; Fbg, fibrinogen; FoV, field of view; JON/A, active integrin $\alpha_{IIb}\beta_3$; KO, knockout; PAR4, protease-activated receptor 4; PGI_2, prostacyclin; WT, wild type.

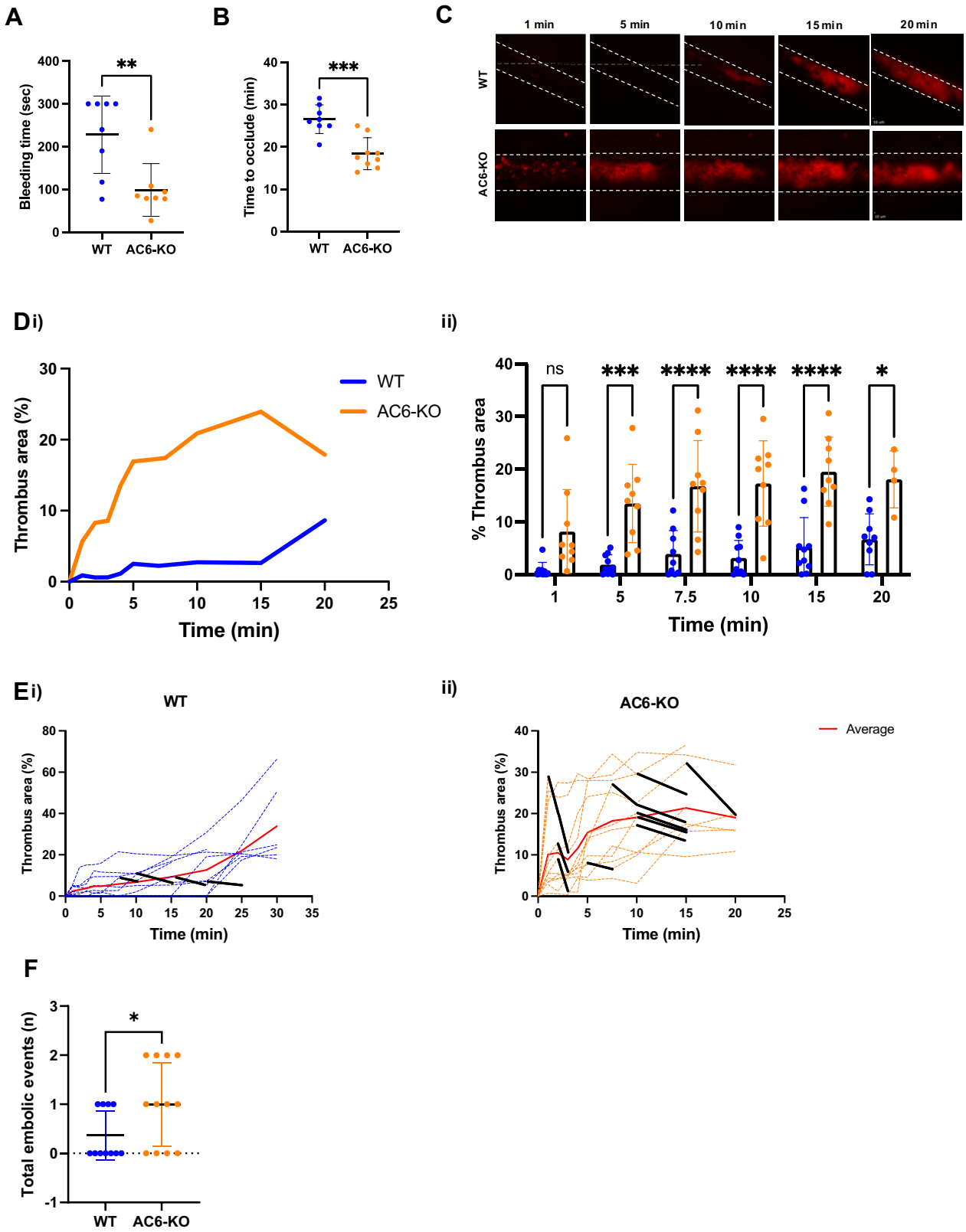

FIGURE 5 AC6 mediates hemostasis, thrombosis, and thrombus stability *ex vivo* and *in vivo*. (A) To assess hemostasis, tail clip bleeding time assays were performed and compared using an unpaired Student's *t*-test with Welch's correction (WT, $n = 8$; AC6-KO, $n = 8$; $P < .05$). (B–D) *In vivo* thrombus formation after $FeCl_3$ injury to mesenteric vessels of WT mice ($n = 8$) was compared with AC6-KO mice ($n = 9$). Data presented as (B) vessel occlusion time, (C) representative images, (D) (i) representative graph of thrombus area over time, (D) (ii) percent thrombus area over time expressed as means ± SD per mouse from an average of up to 2 vessels (WT, $n = 8$; AC6-KO, $n = 9$). Mean vessel occlusion time data were compared

speed of platelet deposition, fibrin generation, or clot retraction. Taken together, we propose that the diminished bleeding time is related to accelerated thrombosis in the absence of AC6. This is likely due to a reduced downstream effect of PGI_2 in controlling thrombosis. While it is difficult to measure platelet sensitivity to PGI_2 *in vivo* because of the constant production and release by the endothelium, its short half-life, and the presence of platelet PDEs, we believe that other pieces of evidence point to this being a probable explanation: first, the reduced inhibitory effect of PGI_2 on whole blood *ex vivo* thrombosis, and second, the diminished PKA signaling events measured by phosphoflow in whole blood. The nature of the hyperthrombotic response is likely multifactorial, including unchecked platelet secretion and procoagulant function supported by our flow cytometry findings. The increased generation of procoagulant platelets leads to exposure of PS on their surface to support the formation of both tenase and prothrombinase complexes required for thrombin and fibrin generation. Potent platelet activation leads to the generation of 2 core subpopulations that may enact distinct functional roles, and we have previously shown that PGI_2 plays a key role in modifying these subpopulations in whole blood and, in particular, preventing PS exposure at the cell surface [26]. Our observation that in the absence of AC6, the ability of PGI_2 to modulate PS in response to dual stimulation is compromised may render these platelets more thrombogenic. Due to the broader nature of *Pf4-Cre* recombination beyond the megakaryocyte lineage, low-level *Pf4-Cre* recombination may occur in circulating leukocytes, which may lead to the excision of the *Adcy6* gene in leukocytes [58–60]. While this may contribute to the thrombotic phenotype demonstrated in our AC6-KO mouse during *in vivo* thrombosis experiments, data regarding the expression of ACs and, in particular, AC6 in leukocytes is lacking [61,62].

A general problem in studying AC biology is the lack of isoform-specific inhibitors or antibodies. This key issue is exemplified here where we were unable to precisely confirm the absence of AC6 through immunoblotting and relied on real-time quantitative polymerase chain reaction. Nevertheless, the genetic approach has allowed us for the first time to describe functional links between an AC isoform and the regulation of activation by an individual agonist. Given that only activation through PARs is affected, it suggests that AC6-generated cAMP is not linked to general G-protein coupled activation but is compartmentalized. In airway smooth muscle cells, AC2 and AC6 generate distinct pools of cAMP, suggesting that they may regulate different cellular responses in separate compartments [42,63–65]. It is unclear if a similar model is present in platelets expressing AC5 and AC6, but there is evidence in cardiomyocytes for differential localization [66]. This compartmentalization occurs through the formation of macromolecular complexes with kinases and phosphodiesterases, coupled by A-kinase anchoring proteins (AKAPs). In cardiomyocytes, AC5 forms an AKAP-scaffolded complex with the β-adrenergic receptor, PKA, and PDE4D3, from which AC6 is excluded, leading to AC5-specific actions. The role of AKAPs in platelet biology is unclear, although their presence is suggested by transcriptomic studies and our own work identifying the presence of AKAPs 7, 9, 12, 13, 79, 95, 149, and moesin (Khalil and Naseem, unpublished) [23,24]. Of potential interest is AKAP9, which is an AC-associated AKAP [67,68]. Previously, we showed that AC5/6 is localized to lipid raft fractions in human platelets but were unable to determine if the 2 isoforms were in distinct lipid-enriched fractions or were colocalized [69]. It is possible that the colocalization of thrombin receptors with AC6 could account for our observations and open the possibility that partitioned pools of cAMP downstream of individual AC isoforms control specific aspects of platelet function in a coordinated manner. This may be a key area for understanding how cAMP and cGMP signaling pathways are coordinated to regulate platelet-driven hemostasis.

In summary, the study provides, to our knowledge, the first direct evidence that AC6, which is expressed in both mouse and human platelets at similar levels, plays a primary role in regulating hemostasis and thrombosis. Despite the presence of AC5 and soluble AC9 and their potential role in platelet cAMP generation, AC6 may be the key mediator of thrombosis in mice. The potential functional redundancy between AC5, AC6, and soluble AC9 in controlling platelet responses to specific agonists requires further investigation.

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AUTHOR CONTRIBUTIONS

B.A.W. designed and performed experiments, analyzed data, and wrote and edited the manuscript. M.S.H., J.S.K., and L.T.C. performed experiments, analyzed data, and edited the manuscript. C.D. analyzed data and edited the manuscript. R.A.S.A., N.A.T., and M.T.K. provided essential materials and edited the manuscript. K.M.N. designed the research, acquired the funding, and wrote the manuscript.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

DATA AVAILABILITY

All raw data and protocols can be made available by emailing the corresponding author.

between WT and AC6-KO mice using an unpaired Student's *t*-test with Welch's correction (***$P < .001$). Percentage thrombus size over time was compared between WT and AC6-KO mice using 2-way ANOVA with Šídák's multiple comparisons test (ns = not significant, *$P < .05$, ***$P < .001$, ****$P < .0001$). (E) The percentage of reduction in the thrombus area was calculated for each vessel. Black bars indicate a percentage reduction in thrombus size >15%, while the red line indicates the average percentage area for WT vs AC6-KO. Data presented as individual injuries per group (WT, $n = 11$ vessels from 8 mice; AC6-KO, $n = 12$ vessels from 9 mice). (F) Total number of embolic events was compared between WT and AC6-KO using an unpaired Student's *t*-test with Welch's correction (*$P < .05$). AC6, adenylyl cyclase 6; KO, knockout; WT, wild type.

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SUPPLEMENTARY MATERIAL

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