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FUNDC2 regulates platelet activation through AKT/GSK-3β/cGMP axis

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Aims AKT kinase is vital for regulating signal transduction in platelet aggregation. We previously found that mitochondrial protein FUNDC2 mediates phosphoinositide 3-kinase (PI3K)/phosphatidylinositol-3,4,5-trisphosphate (PIP3)-dependent AKT phosphorylation and regulates platelet apoptosis. The aim of this study was to evaluate the role of FUNDC2 in platelet activation and aggregation.

Methods and results We demonstrated that FUNDC2 deficiency diminished platelet aggregation in response to a variety of agonists, including adenosine 5′-diphosphate (ADP), collagen, ristocetin/VWF, and thrombin. Consistently, *in vivo* assays of tail bleeding and thrombus formation showed that FUNDC2-knockout mice displayed deficiency in haemostasis and thrombosis. Mechanistically, FUNDC2 deficiency impairs the phosphorylation of AKT and downstream GSK-3β in a PI3K-dependent manner. Moreover, cGMP also plays an important role in FUNDC2/AKT-mediated platelet activation. This FUNDC2/AKT/GSK-3β/cGMP axis also regulates clot retraction of platelet-rich plasma.

Conclusion FUNDC2 positively regulates platelet functions via AKT/GSK-3β/cGMP signalling pathways, which provides new insight for platelet-related diseases.

Keywords FUNDC2 • Platelet activation • AKT

1. Introduction

Platelets play essential roles in haemostasis, angiogenesis, inflammation, and metastasis.[1] Phosphoinositide 3-kinase (PI3K) and serine-threonine kinase AKT (also known as protein kinase B, PKB) are essential for regulating signal transduction in platelet aggregation and activation.[2–8] PI3K is activated downstream of a diversity of platelet receptors, including G protein-coupled receptors (GPCRs), αIIbβ3, GPIb-IX-V, and collagen receptor glycoprotein VI (GPVI).[4,6,9–11] Activated PI3K can phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP2) to produce phosphatidylinositol-3,4,5-trisphosphate (PIP3), which is required for AKT phosphorylation. PIP3 directly binds AKT to its pleckstrin homology (PH) domain, facilitates AKT recruitment to the plasma membrane, and mediates AKT activation.[12–14] AKT is also the primary substrate activated by PI3K/PIP3 and is critical for platelet aggregation induced by GPCR, αIIbβ3, GPIb-IX-V, and GPVI.[3,4,6]

Recently, we identified a mitochondrial outer membrane protein FUNDC2. Our previous report showed that FUNDC2 directly and specifically binds PIP3, thereby facilitates the recruitment of PIP3 to mitochondria.[15] It is well known that AKT is evoked by binding to PIP3 and PI3K is essential for PIP3/AKT stimulation.[16] Our previous data1 also showed that FUNDC2 enhanced AKT phosphorylation in a PIP3/PI3K-dependent manner.[15] FUNDC2/PIP3 enhances AKT phosphorylation on mitochondria, which is required for platelet maintenance. Loss of FUNDC2 decreases PIP3 level on mitochondria, impairs AKT activation, and shortens platelet lifespan. Thus, FUNDC2-knockout (KO) mice display thrombocytopenia in response to hypoxia stress.[15] In the present study, we further evaluated the role of FUNDC2 in platelet aggregation and thrombus formation. Our data demonstrated that FUNDC2 positively regulates platelet activation via AKT/GSK-3β/cGMP signalling pathways.

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Key points

- Loss of FUNDC2 impairs platelet activation.
- FUNDC2 regulates platelet functions via AKT/GSK-3β/cGMP signalling pathways.

2. Methods

2.1 Materials

FUNDC2 antibody (Ab) was generated by immunizing rabbits with recombinant FUNDC2 protein and affinity purified by our laboratory. Abs (1 µg/mL final concentration) were used for immunoblotting. Unless otherwise noted, all Abs were purchased from Cell Signaling and all chemicals were from Sigma Aldrich.

2.2 Mice

All animal experiments conform to the rules of the American Association for the Accreditation of Laboratory Animal Care (AAALAC) International and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All procedures were approved by the Animal Care Committee of Peking University accredited by AAALAC International (IMM-ChengHP-15). Adult (3∼5-month-aged) male animals were used in this study. After anaesthesia, blood and mouse tissues were harvested for further analyses. During the experiments, mice were anaesthetized with 150 mg/kg intraperitoneal (i.p.) sodium pentobarbital before euthanasia by CO_2 inhalation.

2.3 Platelet preparation

Whole blood from mice was mixed with 1/10 volume of White's anticoagulant (2.94% sodium citrate, 136 mM glucose, pH 6.4). Platelet-rich plasma (PRP) was obtained by centrifugation at 150 *g* for 10 min. Washed platelets were obtained by centrifugation at 800 *g* for 10 min and resuspended in modified Tyrode's buffer (12 mM $NaHCO_3$, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 2 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 10 mM HEPES, pH 7.4). Washed platelets were left to rest for 30 min before use. Human platelets were prepared from healthy donors, as Institutional Review Board approval was obtained from Peking University, and informed consent was obtained from volunteers in accordance with the Declaration of Helsinki.

2.4 Immunoblotting

Tissues or cells were lysed in lysis buffer (30 mM HEPES, 100 mM NaCl, 0.5% Nonidet P (NP)-40, protease inhibitors mixture, pH7.6) on ice for 10 min and the lysates were centrifuged at 13 000 rpm for 10 min. For immunoblotting, proteins were resolved by SDS-PAGE transferred onto nitrocellulose membranes and detected with the indicated antibodies.

2.5 Electron microscopy

Platelets were fixed with 2% paraformaldehyde at 4°C overnight and dehydrated in a graded ethanol series and embedded in acrylic resin (LR White). A 70 nm ultrathin sections were mounted on nickel grids. Grids were washed five times for 5 min in 0.5% BSA, incubated for 15 min in 1% glutaraldehyde, washed twice for 5 min in PBS then three times in distilled water, stained, and dried at room temperature. The samples were visualized using a 120 kV Jeol electron microscope at 80 kV and images were captured using an AMT digital camera.

2.6 Platelet aggregation

Washed platelets (3×10^8/mL) were pretreated with inhibitors for 3 min, and then the aggregation of platelets was stimulated with thrombin (0.05 U/mL). Aggregation was measured in a turbidometric aggregometer (Precil LBY-NJ, Beijing, China) with a stirring speed of 1000 rpm at a temperature of 37°C.

2.7 Flow cytometry analysis

Platelets were labelled with FITC-conjugated annexin V for apoptotic analysis; with FITC-conjugated anti-P-selectin (CD62P), GPIbα (CD42b), GPV (CD42d), GPVI, and GPIIb/IIIa (CD41/CD61) antibodies, respectively for platelet receptors measurement, before quantitative analysis by flow cytometry.

2.8 Tail bleeding time

The tail of the mouse was amputated at distal 3 mm and the 1.5 cm of tail was immersed in 37°C saline. Time to complete cessation of bleeding was recorded.

2.9 Ferric chloride-induced carotid artery injury

A ferric chloride-induced carotid artery injury murine thrombosis model was processed. Carotid artery was separated from other tissue in isofluorane-anaesthetized mice, and injury was induced by 10% $FeCl_3$. Monitoring of carotid artery blood flow was initiated at the time of $FeCl_3$ treatment and continuously monitored for 13 min. Carotid artery blood flow <0.06 mL/min was scored as occlusion, allowing the time to first occlusion to be determined.

2.10 Clot retraction

PRP was anticoagulated with 0.38% sodium citrate, preincubated with inhibitors, and induced to coagulate with 0.2 U/mL thrombin. The clots were allowed to retract at 37°C. Two-dimensional sizes of retracted clots on photographs were quantified using NIH ImageJ software, and retraction was expressed as retraction ratio [1 - (final clot size/initial clot size)].

2.11 Statistical analysis of data

Statistical data are presented as mean ± SEM. Significance was calculated by Student's *t*-test. NS, no statistical significance; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. The number of independent experiments or mice was clarified in figure legends or in column of each figure.

3. Results

3.1 Normal mitochondrial morphology and functions in FUNDC2-knockout platelets

Our previous data characterized that FUNDC2, which is localized in the outer mitochondrial membrane has a high protein level in mouse platelets.[15] Herein, we tested the protein level of FUNDC2 in human platelets. Immunoblotting assays confirmed the presence of FUNDC2 in human platelets and showed the similar levels of this protein in human and mouse (*Figure 1A*). Conventional FUNDC2-KO mice[15] was used and the absence of FUNDC2 in platelets was confirmed by immunoblotting (*Figure 1B*). Consistent with our previous report,[15] the platelet numbers of FUNDC2-deficient mice are similar as the wild-type littermates in normal conditions (*Figure 1C*). Moreover, Fundc2-deficient platelets

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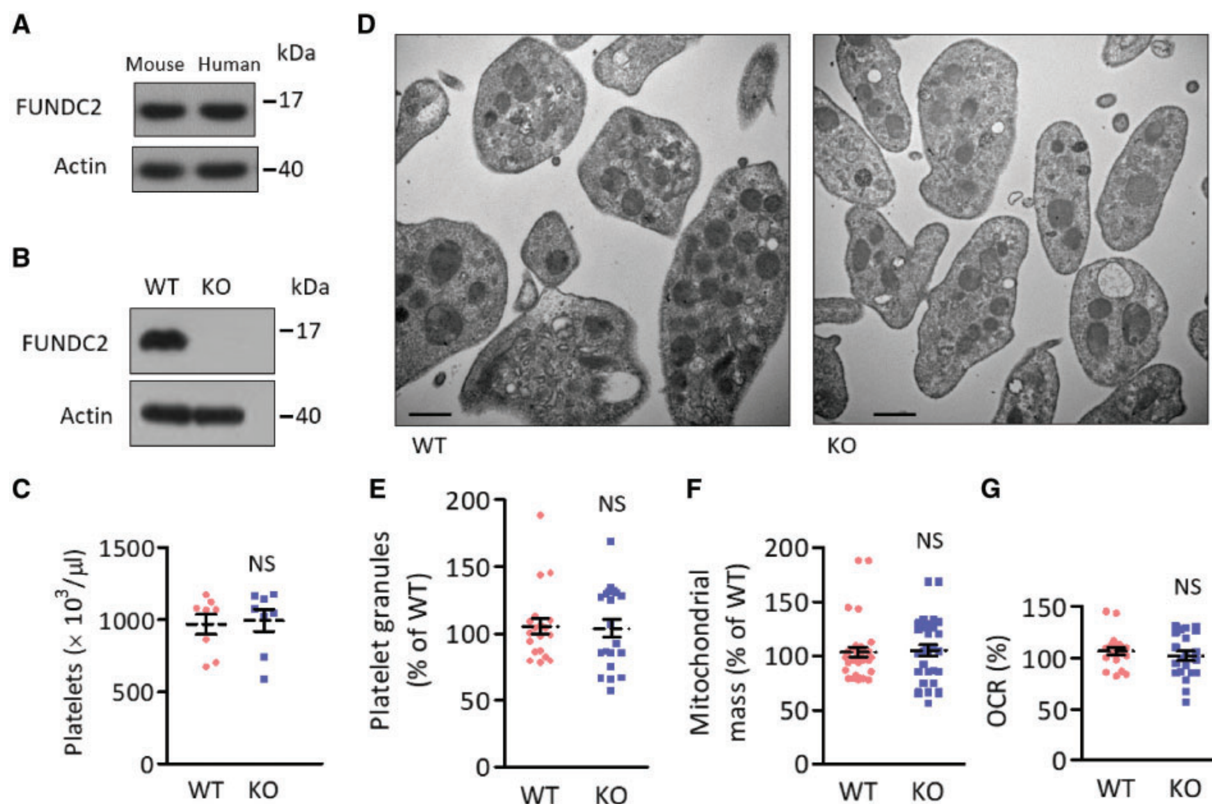

Figure 1 Normal mitochondrial morphology and functions in FUNDC2-KO platelets. (*A*) FUNDC2 was immunoblotted in human and mouse platelets. (*B*) Immunoblotting results showed that FUNDC2 was depleted in KO platelets. (*C*) Platelet counts were determined from FUNDC2-KO mice and the wild-type (WT) littermates; $n = 8$ mice. (*D*) Transmission electron microscopy imaging showed platelets isolated from WT or KO mice; scale bar, 500 nm. (*E*) The number of platelet granules was normalized to the percentage of WT platelets; $n = 20$ platelets from five mice. (*F*) Mitochondrial mass was measured by flow cytometry after staining with NAO (20 nM); $n = 33$ platelets from five mice. (*G*) Platelets were isolated and the oxygen consumption rate (OCR) was measured; $n = 20$ independent experiments from five mice. Data are mean ± SEM; Student's *t*-test; NS, no statistical significance.

exhibited morphological normality similarly as wild-type ones (*Figure 1D*). The deficiency of this mitochondrial protein had no effect on the number of platelet granules (*Figure 1E*), mitochondrial mass (*Figure 1F*), or mitochondrial functions (*Figure 1G*).

3.2 FUNDC2 deficiency inhibited platelet aggregation in response to a diversity of stimulation

Firstly, we measured platelet receptor expression levels in FUNDC2-KO mice. Platelets were isolated from FUNDC2-deficient mice and the wild-type littermates and then labelled with FITC-conjugated antibodies, respectively, before quantitative analysis by flow cytometry. The baseline levels of P-selectin (CD62P), GPIbα (CD42b), GPV (CD42d), GPVI, and GPIIb/IIIa (CD41/CD61) were not significantly different between the KO and WT mice (*Figure 2A* and Supplementary material online, *Figure S1A*). However, GPIIb/IIIa activation was impaired in FUNDC2-KO platelets in response to GPCR agonist (adenosine 5′-diphosphate, ADP) stimulation (*Figure 2B* and Supplementary material online, *Figure S1B*).

Next, we stimulated platelets with thrombin. The aggregation of FUNDC2-deficient platelets was severely diminished, compared with the wild-type platelets. FUNDC2-deficient platelets were also tested with ADP, collagen, and ristocetin/von Willebrand factor (VWF). Similarly, the aggregation of FUNDC2-KO platelets was attenuated in response to these soluble agonists stimulation (*Figure 2C*). Therefore, FUNDC2 plays a critical role in agonist-induced platelet activation.

Our previous data characterized that FUNDC2 regulate platelet apoptosis in hypoxic stress.[15] Herein, we tested annexin V binding levels of FUNDC2-KO platelets in response to thrombin stimulation. There were no significant difference between the KO and WT platelets (*Figure 2D*).

3.3 FUNDC2 deficiency caused a prolonged bleeding time *in vivo*

Next, the function of FUNDC2 in haemostasis was examined by measuring bleeding times *in vivo* (*Figure 3A*). The average bleeding time of FUNDC2-KO mice (116 ± 9 s, $n = 10$) was significantly longer ($P = 0.019$) than that of the wild-type littermates (87 ± 8 s, $n = 12$). Thus, FUNDC2 deficiency increased the bleeding time. Given the conventional knockout of FUNDC2 in whole body of mouse, we performed the transplantation of bone marrow to exclude the effects of the interactions between platelets and other cells such as vascular endothelial cells.

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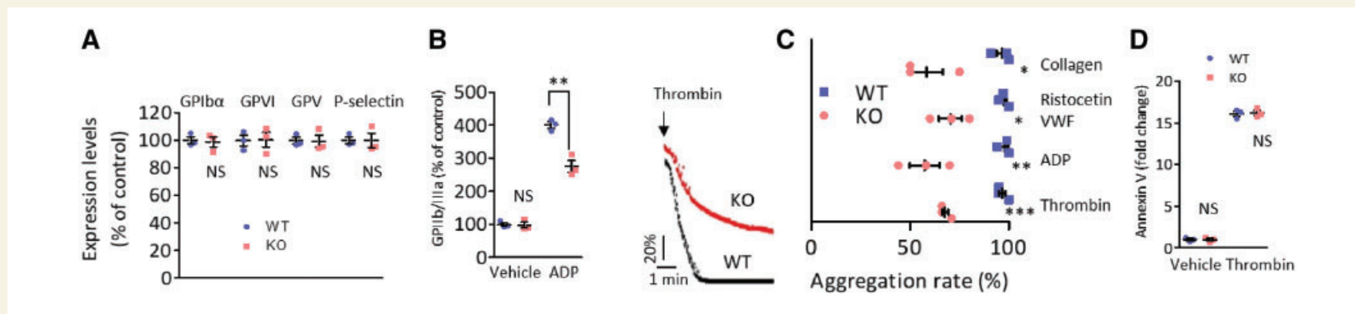

Figure 2 FUNDC2 deficiency inhibited platelet aggregation. (*A*) The baseline levels of platelet receptors were measured by flow cytometry. (*B*) GPIIb/IIIa activation was impaired in FUNDC2-KO platelets in response to10 μM ADP. (*C*) The aggregation of FUNDC2-deficient platelets was diminished in response to 0.05 U/mL thrombin, 10 μM ADP, 1.25 mg/mL ristocetin and 35 μg/mL VWF, or 0.5 μg/mL collagen. (*D*) There were no significant difference of annexin V binding levels between the KO and WT platelets in response to 0.05 U/mL thrombin. Data are mean ± SEM; $n = 3$ mice; Student's *t*-test; NS, no statistical significance; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

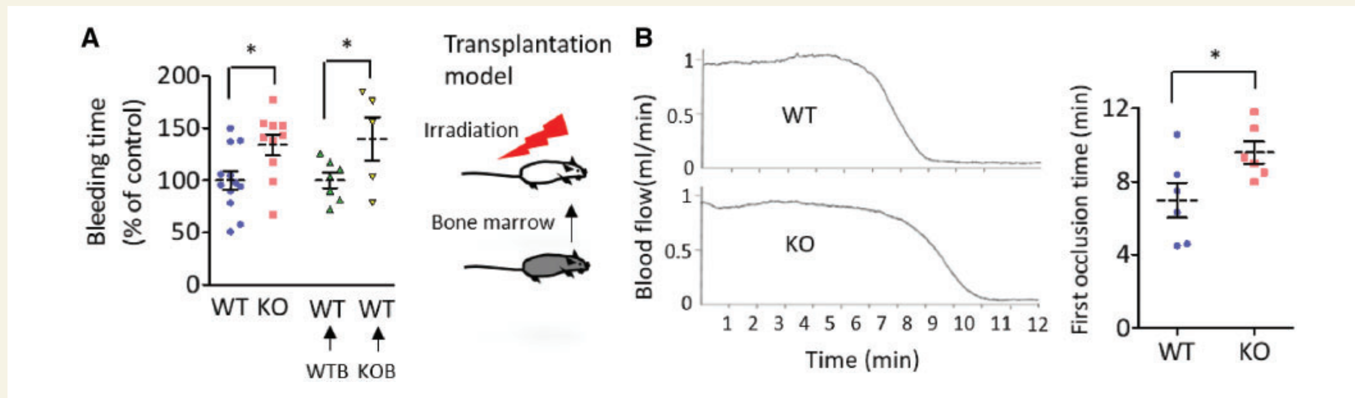

Figure 3 FUNDC2 deficiency caused a prolonged bleeding time and delayed $FeCl_3$-induced occlusion of the carotid artery. (*A*) Bleeding time was quantified following 3-mm tail transection. Bone marrow derived from FUNDC2-KO animals were transplanted into recipient mice (wild-type), which were pretreated with X-ray irradiation. These WT mice with KO bone marrow showed prolonged bleeding times, similar to KO mice. (*B*) The mouse carotid artery was treated with 10% $FeCl_3$. Traces of blood flow in the carotid arteries of wild type (WT) and KO mice were presented, respectively. The times to occlusion were measured. Data are mean ± SEM; Student's *t*-test; $*P < 0.05$.

Bone marrow derived from FUNDC2-KO animals were transplanted into recipient mice (wild-type), which were pretreated with X-ray irradiation at a single dose of 8.5 Gy. As expected, these WT mice with KO bone marrow showed prolonged bleeding times, similar to KO mice (*Figure 3A*).

3.4 FUNDC2 deficiency delayed FeCl3-induced occlusion of the carotid artery *in vivo*

To further investigate the physiological role of FUNDC2 in thrombosis *in vivo*, we examined thrombus formation in WT and KO mice using the $FeCl_3$-induced carotid artery thrombosis model (*Figure 3B*). The average time to first occlusion for *Fundc2*-deleted mice was 9.60 ± 0.60 min, in contrast to 6.98 ± 0.96 min in WT littermates ($P < 0.05$, $n = 6$). These results revealed that FUNDC2 is important in arterial thrombosis.

3.5 FUNDC2 positively regulated platelet activation through AKT/GSK-3β signalling pathways

Then we studied the mechanistic mechanism by which FUNDC2 regulates platelet aggregation. Notably, the protein levels of the ADP receptors $G\alpha_{12/13}$ (*Figure 4A*) and Gα(i) (*Figure 4B*) were unaffected by FUNDC2 deletion. Our previous report showed that FUNDC2 can mediate AKT activation and FUNDC2 deficiency impairs AKT phosphorylation during platelet apoptosis.[15] Hence, we tested the role of AKT signalling in platelet aggregation. Both sites of AKT-Ser473 and Thr308 were phosphorylated in wild-type platelets after the stimulation of ADP. However, the phosphorylation of AKT-Ser473 was attenuated, and AKT-Thr308 phosphorylation was totally abrogated in KO platelets, in response to ADP (*Figure 4C*). GSK-3β, which negatively regulates platelet activation, has been known as a downstream signalling molecule of AKT pathway.[7,17] The phosphorylation of GSK-3β at Ser9 site by AKT

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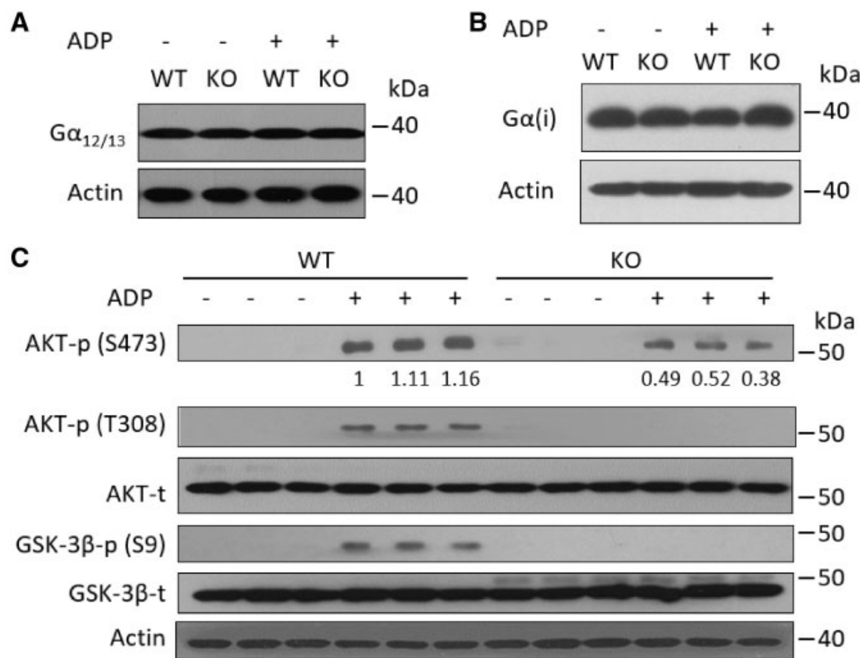

Figure 4 FUNDC2 positively regulated platelet activation through AKT/GSK-3β signalling pathways. (*A*) and (*B*) The protein levels of the ADP receptors $G\alpha_{12/13}$ (*A*) and Gα(i) (*B*) were unchanged in response to 10 μM ADP. (*C*) FUNDC2 deficiency in platelets abolished 10 μM ADP-induced phosphorylation of Akt at Ser473 and Thr308, and Gsk-3β at Ser9. The grayscale values of the bands were analysed with ImageJ software and are presented below the corresponding bands to show the band intensities.

inhibited the kinase activity of GSK-3β.[4] Therefore, GSK-3β phosphorylation is essential for platelet function, which is consistent with the fact that ADP stimulated GSK-3β phosphorylation in wild-type platelets (*Figure 4C*). Our data also demonstrated that FUNDC2 deficiency completely abrogated the phosphorylation of GSK-3β-Ser9 in ADP-treated platelets. In contrast, loss of FUNDC2 had no effect on the expression of AKT or GSK-3β (*Figure 4C*).

3.6 FUNDC2 modulates AKT/GSK-3β signalling axis in a PI3K-dependent manner

Moreover, AKT inhibitor (SH6) totally abolished the phosphorylation of AKT-Ser473, AKT-Thr308, and GSK-3β-Ser9, both in wild-type and knockout platelets in response to thrombin stimulation (*Figure 5A*). Consistently, SH6 totally inhibited the aggregation of wild-type and FUNDC2-deficient platelets induced by thrombin (*Figure 6A*). All these results unequivocally demonstrated that FUNDC2 is required for AKT phosphorylation and the downstream GSK-3β phosphorylation in platelet activation. Furthermore, as shown in *Figure 6A*, the inhibition of GSK-3β by its inhibitor, SB216763 (SB), fully restored the aggregation of FUNDC2-deficient platelets in response to thrombin, which also demonstrated that GSK-3β phosphorylation apparently caused the positive regulation of the signalling initiated by FUNDC2-mediated AKT phosphorylation.

In our previous report,[15] we have demonstrated that FUNDC2 can bind PIP3 directly and specifically. It is well known that AKT is evoked by binding to PIP3 and PI3K is essential for PIP3/AKT stimulation.[16] Our previous data1 also showed that FUNDC2 facilitated the recruitment of PIP3 to mitochondria and enhanced AKT phosphorylation, which was abrogated by PI3K inhibitor (LY294002). Thus, FUNDC2 activates AKT signalling in a PIP3/PI3K-dependent manner. To confirm the role of PI3K in FUNDC2-mediated AKT signalling pathways in platelet aggregation, we pretreated platelets with LY294002. LY294002 totally blocked AKT phosphorylation during collagen stimulation (*Figure 5B*). In contrast, the PIP3-independent activation of Src (Supplementary material online, *Figure S2A*) and Pten (Supplementary material online, *Figure S2B*) in response to collagen remained intact in FUNDC2-KO platelets.

3.7 NO/cGMP function in FUNDC2/AKT/GSK-3β signalling pathways during platelet aggregation

AKT/nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) signalling pathway plays a key role in platelet activation, as the positive regulation of cGMP in platelet function has been well established.[6,7,18] 8-Bromo-guanosine 3′,5′-cyclic monophosphate (8-Br-cGMP), a cGMP analog, rescued the aggregation of FUNDC2-deficient platelets treated with thrombin (*Figure 6A*), which suggests that the NO/cGMP pathway is also involved in FUNDC2 and AKT-mediated platelet activation.

3.8 FUNDC2/AKT/GSK-3β/cGMP axis regulates clot retraction of platelet-rich plasma

A number of studies have shown that AKT is vital for integrin αIIbβ3-mediated outside-in signalling that drive clot retraction.[4,11,19] Therefore, we investigated the function of FUNDC2 in clot retraction. As shown in *Figure 6B*, the average ratio of clot retraction of PRP containing wild-type platelets was 0.8 ± 0.06 versus 0.43 ± 0.03 in PRP containing

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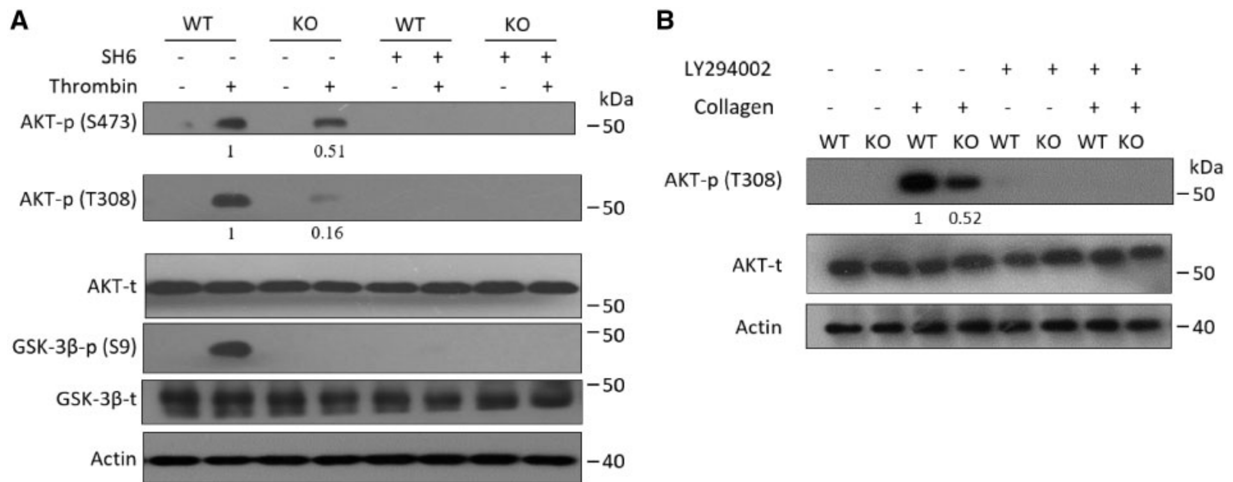

Figure 5 FUNDC2 regulates AKT/GSK-3β signalling axis in a PI3K-dependent manner. (*A*) The phosphorylation of AKT and GSK-3β in response to 0.05 U/mL thrombin were completely abrogated by AKT inhibitor (10 μM SH6). (*B*) AKT phosphorylation in response to 0.5 μg/mL collagen was totally abolished by PI3K inhibitor (5 μM LY294002).

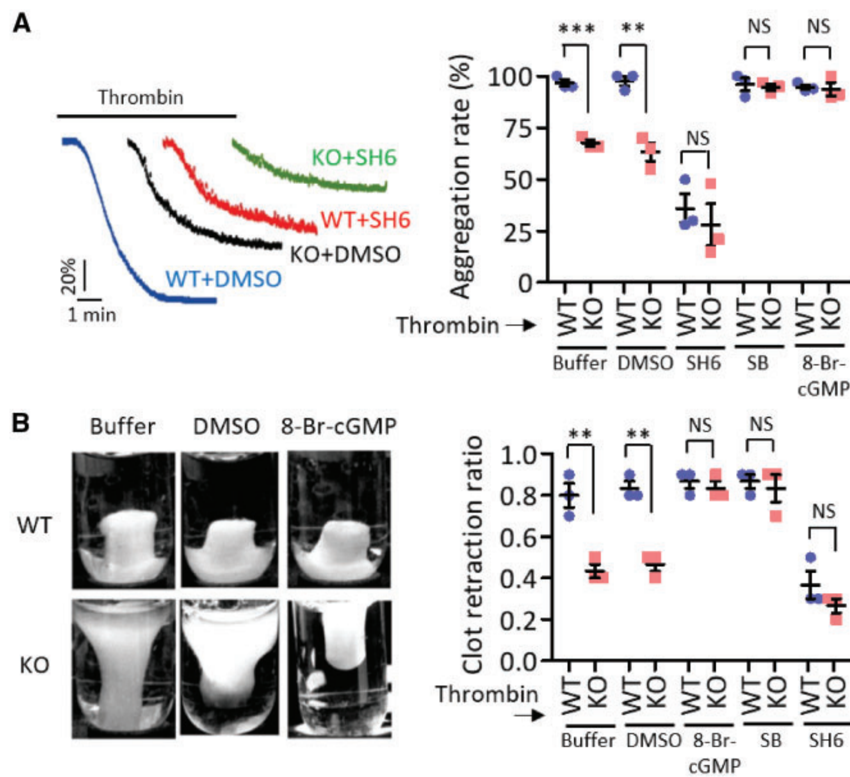

Figure 6 FUNDC2/AKT/GSK-3β/cGMP axis regulates clot retraction of PRP. (*A*) AKT inhibitor (10 μM SH6) blocked thrombin-induced platelet aggregation. GSK-3β inhibitor (10 μM SB216763, SB) or cGMP analog (1 μM 8-Br-cGMP) restored the aggregation of FUNDC2-deficient platelets in response to 0.05 U/mL thrombin. (*B*) Clot retraction of PRP containing WT or KO platelets was analysed in the presence of dimethylsulfoxide (DMSO), 10 μM SB, 10 μM SH6, or 1 μM 8-Br-cGMP. Data are mean ± SEM; $n = 3$ mice; Student's *t*-test; NS, no statistical significance; $^{**}P < 0.01$; $^{***}P < 0.001$.

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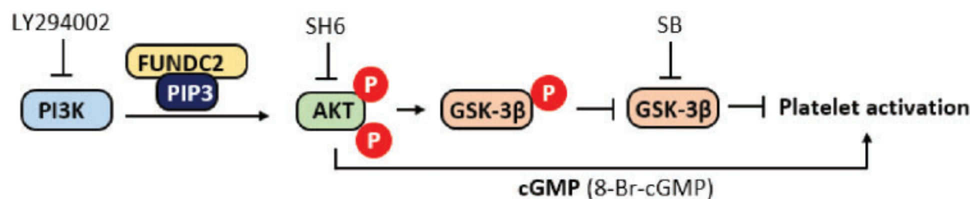

Figure 7 Schematic model shows FUNDC2 regulates platelet activation. FUNDC2 binding to PIP3 mediates AKT activation in a PI3K-dependent manner. GSK-3β acts as an inhibitor of platelet aggregation. AKT stimulates the phosphorylation of GSK-3β, which inhibits its activity and induces platelet aggregation. Moreover, cGMP is involved in the axis and positively regulates platelet function.

FUNDC2-KO platelets. Thus, FUNDC2 deficiency in platelets severely delayed clot retraction ($P = 0.005$, $n = 3$). Furthermore, GSK-3β inhibitor SB and cGMP analog 8-Br-cGMP completely restored clot retraction in PRP containing FUNDC2-deficient platelets. In contrast, AKT inhibitor SH6 totally inhibited clot contraction in PRP containing wild-type or FUNDC2-deficient platelets. These data revealed that platelets participating in clot retraction requires activation of FUNDC2/AKT/cGMP and inhibition of GSK-3β function.

4. Discussion

Recently, we identified FUNDC2 as a novel mitochondrial outer membrane protein.[15] We found that FUNDC2 directly and specifically binds PIP3, via its unique and highly conserved N-terminal motif which was named as PIP3-binding (PB) motif. FUNDC2 can recruit PIP3 to mitochondria and possibly plays as an anchor mediating the transfer of PIP3 from cell membrane to mitochondrial surface. The binding of FUNDC2-PIP3 stabilized PIP3 levels, which is required for AKT phosphorylation in mitochondria. Loss of FUNDC2 decreases PIP3 level on mitochondria and impairs AKT activation.[15] Since AKT kinase is vital for regulating signal transduction in platelet aggregation, we proposed that FUNDC2 may regulate platelet function through AKT signalling pathway. Herein, we evaluated the role of FUNDC2 in platelet activation and thrombus formation. In summary, FUNDC2 is required for the phosphorylation of AKT and downstream GSK-3β, which plays an important role in platelet activation. Moreover, cGMP also acts as a positive regulator in FUNDC2/AKT-mediated platelet aggregation (*Figure 7*).

Previous reports including us[4,5,7] have demonstrated that AKT plays an important role in GPCRs-mediating platelet activation. Lower concentration (10–20 μM) of ADP can induce the activation of wild-type platelets but not AKT-deficient platelets, which indicates the essential role of AKT in ADP-induced platelet aggregation. However, high concentration (40 μM) of ADP can induce normal aggregation of AKT-deficient platelets. Thus, the aggregation of AKT-deficient platelets is abnormal in response to low level of ADP.[7] The possible reason is that other compensatory signalling pathways such as Src may be involved in ADP-induced platelet aggregation.[20] Moreover, AKT kinase includes three isoforms (Akt1, Akt2, and Akt3).[5] Knockout of one isoform or two of AKT may not completely abolish the kinase activities.

In our previous study, we found that mitochondrial protein FUNDC1 (a homolog of FUNDC2) regulates mitochondrial degradation and metabolism during platelet aggregation.[21,22] FUNDC1 contains the LC3-interacting region (LIR) motif, which is essential for the binding of FUNDC1 to LC3 in regulatory mitophagy in platelets.[22] However, FUNDC2 does not contain the LIR motif and no mitophagy can be detected in FUNDC2-KO platelets or in cultured HeLa cells.[15] In the present study, our data also showed that FUNDC2 deficiency had no effects on the mitochondrial mass and metabolic functions.

Platelets play essential roles for blood haemostasis, wound healing, and immunity. Thus, our study paves the new avenue for the regulation of platelet aggregation and hold promises for fighting these diseases. Our *in vivo* data of tail bleeding assays and carotid artery thrombus formation assays have highlighted the physiological relevance of FUNDC2 in haemostasis and thrombosis. Moreover, haemostatic function of platelets in bleeding is also critical for cardiovascular diseases. Therefore, whether is FUNDC2-mediated platelet activation involved in the pathogenesis of acute myocardial infarction (MI) or ischaemia/reperfusion (I/R) injury? It will be interesting to evaluate the pathological significance of FUNDC2 in cardiovascular diseases including MI and I/R models in the future studies.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Authors' contributions

Q.M. and Q.C. designed the experiments. Q.M., C.Z., and W.Z. carried out the experiments. Q.M. and W.Z. analysed and organized the data. Q.M., J.L., and Q.C. wrote the manuscript. All authors discussed the results and commented on the manuscript.

Conflict of interest: none declared.

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