Article type : Original Article - Platelets

Extracellular Signal-regulated Kinase 5 Associates with Casein Kinase II to Regulate GPIb-IX-mediated Platelet Activation via the PTEN/PI3K/Akt Pathway

Z. Cheng,* W. Gao,† X. Fan,‡ X. Chen,‡ H. Mei,*§ J. Liu,‡ X. Luo† and Y. Hu*§

*Institute of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

†Department of Cardiology, Huashan Hospital, Fudan University, Shanghai, China

‡Department of Biochemistry and Molecular Cell Biology, Shanghai Jiao Tong University School of Medicine, Shanghai, China

§Collaborative Innovation Center of Hematology, Huazhong University of Science and Technology, Wuhan, China.

Running head

ERK5 regulates the GPIb/V/IX signaling pathway

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jth.13755

Corresponding author

Yu Hu, Institute of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, 430022, China; email: dr_huyu@126.com

Essentials

The mechanisms of extracellular signal-regulated kinase 5 (ERK5) in GPIb-IX signaling are unclear.

Function of ERK5 in GPIb-IX was tested using aggregation, western blotting, and mass spectrometry.

The protein interacting with ERK5 in human platelets was identified as casein kinase II (CKII).

ERK5 associates with CKII to regulate the activation of the PI3K/Akt pathway in GPIb-IX signaling.

Summary

Background: The platelet glycoprotein (GP) Ib-IX complex plays essential roles in thrombosis and hemostasis. The mitogen-activated protein kinases (MAPKs) ERK1/2 and p38 have been shown to be important in the GPIb-IX-mediated signaling leading to integrin activation. However, the roles of the MAPK extracellular signal-regulated kinase 5 (ERK5) in GPIb-IX-mediated platelet activation are unknown.

***Objective*:**

To reveal the function and mechanisms of ERK5 in GPIb-IX-mediated platelet activation.

***Methods*:**

The functions of ERK5 in GPIb-IX-mediated human platelet activation were assessed using botrocetin/VWF, ristocetin/VWF, or platelet adhesion to VWF under shear stress in the presence of a specific inhibitor of ERK5. ERK5-associated proteins were pulled down from Chinese hamster ovary (CHO) cells transfected with HA-tagged-ERK5, identified by mass spectrometry, and confirmed in human platelets. Roles of ERK5-associated proteins in GPIb-IX-mediated platelet activation were clarified using specific inhibitors.

***Results*:**

The phosphorylation levels of ERK5 were significantly enhanced in human platelets stimulated with botrocetin/VWF or ristocetin/VWF. The ERK5 inhibitor XMD8-92 suppressed the second wave of human platelet aggregation induced by botrocetin/VWF or ristocetin/VWF and inhibited human platelet adhesion on immobilized VWF under shear stress. Casein kinase II (CKII) was identified as an ERK5-associated protein in human platelets. The CKII inhibitor TBB, similar to the ERK5 inhibitor XMD8-92, specifically restrained PTEN phosphorylation, therefore suppressing Akt phosphorylation in human platelets treated with botrocetin/VWF.

***Conclusion*:**

ERK5 associates with CKII to play essential roles in GPIb-IX-mediated platelet activation via the PTEN/PI3K/Akt pathway.

Keywords:

platelet glycoprotein GPIb-IX complex; extracellular signal-regulated kinase 5; casein kinase II; PTEN; platelet activation.

Introduction

Platelets, which are derived from megakaryocytes, circulate in mammalian blood and play essential roles in hemostasis, angiogenesis, inflammation, tumor growth, and metastasis [1-3]. Compared with other cells, platelets have a relatively small size, but they have abundant membrane proteins [4, 5]. Platelet membrane proteins include a variety of receptors, such as the leucine-rich repeat (LRR) family, integrins, GPCRs, the C-type lectin receptor family, and many other receptors [1].

The glycoprotein (GP) Ib-IX complex, which belongs to the LRR family, not only mediates platelet adhesion but also transmits signals, leading to platelet activation [6, 7]. The GPIb-IX-mediated integrin activation signal has been well studied [8, 9]. Among these signaling pathways, mitogen-activated protein kinases (MAPKs) play crucial roles in GPIb-IX-mediated platelet aggregation and adhesion [10, 11]. Four distinct subgroups within

MAPKs have been identified in platelets: extracellular signal-regulated kinase 1/2 (ERK1/2), the p38 group of protein kinases (p38), the c-Jun NH2-terminal kinases 1/2 (JNK1/2), and extracellular signal-regulated kinase 5 (ERK5) [12]. Among these MAPKs, p38 and ERK1/2 have been shown to be important in the GPIb-IX mediated signaling leading to integrin activation [13].

ERK5, also named big mitogen-activated protein kinase-1 (BMK1), is the most recently identified member of the MAPKs. ERK5 has been reported to be a platelet activator in ischemic conditions, and it regulates the expression of multiple platelet proteins after myocardial infarction (MI) [14]. However, the roles of ERK5 in GPIb-IX-mediated platelet activation are unknown. In this study, the functions of ERK5 in GPIb-IX-mediated human platelet activation and possible underlying mechanisms were studied.

Material and methods

Materials

Apyrase, PGE1, adenosine 5′-diphosphate (ADP), and U46619 (thromboxane A2 mimetic) were obtained from Sigma-Aldrich (St Louis, MO, USA). α-thrombin was obtained from Enzyme Research Laboratories (South Bend, IN, USA). Collagen was purchased from Chrono-Par Aggregation Reagents (Chrono-Log Corporation, Havertown, PA, USA). Human von Willebrand factor (VWF) was purchased from Haematologic Technologies (Essex Junction, VT). Botrocetin was prepared as previously described [15]. Ristocetin was obtained from Helena Biosciences (Gateshead, United Kingdom). Protease and phosphatase inhibitor

cocktails and IgG-agarose beads were purchased from Roche (Indianapolis, IN, USA). MAPKs inhibitors (SP600125, XMD8-92, ERK5-IN-1, and SB203580), the MEK1/2 inhibitor U0126, the Raf inhibitor LY3009120, the MEK5 inhibitor BIX02189, and the PI3K inhibitor wortmannin were obtained from Selleck Chemicals (Houston, TX, USA). The Src kinase inhibitor PP2, Akt inhibitor Akt Inhibitor III (SH6), and casein kinase II inhibitor TBB were purchased from CalBiochem (Darmstadt, Germany). Anti-phospho-p44/42 MAPK (ERK1/2), anti-phospho-p38 MAPK (Thr180/Tyr182), anti-phospho-SAPK/JNK (JNK1/2) (Thr183/Tyr185), anti-phospho-ERK5 (Thr218/Tyr220), anti-phospho-PTEN, anti-phospho-Akt (Thr308), anti-phospho-Akt (Ser473), and anti-GAPDH antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-CKIIα (T251) antibody was from Bioword Technology (Minneapolis, MN, USA). Anti-HA-conjugated agarose beads were purchased from Thermo Pierce (Rockford, IL, USA).

Preparation and aggregation of human washed platelets

After informed consent was obtained, peripheral blood was collected from five different healthy donors (the work was approved by the ethics committee of Tongji Medical College, Huazhong University of Science and Technology, and complied with the principles in the Declaration of Helsinki). Human washed platelets were prepared as described previously [16]. Inhibitors were incubated with the platelets for 3 min before stimulation. VWF (5 μg mL^{-1}) with botrocetin (1.5 μg mL^{-1}) or ristocetin (0.25 mg mL^{-1}) was used to investigate GPIb-IX-mediated human platelet activation. ADP (5 μM), collagen (0.1 μg mL^{-1}),

α-thrombin (0.1 U), and U46619 (0.1 μg mL^{-1}) were also used in platelet aggregation test. Platelet aggregation was monitored using an aggregometer at 37°C with 1000 rpm stirring speed.

Western blotting analysis

For western blotting, the platelet aggregation reaction was stopped by adding an equal volume of 2×SDS sample buffer containing 1 M Tris-HCl (pH 6.8), 4% SDS, 10% β-mercaptoethanol, 10% glycerol, and 2% bromophenol blue. Then, samples were boiled at 100 °C for 10 min, separated by 10% SDS-PAGE, transferred to a PVDF membrane, and blotted with the indicated antibodies as described previously [17]. After detection of target proteins, the membranes were stripped and then incubated with anti-GAPDH antibodies to demonstrate equal loading. Blots were developed using the SuperSignal chemiluminescent substrate. The density of protein was measured with NIH Image J software (National Institutes of Health, Bethesda, MD), and statistical significance was evaluated by Student's *t*-test.

DNA constructs, cell cultures, and transfection

DNA constructs were made using standard genetic manipulations as previously described [18]. Human ERK5 cDNA was cloned into the PXJ40-hemagglutinin (HA) vector, and then, HA-ERK5 was sub-cloned into the PLVX-IRES-ZsGreen1 expression vector (Clontech,

Mountain View, CA, USA). Chinese hamster ovary (CHO) cells (from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM (with 4.5 g L^{-1} glucose, 4 mM L-glutamine, and 100 μg mL^{-1} penicillin/streptomycin) supplemented with 10% fetal bovine serum (FBS). DNA constructs were transfected into CHO cells using Lipofectamine 2000 (Life Technologies Inc, Ontario, Canada). Cells expressing the HA-tagged-ERK5 fusion protein were obtained by selection for neomycin resistance using G418 at 2 mg mL^{-1}.

Immunoprecipitation and mass spectrometry

For identification of ERK5 interactors, CHO cells expressing HA-ERK5 proteins were lysed and pre-absorbed with IgG-agarose beads, followed by treatment with anti-HA-conjugated agarose beads at 4°C overnight. Each pull-down sample was separated by 10% SDS-PAGE and stained with Coomassie brilliant blue. Stained bands containing one or more protein species were excised. In-gel digestion, mass spectrometry analysis, and database searching were carried out by Shanghai Applied Protein Technology Ltd (Shanghai, China) as previously described [18]. The presence of ERK5-associated proteins was verified by immunoblotting.

For immunoprecipitation, resting platelets or DMSO- or XMD8-92-pre-incubated activated platelets were lysed using Pierce immunoprecipitation lysis buffer on ice for 30 min, followed by binding to 2 μg mL^{-1} rabbit anti-HA antibody or rabbit anti-CKIIα antibody or rabbit anti-ERK5 antibody or a non-specific rabbit IgG control for 2 h at 4°C. Protein G

agarose beads (30 μL) were added to each sample prior to incubation at 4°C overnight. The beads were harvested by centrifugation at 3000 × g for 1 min and washed three times with lysis buffer. After boiling with sample buffer, immunoprecipitation samples or direct lysates were analyzed by SDS-PAGE and western blotting as previously described [19]. After detection of target proteins, the membranes were stripped and incubated with anti-rabbit IgG or anti-GAPDH antibodies to demonstrate equal loading.

Platelet adhesion under shear stress assays

Platelet adhesion under shear stress was performed as previously described [20]. Briefly, microfluidic channels were coated with VWF (30 μg mL^{-1}) overnight, washed with PBS, and blocked with 2% BSA. Blood samples from healthy donors were labeled with calcein (4 μM) for 30 min and then pretreated with DMSO or XMD8-92 (5 μM) for 5 min. Each group was perfused through the microfluidic channels at wall shear rates of 1500 s^{-1} for 5 min. Channels were then quickly rinsed with PBS 3 times to wash out platelets that were not stably adhered. Microfluidic channels were viewed with a Leica DMI RB fluorescence microscope (Leica Microsystems) using an N PLAN L lens at 40×/0.55 NA objective with 1.5× magnification as previously described [7]. Stable adherent platelets were counted in 9 randomly selected microscope fields. Relative area and mean fluorescence intensity were quantitated. Statistical significance was analyzed using Student's *t* test.

Results

The roles of MAPKs in botrocetin/VWF-induced human platelet aggregation

Although p38 and ERK1/2 have been shown to be important regulators in GPIb-IX mediated platelet activation, there is a lack of systematic studies on the roles of MAPKs in GPIb-IX-mediated platelet activation. First, the activation of 4 major MAPK members in human platelets stimulated with botrocetin/VWF was investigated. The data presented in Fig. 1(A) showed that the phosphorylation levels of the main MAPK members, including ERK5, JNK1/2, p38, and ERK1/2, were significantly enhanced in human platelets in response to botrocetin/VWF stimulation (Fig. 1B). These results suggested that ERK5 and JNK1/2 might also be involved in the regulation of GPIb-IX mediated platelet activation.

To compare the functions of the 4 major MAPK members in GPIb-IX-mediated platelet activation, washed human platelets were stimulated with botrocetin/VWF in the presence of DMSO or different specific MAPK inhibitors. The results in Fig. 1(C) showed that the ERK5 inhibitor XMD8-92, p38 inhibitor SB203580, and MEK1/2 inhibitor U0126 dose-dependently and significantly suppressed the second wave of botrocetin/VWF-induced platelet aggregation. The JNK1/2 inhibitor SP600125 only slightly affected human platelet aggregation induced by botrocetin/VWF. These results demonstrated that ERK5 is another important regulator in GPIb-IX-mediated platelet activation.

ERK5 plays critical roles in GPIb-IX-mediated platelet activation

To further verify the roles of ERK5 in GPIb-IX-mediated platelet activation, the effects of the ERK5 inhibitor XMD8-92 on ristocetin/VWF-induced human platelet aggregation were studied [21]. The results in Fig. 2(A) showed that the second wave of human platelet aggregation induced by ristocetin/VWF was inhibited by XMD8-92 in a dose-dependent manner. The phosphorylation levels of ERK5 in human platelets were obviously enhanced in response to ristocetin/VWF (Fig. 2B), further indicating that ERK5 can be activated and plays important roles in GPIb-IX-mediated platelet activation.

In addition, the roles of ERK5 in platelet adhesion to VWF under shear stress were also assessed. The relative area and mean fluorescence intensity of human platelets stably adhered to immobilized VWF under shear stress were analyzed (Fig. 2C). The results showed that the relative area of human platelets in the ERK5 inhibitor XMD8-92 group was 37.98 ± 12.53% compared to a value of 100.00 ± 13.01% in the DMSO group. In addition, the fluorescence intensity was 64.82 ± 2.77 for the DMSO group vs 46.24 ± 3.85 for the XMD8-92 group. These results suggested that ERK5 also plays an essential role in shear stress-induced GPIb-IX-mediated platelet activation.

Src, Raf, and MEK5 are upstream activators of ERK5

MAPKs are activated through a classical MAPK kinase kinase (MAPKKK)-MAPK kinase (MAPKK)-MAPK signaling cascade. Previous studies have reported that MEKK2, MEKK3, Raf, and MEK5 are upstream activators of ERK5 in tumor cells [22, 23]. Moreover, the Src family kinases (SFKs) are involved in GPIb-mediated signal transduction and play an early

role in the signaling pathway of platelet activation induced by shear stress or by ristocetin or botrocetin [24, 25]. To explore whether they are upstream activators of ERK5 in platelets, the effects of their inhibitors on botrocetin/VWF-induced human platelet aggregation were measured. The results presented in Fig. 3(A) demonstrate that the SFKs inhibitor PP2, Raf inhibitor LY3009120, and MEK5 inhibitor BIX02189 dose-dependently inhibited the second wave of human platelet aggregation stimulated with botrocetin/VWF. Moreover, the phosphorylation levels of ERK5 in human platelets in response to botrocetin/VWF were significantly suppressed by PP2 and BIX02189 and slightly inhibited by LY3009120 (Fig. 3B, C). These results suggested that Src and MEK5 are upstream regulators of ERK5 and that Raf partially contributes to ERK5 activation in GPIb-IX-mediated platelet activation.

CKII is one of ERK5-associated proteins

Although upstream activators of ERK5 have been verified, the precise molecular mechanisms of ERK5 in GPIb-IX-mediated platelet activation remain unclear. First, the proteins interacting with ERK5 were pulled down by immunoprecipitation in Chinese hamster ovary (CHO) cells transfected with HA-ERK5 and subjected to mass spectrometry analysis (Fig. 4A). CKII was identified as one of ERK5-associated proteins, which was confirmed by co-immunoprecipitation and immunoblotting in CHO cells expressing HA-ERK5 (Fig. 4 B,C).

The status of ERK5 binding to CKII in resting platelets and activated platelets was also studied. The results presented in Fig. 4(D) demonstrated that ERK5 constitutively associates with CKII in human platelets. More interesting is that the association of ERK5 with CKII was significantly blocked by the ERK5 inhibitor XMD8-92 in human platelets (Fig. 4D). Therefore, XMD8-92 inhibits GPIb-mediated platelet activation probably via blockage of the association of ERK5 with CKII.

The CKII inhibitor TBB was used to study the functions of CKII in GPIb-IX-mediated human platelet activation. Similar to the ERK5 inhibitor XMD8-92, TBB dose-dependently inhibited the second wave aggregation of human platelets stimulated with botrocetin/VWF (Fig. 4E). These results suggested that ERK5 associates with CKII to regulate GPIb-IX-mediated platelet activation.

ERK5/CKII is involved in GPIb-IX-mediated platelet activation via regulation of the PTEN/PI3K/Akt pathway

CKII is composed of two α subunits and two β subunits, and it phosphorylates the serine/threonine/tyrosine of its substrate [26, 27]. It has been reported that CKII can phosphorylate and attenuate the activity of phosphatase and tensin homolog deleted on chromosome ten (PTEN) [28]. Whether ERK5/CKII was involved in GPIb-IX-mediated platelet activation through regulation of PTEN phosphorylation requires investigation. The data presented in Fig. 5(A) showed that PTEN phosphorylation was significantly enhanced in human platelets in response to botrocetin/VWF. Moreover, the phosphorylation levels of

PTEN in human platelets were dose-dependently inhibited by the ERK5 inhibitor XMD8-92 or CKII inhibitor TBB (Fig. 5A), indicating that ERK5/CKII did regulate PTEN activity in GPIb-IX-mediated signaling. Inhibitors of JNK1/2, p38, and ERK1/2 had no effects on PTEN phosphorylation (Fig. 5B), suggesting the specificity of the ERK5/CKII-PTEN signaling axis. Taken together, the results indicated that ERK5/CKII is involved in GPIb-mediated human platelet activation through regulation of PTEN activity.

PTEN, as a lipid phosphatase, negatively regulates the PI3K/Akt signaling pathway in platelets [29, 30]. The regulatory roles of the ERK5/CKII-PTEN axis in the GPIb-IX-mediated PI3K/Akt signaling pathway were elucidated. The results presented in Fig. 5(C) showed the phosphorylation levels of Akt at thr308 and ser473 sites were significantly suppressed in human platelets stimulated with botrocetin/VWF in the presence of the ERK5 inhibitor XMD8-92, CKII inhibitor TBB, or PI3K inhibitor wortmannin (Fig. 5C). The aggregation experiments confirmed that both the PI3K inhibitor wortmannin and Akt inhibitor SH6 dose-dependently inhibited botrocetin/VWF-induced human platelet aggregation (Fig. 5D). These results suggested that ERK5/CKII regulates GPIb-IX-mediated platelet activation via the PTEN/PI3K/Akt signaling pathway (Fig. 5E).

Discussion

The binding of VWF to the GPIb-IX complex initiates a signaling cascade that causes integrin αIIbβ3 activation and platelet aggregation. GPIb-IX signaling is mediated by the SFKs, PLC/PKD, calcium elevation, PI3K/Akt, MAPK p38 and ERK1/2, and secondary amplifying ADP and thromboxane A2 pathways [10]. Cameron et al. reported that ERK5 deficiency inhibited thrombin- and U46619-induced platelet aggregation, but had no effects on ADP- and collagen-induced platelet aggregation [14]. In our study, we showed that the specific ERK5 inhibitor XMD8-92 had similar inhibitory effects as ERK5 deficiency on platelet aggregation (Fig. S2). We also found that ERK5 is a critical MAPK involved in GPIb-IX-mediated platelet activation using the ERK5 inhibitor XMD8-92 or ERK5-IN-1 (Fig. S1) [31, 32]. It has been reported that GPIb-IX-mediated signaling is amplified by the activation of the TxA2 receptor [19]. Therefore, the inhibitory effect of the ERK5 inhibitor on human platelet aggregation or stable platelet adhesion could result from GPIb-IX-mediated direct signaling and secondary amplifying signals mediated by the TxA2 receptor.

Extracellular stimuli induce the sequential activation of the MAPKKK-MAPKK-MAPK signaling cascade to regulate abundant cell activities [33, 34]. It was believed that three MAPKKKs, MEKK2, MEKK3 or Raf are upstream signaling molecules to activate a specific MAPKK, MEK5. Then, MEK5 further phosphorylates and activates ERK5 and its substrates [23, 35, 36]. Our results showed that MEK5 is an upstream activator of ERK5 in GPIb-IX-mediated human platelet activation. Raf inhibitor slightly inhibited GPIb-IX-mediated platelet aggregation and ERK5 phosphorylation, suggesting that there

could be other MAPKKKs, such as MEKK2 or MEKK3, also involved in the regulation of ERK5 activation. The SFKs Src, Lyn, and Fyn have all been implicated in the early stages of GPIb-IX signaling, and our results demonstrated that SFKs are also upstream kinases to activate the MAPKKKs (Raf)/MEK5/ERK5 pathway.

ERK5 has been associated with a wide range of cellular processes, including cellular proliferation, migration, survival, and angiogenesis [37]. The roles of ERK5 in the regulation of Akt activation have been reported in mouse embryonic fibroblasts under osmotic stress and in osteoblast apoptosis induced by TNF-α [38, 39]. ERK5 was also shown to be involved in PDGFRβ-mediated Akt activation in porcine aortic endothelial cells [40]. Our results also indicated that ERK5 was able to regulate Akt activation in human platelets in response to botrocetin/VWF. These observations suggested that ERK5 is an important regulator of the PI3K/Akt pathway, but the underlying mechanisms were unclear. We found that that ERK5 constitutively associates with CKII in human platelets. And we also revealed that inhibitory effects of the specific ERK5 inhibitor XMD8-92 on GPIb-IX-mediated platelet aggregation probably occur through the breakage of the ERK5 and CKII association. CKII is a highly pleiotropic and ubiquitous active serine/threonine kinase that has been implicated in neoplasia, cell survival, and viral infection [28, 41]. CKII combines with itself and forms inactive multimers in the resting state [42]. We hypothesized that phosphorylation of ERK5 might depolymerize the multimers of CKII, associate with CKII, and activate it. Further studies are needed to clarify the specific region of CKII binding to ERK5.

It has been reported that CKII can mediate PTEN tail phosphorylation and induce the "tail-closed" conformation of PTEN, which prevents the phosphatase activity of PTEN toward phosphatidylinositol (3,4,5)-triphosphate (PIP3) [43, 44]. Our study showed that the inhibition of ERK5 or CKII significantly attenuated PTEN phosphorylation, which may promote PTEN reactivation and the de-phosphorylation of PIP3 to block the PI3K/Akt pathway. Therefore, we propose a novel ERK5/CKII/PTEN axis as a potential pathway in the regulation of GPIb-IX-mediated PI3K/Akt pathway activation.

In summary, we identified a new Src-Raf-MEK5-ERK5/CKII-PTEN pathway that plays a critical role in GPIb-IX-mediated platelet activation via regulation of PI3K/Akt activation. Moreover, the inhibition of the association of ERK5 with CKII may serve as a promising anti-platelet target.

Addendum

Y. Hu and J. Liu designed the study. Z. Cheng, W. Gao, X. Fan, and X. Chen collected and analyzed the data. Z. Cheng, Y. Hu, and J. Liu wrote the manuscript. H. Mei and X. Luo helped perform the experiments. All authors read and approved the manuscript.

Acknowledgements

We would like to thank S. P. Watson for his knowledgeable suggestions. This study was supported by grants from the National Natural Science Foundation of China (31620103909 to Y. Hu, 81270278 to X. Luo, 81600104 to X. Fan) and China Postdoctoral Science Foundation (2015M580336 to X. Chen and 2016T90374 to X. Fan).

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

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

Fig. S1. The effect of ERK5-IN-1 on botrocetin/VWF-induced human platelet two-wave aggregation.

Fig. S2. Roles of ERK5 in platelet aggregation induced by thrombin, U46619, ADP, and collagen.

References

1 George JN. Platelets. *Lancet (London, England)*. 2000; **355**: 1531-9. 10.1016/s0140-6736(00)02175-9.

2 Boilard E, Nigrovic PA, Larabee K, Watts GF, Coblyn JS, Weinblatt ME, Massarotti EM, Remold-O'Donnell E, Farndale RW, Ware J, Lee DM. Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. *Science (New York, NY)*. 2010; **327**: 580-3. 10.1126/science.1181928.

3 Nash GF, Turner LF, Scully MF, Kakkar AK. Platelets and cancer. *The Lancet Oncology*. 2002; **3**: 425-30.

4 Yang H, Kim A, David T, Palmer D, Jin T, Tien J, Huang F, Cheng T, Coughlin SR, Jan YN, Jan LY. TMEM16F forms a Ca2+-activated cation channel required for lipid scrambling in platelets during blood coagulation. *Cell*. 2012; **151**: 111-22. 10.1016/j.cell.2012.07.036.

5 Gieger C, Radhakrishnan A, Cvejic A, Tang W, Porcu E, Pistis G, Serbanovic-Canic J, Elling U, Goodall AH, Labrune Y, Lopez LM, Magi R, Meacham S, Okada Y, Pirastu N, Sorice R, Teumer A, Voss K, Zhang W, Ramirez-Solis R, *et al*. New gene functions in

megakaryopoiesis and platelet formation. *Nature*. 2011; **480**: 201-8. 10.1038/nature10659.

6 Liu J, Pestina TI, Berndt MC, Steward SA, Jackson CW, Gartner TK. The roles of ADP and TXA in botrocetin/VWF-induced aggregation of washed platelets. *Journal of thrombosis and haemostasis : JTH*. 2004; **2**: 2213-22. 10.1111/j.1538-7836.2004.01023.x.

7 Yin H, Liu J, Li Z, Berndt MC, Lowell CA, Du X. Src family tyrosine kinase Lyn mediates VWF/GPIb-IX-induced platelet activation via the cGMP signaling pathway. *Blood*. 2008; **112**: 1139-46. 10.1182/blood-2008-02-140970.

8 Wang Y, Sakuma M, Chen Z, Ustinov V, Shi C, Croce K, Zago AC, Lopez J, Andre P, Plow E, Simon DI. Leukocyte engagement of platelet glycoprotein Ibalpha via the integrin Mac-1 is critical for the biological response to vascular injury. *Circulation*. 2005; **112**: 2993-3000. 10.1161/circulationaha.105.571315.

9 Fan X, Chen X, Wang C, Dai J, Lu Y, Wang K, Liu J, Zhang J, Wu X. Drospirenone enhances GPIb-IX-V-mediated platelet activation. *Journal of thrombosis and haemostasis : JTH*. 2015; **13**: 1918-24. 10.1111/jth.13109.

10 Garcia A, Quinton TM, Dorsam RT, Kunapuli SP. Src family kinase-mediated and Erk-mediated thromboxane A2 generation are essential for VWF/GPIb-induced fibrinogen receptor activation in human platelets. *Blood*. 2005; **106**: 3410-4. 10.1182/blood-2005-05-1933.

11 Li Z, Zhang G, Feil R, Han J, Du X. Sequential activation of p38 and ERK pathways by cGMP-dependent protein kinase leading to activation of the platelet integrin alphaIIb beta3. *Blood*. 2006; **107**: 965-72. 10.1182/blood-2005-03-1308.

12 Oury C, Daenens K, Hu H, Toth-Zsamboki E, Bryckaert M, Hoylaerts MF. ERK2 activation in arteriolar and venular murine thrombosis: platelet receptor GPIb vs. P2X. *Journal of thrombosis and haemostasis : JTH*. 2006; **4**: 443-52. 10.1111/j.1538-7836.2006.01745.x.

13 Du X. Signaling and regulation of the platelet glycoprotein Ib-IX-V complex. *Current opinion in hematology*. 2007; **14**: 262-9. 10.1097/MOH.0b013e3280dce51a.

14 Cameron SJ, Ture SK, Mickelsen D, Chakrabarti E, Modjeski KL, McNitt S, Seaberry M, Field DJ, Le NT, Abe J, Morrell CN. Platelet Extracellular Regulated Protein Kinase 5 Is a Redox Switch and Triggers Maladaptive Platelet Responses and Myocardial Infarct Expansion. *Circulation*. 2015; **132**: 47-58. 10.1161/circulationaha.115.015656.

15 Andrews RK, Booth WJ, Gorman JJ, Castaldi PA, Berndt MC. Purification of botrocetin from Bothrops jararaca venom. Analysis of the botrocetin-mediated interaction between von Willebrand factor and the human platelet membrane glycoprotein Ib-IX complex. *Biochemistry*. 1989; **28**: 8317-26.

16 Fan X, Shi P, Dai J, Lu Y, Chen X, Liu X, Zhang K, Wu X, Sun Y, Wang K, Zhu L, Zhang CC, Zhang J, Chen GQ, Zheng J, Liu J. Paired immunoglobulin-like receptor B regulates platelet activation. *Blood*. 2014; **124**: 2421-30. 10.1182/blood-2014-03-557645.

17 Chen X, Zhang Y, Wang Y, Li D, Zhang L, Wang K, Luo X, Yang Z, Wu Y, Liu J. PDK1 regulates platelet activation and arterial thrombosis. *Blood*. 2013; **121**: 3718-26. 10.1182/blood-2012-10-461897.

18 Dai J, Lu Y, Wang C, Chen X, Fan X, Gu H, Wu X, Wang K, Gartner TK, Zheng J, Chen G, Wang X, Liu J. Vps33b regulates Vwf-positive vesicular trafficking in megakaryocytes. *The Journal of pathology*. 2016; **240**: 108-19. 10.1002/path.4762.

19 Liu J, Pestina TI, Berndt MC, Jackson CW, Gartner TK. Botrocetin/VWF-induced signaling through GPIb-IX-V produces TxA2 in an alphaIIbbeta3- and aggregation-independent manner. *Blood*. 2005; **106**: 2750-6. 10.1182/blood-2005-04-1667.

20 Conant CG, Schwartz MA, Beecher JE, Rudoff RC, Ionescu-Zanetti C, Nevill JT. Well plate microfluidic system for investigation of dynamic platelet behavior under variable shear loads. *Biotechnology and bioengineering*. 2011; **108**: 2978-87. 10.1002/bit.23243.

21 Dong JF, Berndt MC, Schade A, McIntire LV, Andrews RK, Lopez JA. Ristocetin-dependent, but not botrocetin-dependent, binding of von Willebrand factor to the platelet glycoprotein Ib-IX-V complex correlates with shear-dependent interactions. *Blood*. 2001; **97**: 162-8.

22 de Jong PR, Taniguchi K, Harris AR, Bertin S, Takahashi N, Duong J, Campos AD, Powis G, Corr M, Karin M, Raz E. ERK5 signalling rescues intestinal epithelial turnover and tumour cell proliferation upon ERK1/2 abrogation. *Nature communications*. 2016; **7**: 11551. 10.1038/ncomms11551.

23 Drew BA, Burow ME, Beckman BS. MEK5/ERK5 pathway: the first fifteen years. *Biochimica et biophysica acta*. 2012; **1825**: 37-48. 10.1016/j.bbcan.2011.10.002.

24 Ozaki Y, Suzuki-Inoue K, Inoue O. Platelet receptors activated via mulitmerization: glycoprotein VI, GPIb-IX-V, and CLEC-2. *Journal of thrombosis and haemostasis : JTH*. 2013; **11 Suppl 1**: 330-9. 10.1111/jth.12235.

25 Ozaki Y, Satoh K, Yatomi Y, Miura S, Fujimura Y, Kume S. Protein tyrosine phosphorylation in human platelets induced by interaction between glycoprotein Ib and von Willebrand factor. *Biochimica et biophysica acta*. 1995; **1243**: 482-8.

26 Nakanishi K, Komada Y, Hayashi T, Suzuki K, Ido M. Protease activated receptor 1 activation of platelet is associated with an increase in protein kinase CK2 activity. *Journal of thrombosis and haemostasis : JTH*. 2008; **6**: 1046-8. 10.1111/j.1538-7836.2008.02955.x.

27 Kang NI, Yoon HY, Kim HA, Kim KJ, Han MK, Lee YR, Hwang PH, Soh BY, Shin SJ, Im SY, Lee HK. Protein kinase CK2/PTEN pathway plays a key role in platelet-activating factor-mediated murine anaphylactic shock. *Journal of immunology (Baltimore, Md : 1950)*. 2011; **186**: 6625-32. 10.4049/jimmunol.1100007.

28 Kalathur M, Toso A, Chen J, Revandkar A, Danzer-Baltzer C, Guccini I, Alajati A, Sarti M, Pinton S, Brambilla L, Di Mitri D, Carbone G, Garcia-Escudero R, Padova A, Magnoni L, Tarditi A, Maccari L, Malusa F, Kalathur RK, L AP, *et al*. A chemogenomic screening identifies CK2 as a target for pro-senescence therapy in PTEN-deficient tumours. *Nature communications*. 2015; **6**: 7227. 10.1038/ncomms8227.

29 Weng Z, Li D, Zhang L, Chen J, Ruan C, Chen G, Gartner TK, Liu J. PTEN regulates collagen-induced platelet activation. *Blood*. 2010; **116**: 2579-81. 10.1182/blood-2010-03-277236.

30 Laurent PA, Severin S, Gratacap MP, Payrastre B. Class I PI 3-kinases signaling in platelet activation and thrombosis: PDK1/Akt/GSK3 axis and impact of PTEN and SHIP1. *Advances in biological regulation*. 2014; **54**: 162-74. 10.1016/j.jbior.2013.09.006.

31 Yang Q, Deng X, Lu B, Cameron M, Fearns C, Patricelli MP, Yates JR, 3rd, Gray NS, Lee JD. Pharmacological inhibition of BMK1 suppresses tumor growth through promyelocytic leukemia protein. *Cancer cell*. 2010; **18**: 258-67. 10.1016/j.ccr.2010.08.008.

32 Deng X, Elkins JM, Zhang J, Yang Q, Erazo T, Gomez N, Choi HG, Wang J, Dzamko N, Lee JD, Sim T, Kim N, Alessi DR, Lizcano JM, Knapp S, Gray NS. Structural determinants for ERK5 (MAPK7) and leucine rich repeat kinase 2 activities of benzo[e]pyrimido-[5,4-b]diazepine-6(11H)-ones. *European journal of medicinal chemistry*. 2013; **70**: 758-67. 10.1016/j.ejmech.2013.10.052.

33 Kovtun Y, Chiu WL, Zeng W, Sheen J. Suppression of auxin signal transduction by a MAPK cascade in higher plants. *Nature*. 1998; **395**: 716-20. 10.1038/27240.

34 Posas F, Saito H. Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK. *Science (New York, NY)*. 1997; **276**: 1702-5.

35 Roberts OL, Holmes K, Muller J, Cross DA, Cross MJ. ERK5 and the regulation of endothelial cell function. *Biochemical Society transactions*. 2009; **37**: 1254-9. 10.1042/bst0371254.

36 Nishimoto S, Nishida E. MAPK signalling: ERK5 versus ERK1/2. *EMBO reports*. 2006; **7**: 782-6. 10.1038/sj.embor.7400755.

37 Zhou G, Bao ZQ, Dixon JE. Components of a new human protein kinase signal transduction pathway. *The Journal of biological chemistry*. 1995; **270**: 12665-9.

38 Wang X, Finegan KG, Robinson AC, Knowles L, Khosravi-Far R, Hinchliffe KA, Boot-Handford RP, Tournier C. Activation of extracellular signal-regulated protein kinase 5 downregulates FasL upon osmotic stress. *Cell death and differentiation*. 2006; **13**: 2099-108. 10.1038/sj.cdd.4401969.

39 Bin G, Bo Z, Jing W, Jin J, Xiaoyi T, Cong C, Liping A, Jinglin M, Cuifang W, Yonggang C, Yayi X. Fluid shear stress suppresses TNF-alpha-induced apoptosis in MC3T3-E1 cells: Involvement of ERK5-AKT-FoxO3a-Bim/FasL signaling pathways. *Experimental cell research*. 2016; **343**: 208-17. 10.1016/j.yexcr.2016.03.014.

40 Lennartsson J, Burovic F, Witek B, Jurek A, Heldin CH. Erk 5 is necessary for sustained PDGF-induced Akt phosphorylation and inhibition of apoptosis. *Cellular signalling*. 2010; **22**: 955-60. 10.1016/j.cellsig.2010.01.020.

41 Loizou JI, El-Khamisy SF, Zlatanou A, Moore DJ, Chan DW, Qin J, Sarno S, Meggio F, Pinna LA, Caldecott KW. The protein kinase CK2 facilitates repair of chromosomal DNA single-strand breaks. *Cell*. 2004; **117**: 17-28.

42 Lolli G, Ranchio A, Battistutta R. Active form of the protein kinase CK2 alpha2beta2 holoenzyme is a strong complex with symmetric architecture. *ACS chemical biology*. 2014; **9**: 366-71. 10.1021/cb400771y.

43 Torres J, Pulido R. The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to proteasome-mediated degradation. *The Journal of biological chemistry*. 2001; **276**: 993-8. 10.1074/jbc.M009134200.

44 van Diepen MT, Parsons M, Downes CP, Leslie NR, Hindges R, Eickholt BJ. MyosinV controls PTEN function and neuronal cell size. *Nature cell biology*. 2009; **11**: 1191-6. 10.1038/ncb1961.

Figure legends

Fig. 1. Roles of MAPKs in human platelet two-wave aggregation induced by botrocetin/VWF. (A) Washed human platelets were stimulated with botrocetin (1.5 μg mL^{-1}) or VWF (5 μg mL^{-1}) or both. (B) The platelets in (A) were lysed and immunoblotted with anti-p-ERK5, anti-p-JNK1/2, anti-p-p38, and anti-p-ERK1/2 antibodies. An anti-GAPDH antibody was used to indicate protein loading levels. (C) Washed human platelets were preincubated with DMSO or different concentrations of MAPK inhibitors: the ERK5 inhibitor XMD8-92 (5 μM or 10 μM), JNK1/2 inhibitor SP600125 (5 μM or 10 μM), p38 inhibitor SB203580 (2.5 μM or 5 μM), and MEK1/2 inhibitor U0126 (5 μM or 10 μM). Then, platelets were stimulated with botrocetin (1.5 μg mL^{-1}) and VWF (5 μg mL^{-1}). Traces shown are representative of three independent experiments with platelets from different donors.

Fig. 2. The effects of ERK5 on ristocetin/VWF-induced human platelet two-wave aggregation and on stable platelet adhesion to VWF under shear stress. (A) Washed human platelets were pre-incubated with DMSO or different concentrations of the ERK5 inhibitor XMD8-92 (2.5 μM or 5 μM). The aggregation of platelets was treated with ristocetin (0.25 mg mL^{-1}) and VWF (5 μg mL^{-1}). (B) Resting platelets and platelets in (A) were lysed and immunoblotted with anti-p-ERK5 antibody. An anti-GAPDH antibody was used to indicate protein loading levels. ** $P < 0.01$. Traces are representative of three independent experiments with platelets from different donors. (C) Calcein-labeled human blood samples were pretreated with DMSO or XMD8-92 (5 μM) for 5 min, loaded onto the VWF-coated

glass microfluidic channels, and then subjected to a constant shear rate (1500 s^{-1}) for 5 min. After washing, stably adherent platelets were photographed under a fluorescent microscope. Relative area under coverage and mean fluorescence intensity were quantitated. Data (mean±SD) were obtained from 9 randomly selected fields from each of 3 experiments. ### P < 0.001.

Fig. 3. Upstream activators of ERK5 in human platelets. (A) Washed human platelets were pre-incubated with DMSO or different concentrations of inhibitors: the Src inhibitor PP2 (5 μM or 10 μM), Raf inhibitor LY3009120 (0.5 μM or 1 μM), and MEK5 inhibitor BIX02189 (5 μM or 10 μM). Then, platelets were treated with botrocetin (1.5 μg mL-1) and VWF (5 μg mL-1). (B, C) Resting platelets and platelets in (A) were lysed and immunoblotted with anti-p-ERK5 antibody. Anti-GAPDH antibody was used to indicate protein loading levels. *** P < 0.001 versus resting platelets, ### P < 0.001 versus the DMSO-treated group. Traces shown are representative of three independent experiments with platelets from different donors.

Fig. 4. Composition of the ERK5 interacting protein complexes. (A) CHO cells were transfected with an HA-tagged-ERK5 construct or an HA-unmodified vector. After 24 hours, cells were lysed, and immunoprecipitation was performed with anti-HA-conjugated beads. The proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue. One gel band was excised, the protein/proteins in it were digested in situ, and the digestion

products were subjected to mass spectrometry for protein identification. (B) CHO cells were transfected with the HA-ERK5 vector or the HA-unmodified vector. Expression of ERK5 and CKII was detected by western blotting with specific antibodies. (C) An IP experiment was used to identify the proteins associated with ERK5 in CHO cells. 500 mg of cell lysate proteins was used for each IP sample. IP was performed using a rabbit anti-HA antibody. The proteins were detected by western blotting with specific antibodies. (D) An IP experiment was used to identify the proteins associated with ERK5 in platelets. XMD8-92 (10 μM)- or DMSO-preincubated platelets and resting platelets were tested in the IP experiment. IP was performed using a rabbit anti-ERK5 antibody or rabbit anti-CKIIα antibody or a non-specific rabbit IgG control. The detection of CKIIα associated with ERK5 and ERK5 associated with CKIIα was performed using specific antibodies. Rabbit IgG was used to demonstrate similar loading. (E) Washed human platelets were pre-incubated with DMSO or the CKII inhibitor TBB (5 μM or 10 μM). The aggregation of platelets was treated with botrocetin (1.5 μg mL-1) and VWF (5 μg mL-1). At least 3 independent experiments were performed.

Fig. 5. Downstream of ERK5/CKII in GPIb-IX-mediated platelet activation. (A) Platelets in Fig. 1(C) and Fig. 4(E) were lysed and immunoblotted with an anti-p-PTEN antibody. An anti-GAPDH antibody was used to indicate protein loading levels. (B) Resting platelets and platelets in Fig. 1(C) were lysed and immunoblotted with an anti-p-PTEN antibody. An anti-GAPDH antibody was used to indicate protein loading levels. (C) Platelets in Fig. 4(E) and Fig. 5(D) were lysed and immunoblotted with an anti-p-Akt T308 antibody and

anti-p-Akt S473 antibody. An anti-GAPDH antibody was used to indicate protein loading levels. *** P<0.001 versus resting platelets, ### P<0.001 versus the DMSO-treated group, ## P<0.01 versus the DMSO-treated group, # P<0.05 versus the DMSO-treated group. Traces shown are representative of three independent experiments with platelets from different donors. (D) Washed human platelets were pre-incubated with DMSO, the PI3K inhibitor wortmannin (1 μM or 2 μM), or the Akt inhibitor SH6 (10 μM or 20 μM). Then, the aggregation of platelets was treated with botrocetin (1.5 μg mL^{-1}) and VWF (5 μg mL^{-1}). (E) A working model showing that ERK5 associates with CKII to regulate GPIb-IX-mediated platelet activation via the PTEN/PI3K/Akt pathway.

Fig. S1. The effect of ERK5-IN-1 on botrocetin/VWF-induced human platelet two-wave aggregation. (A) Washed human platelets were pre-incubated with DMSO or different concentrations of the ERK5 inhibitor ERK5-IN-1 (5 μM or 10 μM). The aggregation of platelets was treated with botrocetin (1.5 μg mL-1) and VWF (5 μg mL-1). (B) Resting platelets and platelets in (A) were lysed and immunoblotted with anti-p-PTEN and anti-p-Akt antibodies. An anti-GAPDH antibody was used to indicate protein loading levels. Traces are representative of three independent experiments with platelets from different donors.

Fig. S2. Roles of ERK5 in platelet aggregation induced by thrombin, U46619, ADP, and collagen. Platelets were pre-incubated with DMSO or different concentrations of the ERK5 inhibitor XMD8-92 (10 μM or 20 μM). The aggregation of platelets was treated with (A) 0.1

U thrombin or (B) 0.1 μg mL^{-1} U46619 or (C) 10 μM ADP or (D) 1.0 μg mL^{-1} collagen. (E) Resting platelets and platelets in (A-D) were lysed and immunoblotted with an anti-p-ERK5 antibody. An anti-GAPDH antibody was used to indicate protein loading levels. *** P<0.001, ** P<0.01. Traces are representative of three independent experiments with platelets from different donors.

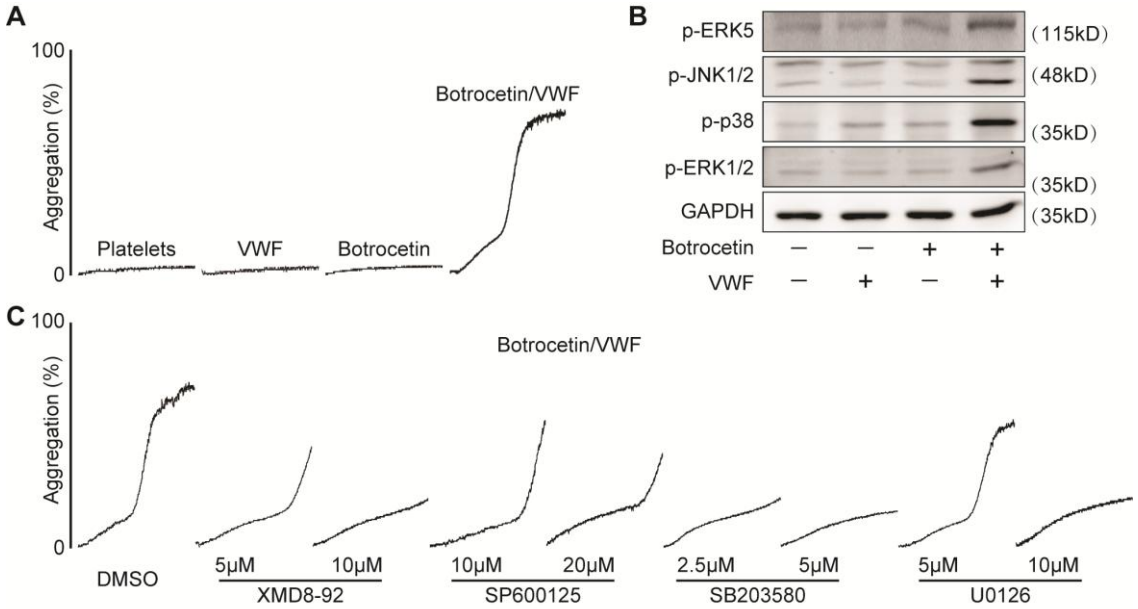

Figure 1

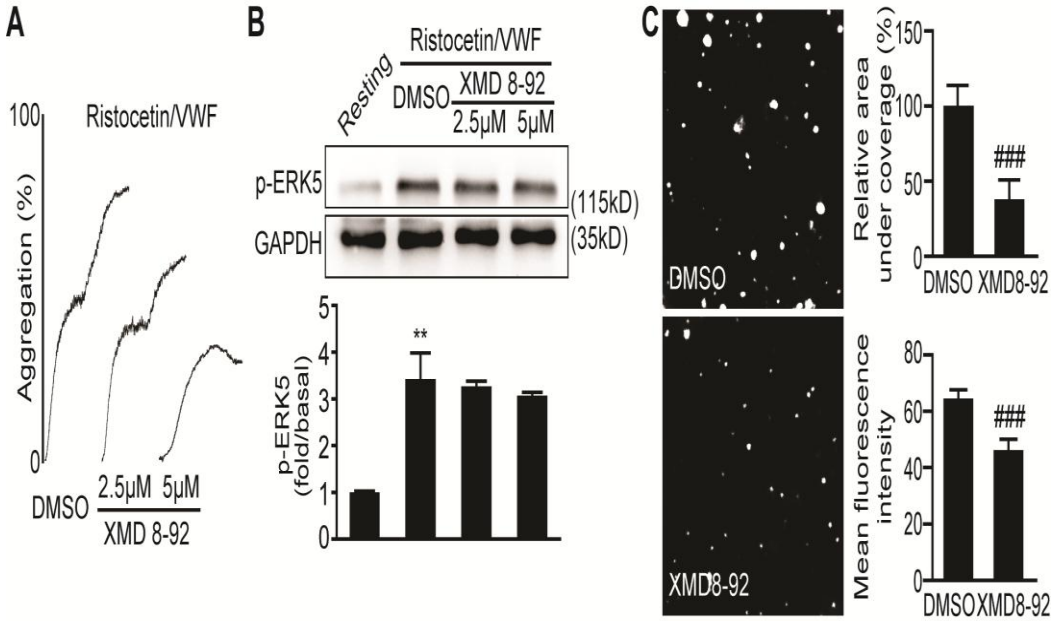

Figure 2

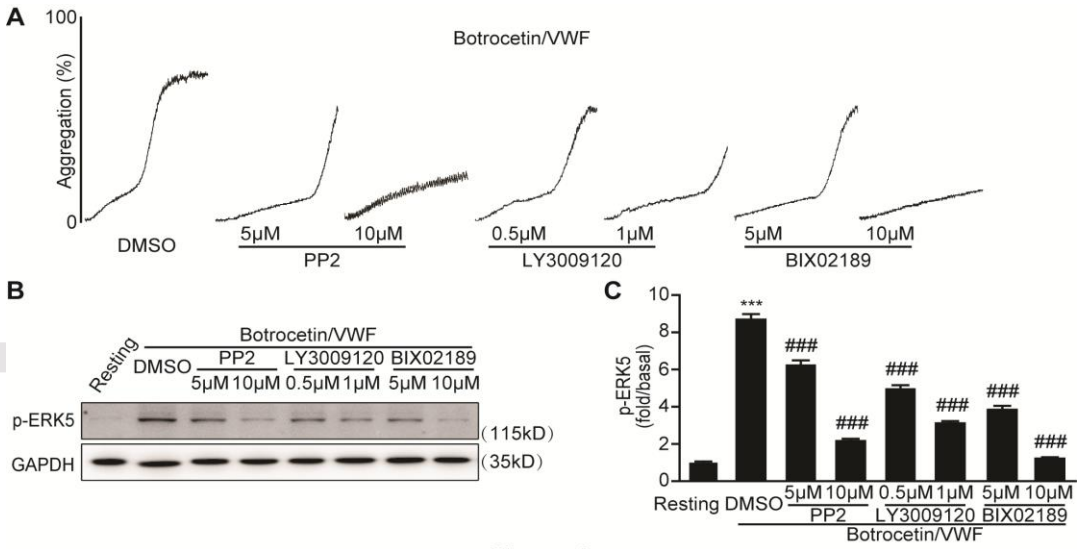

Figure 3

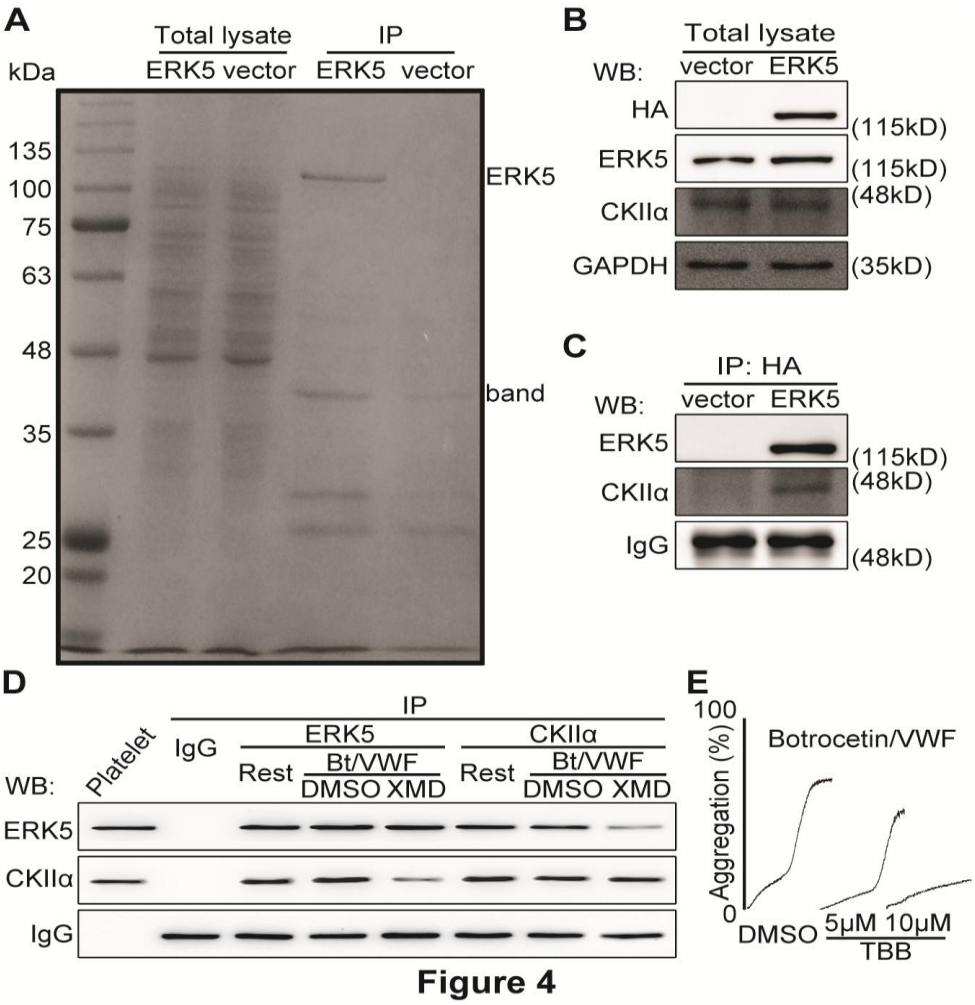

Figure 4

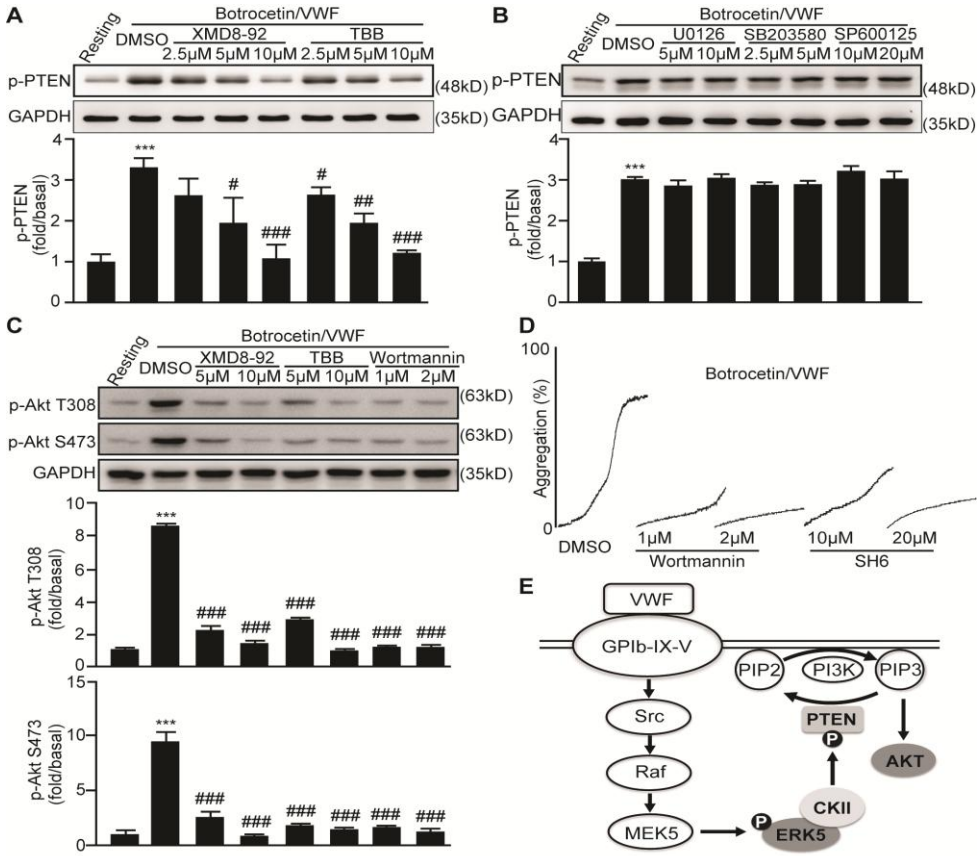

Figure 5