**Vascular Medicine**

**Dual-Specificity Phosphatase 3 Deficiency or Inhibition Limits Platelet Activation and Arterial Thrombosis**

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**Background**—A limitation of current antiplatelet therapies is their inability to separate thrombotic events from bleeding occurrences. A better understanding of the molecular mechanisms leading to platelet activation is important for the development of improved therapies. Recently, protein tyrosine phosphatases have emerged as critical regulators of platelet function.

**Methods and Results**—This is the first report implicating the dual-specificity phosphatase 3 (DUSP3) in platelet signaling and thrombosis. This phosphatase is highly expressed in human and mouse platelets. Platelets from DUSP3-deficient mice displayed a selective impairment of aggregation and granule secretion mediated by the collagen receptor glycoprotein VI and the C-type lectin-like receptor 2. DUSP3-deficient mice were more resistant to collagen- and epinephrine-induced thromboembolism compared with wild-type mice and showed severely impaired thrombus formation on ferric chloride-induced carotid artery injury. Intriguingly, bleeding times were not altered in DUSP3-deficient mice. At the molecular level, DUSP3 deficiency impaired Syk tyrosine phosphorylation, subsequently reducing phosphorylation of phospholipase Cγ2 and calcium fluxes. To investigate DUSP3 function in human platelets, a novel small-molecule inhibitor of DUSP3 was developed. This compound specifically inhibited collagen- and C-type lectin-like receptor 2–induced human platelet aggregation, thereby phenocopying the effect of DUSP3 deficiency in murine cells.

**Conclusions**—DUSP3 plays a selective and essential role in collagen- and C-type lectin-like receptor 2–mediated platelet activation and thrombus formation in vivo. Inhibition of DUSP3 may prove therapeutic for arterial thrombosis. This is the first time a protein tyrosine phosphatase, implicated in platelet signaling, has been targeted with a small-molecule drug. (Circulation. 2015;131:656-668. DOI: 10.1161/CIRCULATIONAHA.114.010186.)

Key Words: antagonists and inhibitors • blood platelets • signal transduction • thrombosis
Antiplatelet therapy has been effective in reducing the mortality and morbidity of acute myocardial infarction, the most common cause of death in developed countries. However, US Food and Drug Administration–approved antiplatelet agents have serious side effects, including gastrointestinal toxicity, neutropenia, thrombocytopenia, and common bleeding. There also remains a considerable incidence of arterial thrombosis in patients receiving currently available antiplatelet therapy. A better understanding of the molecular mechanisms leading to platelet activation is essential for the development of new therapeutics.

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Platelet activation depends on rapid phosphorylation and dephosphorylation of key signaling proteins, in particular tyrosine. Although the repertoire of protein tyrosine kinases has been well described in platelet activation, the expression, regulation, specificity, and function of platelet-expressed protein tyrosine phosphatases (PTPs) are largely unknown. A recent proteomic analysis found that 14 of 37 classic phosphotyrosine-specific PTPs are expressed in human platelets. Expression and function of the dual-specificity phosphatases, the largest subgroup of the PTP superfamily, are unexplored.

Dual-specificity phosphatase 3 (DUSP3), also known as Vaccinia H1–related phosphatase, is a dual-specificity phosphatase encoded by the DUSP3/Dusp3 gene. DUSP3 (185 amino acids; Mr, 21 kDa), which contains only a catalytic domain, has been reported to dephosphorylate the mitogen-activated protein kinases extracellular signal-regulated kinase 1/2 (ERK1/2) and Jun N-terminal kinase 1/2 (JNK1/2). Additional reported substrates include epidermal growth factor receptor and ErbB2. DUSP3 is implicated in cell cycle regulation, and its expression is altered in human cancer. However, because all of these studies were performed either in vitro with recombinant proteins or in cell lines with transient overexpression or siRNA knockdown, the true physiological function of DUSP3 has remained elusive. We recently generated a full Dusp3-knockout (Dusp3-KO) mouse. Dusp3-KO mice were healthy and fertile and showed no spontaneous phenotypic abnormality. However, DUSP3 deficiency prevented neangiogenesis and basic fibroblast growth factor–induced microvessel outgrowth. In the present study, we identified DUSP3 as a key and nonredundant player in glycoprotein (GP) VI– and C-type lectin–like receptor 2 (CLEC-2)–mediated signaling pathways in mouse and human platelets. We show that DUSP3 deficiency limits platelet activation and arterial thrombosis. Moreover, we developed a specific small-molecule inhibitor of DUSP3 that was able to phenocopy DUSP3 deficiency in platelets.

Methods
Platelet RNA Sampling and Microarray
Platelets from 256 healthy volunteers were isolated from citrate-anticoagulated blood. Donors were informed about the objectives of the study and signed an informed consent. The study was approved by the ethics committee review board of the Liège University Hospital. RNA extraction and microarray procedures are described in the online-only Data Supplement.

Mice
C57BL/6-Dusp3-KO mice were generated by homologous recombination. Heterozygous mice were mated to generate +/+ and −/− litters used for experimentation (male mice 8–12 weeks old). All experiments were approved by the local ethics committee.

Isolation of Human and Mouse Platelets
Human platelets were prepared from peripheral blood freshly drawn from healthy donors, as previously described. Mouse washed platelets were prepared as previously described.

Isolation of Human and Murine B and T Cells
Human B and T cells were sorted from freshly collected blood with the use of EasySep B and T cell–negative selection kits (Stemcell Technologies). Mouse B and T cells were sorted from spleens.

Platelet Aggregation Analyses
Light transmission was recorded during platelet aggregation induced by collagen, convulxin, collagen-related peptide (CRP), rhodocytin, thrombin, U46619, or ADP in the presence of 2 mmol/L CaCl2 on a Lumi-Aggregometer (Chrono-log).

Flow Cytometry
Washed platelets were stimulated for 15 minutes with different concentrations of collagen, CRP, thrombin, or ADP under nonstirring conditions. Saturating concentrations of FITC-conjugated P-selectin and phycoerythrin-conjugated JON/A antibodies were added. Samples were analyzed on a FACSCantoII flow cytometer (BD Biosciences).

Figure 1. Dual-specificity phosphatase 3 (DUSP3) expression in human and mouse platelets. A, Microarray data of mRNA expression of 17 atypical dual-specificity phosphatases (DSPs) in human platelets isolated from 256 healthy volunteers. Each open circle represents 1 individual. DNM3 was used as positive control for platelet-expressed mRNA. Data are presented as ratio of the fluorescence intensity for the DSP probe of interest and the fluorescence intensity for the housekeeping gene of each sample. A negative value corresponds to an expression below background level. Means±SEM values are shown. B through D, DUSP3 protein expression in human B and T lymphocytes and in platelets isolated from peripheral blood (B), in mouse splenic B and T cells and in washed platelets (C), and in wild-type (WT) and Dusp3-knockout (KO) mouse washed platelets (D). Western blot analysis was performed with anti-human (B) and anti-mouse DUSP3 (C and D). GAPDH was used as loading control. Representative blots of 3 independent experiments are shown.
Platelet pellets were fixed for 60 minutes in 2.5% glutaraldehyde in Sörensen buffer (0.1 mol/L, pH 7.4), postfixed for 30 minutes with 1% osmium tetroxide, dehydrated in a series of ethanol concentrations, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined on a Jeol-CX100II transmission electron microscope (60 kV).

Electron Microscopy
Platelet pellets were fixed for 60 minutes in 2.5% glutaraldehyde in Sörensen buffer (0.1 mol/L, pH 7.4), postfixed for 30 minutes with 1% osmium tetroxide, dehydrated in a series of ethanol concentrations, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined on a Jeol-CX100II transmission electron microscope (60 kV).

Figure 2. Dual-specificity phosphatase 3 (DUSP3)–deficient platelets exhibit impaired glycoprotein (GP) VI– and C-type lectin-like receptor 2 (CLEC-2)–mediated platelet aggregation. A through E, Washed platelets prepared from wild-type (WT) or Dusp3-knockout (KO) mice were stimulated with collagen (0.5 and 1 μg/mL; A), collagen-related peptide (CRP; 0.1, 0.3, and 1 μg/mL; B), convulxin (CVX; 5, 10, and 100 ng/mL; C), rhodocytin (2.5, 5, and 10 mmol/L; D), or the thromboxane A2 analog U46619 (1 μmol/L), thrombin (0.05 U/mL), or ADP (20 μmol/L; E). Representative platelet aggregation curves of 3 individual experiments are shown.
Whole-Blood Platelet Aggregate Formation Under Flow

Thrombus formation under flow conditions was assessed with anticoagulated mouse blood (4 U/mL heparin, 20 μmol/L PPACK), as previously described.15 Area coverage from phase-contrast images was analyzed with ImagePro (Media Cybernetics).16 Area coverage by platelets stained with OG488-annexin A5 was determined with Quanticell (Visitech).

Ca²⁺ Flux

Apyrase (0.5 U/mL)-treated murine washed platelets were loaded with 3.5 μmol/L fura-2-acetoxymethyl ester in the presence of Pluronic F-127 for 15 minutes. Fluorescence was recorded on an Aminco spectrofluorimeter (SLM Instruments), as described elsewhere.17

Arterial Thrombosis Models

Pulmonary embolism was induced by injection of a mixture of collagen (170 μg/kg) and epinephrine (60 μg/kg) into the plexus retroorbital veins of anesthetized mice (ketamine, 60 mg/kg; xylazine, 5 mg/kg). Time to death was monitored. Lungs were perfused with 4% formaldehyde solution and collected for histological studies.

Injury of carotid arteries of anesthetized mice was performed by applying a filter paper soaked in 10% ferric chloride (FeCl₃) solution to the exposed artery for 5 minutes.18 Fluorescence of exogenously carboxyfluorescein succinimidyl ester–labeled platelets was monitored with a BX61WI microscope (Olympus). Digital images were captured with a Hamamatsu 9100-13 electron-multplying charge-coupled device camera using a Lambda DG-4 (Sutter instrument) light source and Slidebook software 5.5 (3i).
Mouse Irradiation and Bone Marrow Transplantation

Donor mice (7–8 weeks old) were euthanized by cervical dislocation. Tibia and fibula were collected, and bone marrow was flushed with PBS. Then, 10×10⁶ single bone marrow cells were transplanted to 4- to 5-week-old lethally irradiated (866.3 cGy) recipient mice. Donor mice (7–8 weeks old) were euthanized by cervical dislocation. Tibia and fibula were collected, and bone marrow was flushed with PBS. Then, 10×10⁶ single bone marrow cells were transplanted to 4- to 5-week-old lethally irradiated (866.3 cGy) recipient mice. 

Tail Bleeding

Mice were anesthetized with isoflurane. A 3-mm portion of the tail tip was excised and submerged in a 37°C water bath. Bleeding was monitored for 15 minutes.

Platelet Activation, Cell Lysis, Immunoprecipitation, and Western Blotting

Mouse washed platelets were activated with CRP or rhodocytin in Tyrode buffer for 30, 60, or 90 seconds under 400-rpm stirring conditions at 37°C. Western blotting and immunoprecipitations were performed according to standard procedures¹⁹.

Statistical Analysis

Data are presented as mean±SEM of at least 3 independent experiments. Data were analyzed by use of the unpaired Student t test or ANOVA and the Bonferroni multiple-comparison test, as indicated in each figure legend. Differences in survival were determined with Kaplan-Meier analysis (log-rank Mantel test). Values of P<0.05 were considered significant. Calculations were performed with GraphPad-Prism (GraphPad Software, Inc).

Results

DUSP3 Expression in Platelets

Transcriptomic analysis of platelets from 256 healthy human individuals revealed that DUSP3-encoding mRNA is highly expressed in platelets (Figure 1A). An abundance of DUSP3 in human and mouse platelets was confirmed by Western blot analysis (Figure 1B and 1C). Expression levels of DUSP3 were substantially higher in platelets compared with B and T lymphocytes (Figure 1B and 1C); DUSP3 function in B and T lymphocytes has previously been described. Thus, we set out to investigate the role of DUSP3 in platelets using both genetic deletion in mice and pharmacological inhibition of DUSP3 in isolated human platelets.

Activation and Aggregation of DUSP3-Deficient Mouse Platelets

Using our previously generated Dusp3-KO mice,¹² we confirmed that platelets isolated from these animals do...
not express DUSP3 (Figure 1D). Hematologic parameters were normal except for slight but significant differences in monocytes \((P<0.05)\) and mean platelet volume \((P<0.0001; \text{Table I in the online-only Data Supplement})\). Dusp3-KO mice did not show any spontaneous bleeding or thrombotic disorders. However, in platelet aggregation assays, DUSP3-deficient platelets failed to aggregate in response to low concentrations of collagen \((0.5 \mu g/mL)\) and selective GPVI agonists, including convulxin \((5 \text{ ng/mL}; \text{Figure 2A–2C})\). Additionally, Dusp3-KO platelets exhibited delayed aggregation induced by low concentrations of rhodocytin \((2.5 \text{ and } 5 \text{ nmol/L})\), a selective CLEC-2 receptor agonist (Figure 2D). GPVI and CLEC-2 surface expression on Dusp3-KO platelets was similar to that of wild-type (WT) platelets (Figures IA and IIA in the online-only Data Supplement). In contrast, aggregation induced by ADP \((5–50 \mu mol/L)\), the thromboxane \(A_2\) mimetic U46619 \((0.75–2 \mu mol/L)\), or thrombin \((0.01–0.1 \text{ U/mL})\) occurred normally (Figure 2E and data not shown), indicating normal G-protein–coupled receptor–mediated responses.

To investigate the mechanism responsible for the impairment of collagen- and CRP-induced aggregation of DUSP3-deficient platelets, we analyzed their ability to release granule content by measuring P-selectin surface expression, and we examined their capacity to activate integrin \(\alpha_{\text{IIb}}\beta_3\) by using the JON/A antibody, which is specific for the high-affinity conformation of mouse \(\alpha_{\text{IIb}}\beta_3\). In DUSP3-deficient compared with WT platelets, P-selectin expression was reduced after stimulation with a low concentration of collagen \((0.5 \mu g/mL)\) or various concentrations of CRP \((0.1, 0.3, \text{ and } 1 \mu g/mL; \text{Figure 3A})\). Integrin \(\alpha_{\text{IIb}}\beta_3\) activation
was reduced with low concentrations of collagen (0.5 μg/mL) and CRP (0.1 μg/mL; Figure 3B). Electron microscopy analysis of resting DUSP3-deficient platelets revealed normal ultrastructure but a slightly increased number of α-granules (Figure 3C and 3D). When activated with convulxin, degranulation remained incomplete among the few
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DUSP3-deficient platelet aggregates compared with WT (Figure 3C). These findings indicate that DUSP3 deficiency impairs GPVI- and CLEC-2–dependent mouse platelet activation and aggregation.

GPVI and CLEC-2 Signaling in DUSP3-Deficient Platelets

Earlier studies suggested that DUSP3 dephosphorylates ERK1/2 and JNK1/2 but not p38. Therefore, we evaluated
the activation of these mitogen-activated protein kinases using phospho-specific antibodies at basal levels and after CRP stimulation. No differences in mitogen-activated protein kinase activation between DUSP3-deficient and WT platelets were found (Figure III in the online-only Data Supplement). We then analyzed global tyrosine phosphorylation and found decreased phosphorylation of a ≈70-kDa band in DUSP3-deficient compared with WT platelets after CRP or rhodocytin stimulation (Figure 4A and 4C). Longer exposure of the phosphotyrosine blot revealed additional bands (at ≈12, ≈26, and ≈40 kDa) with decreased phosphorylation in DUSP3-deficient compared with WT platelets after CRP stimulation (Figure 4B). We then tested whether the observed change in phosphotyrosine of the ≈70-kDa band may correspond to phosphotyrosine levels in the tyrosine kinase Syk (Mr, 72.1 kDa), a key signaling molecule in GPVI- and CLEC-2-mediated platelet activation. Indeed, phosphotyrosine of immunoprecipitated Syk was significantly reduced in DUSP3-deficient compared with WT platelets after GPVI and CLEC-2 stimulation (P<0.05; Figure 4D and 4E and Figure IVA and IVB in the online-only Data Supplement). Probing total lysates of DUSP3-deficient or WT platelets with phospho-Syk–specific antibodies revealed that, after activation with CRP, Syk phosphorylation was reduced on the activation residues Tyr-525/526, whereas phosphorylation of the negative regulatory Tyr-323 was not affected (Figure 4F and Figure IVF and IVG in the online-only Data Supplement). In rhodocytin-stimulated platelets, Syk phosphorylation was reduced on both Tyr-525/526 and Tyr-323 in the absence of DUSP3 (Figure 4G and Figure VH and VI in the online-only Data Supplement).

Syk is recruited to the GPVI/Fc receptor γ-chain (FcRγ) complex via phosphorylation of FcRγ-associated immunoreceptor tyrosine-based activation motifs by Src-family kinases (SFKs) and is then activated via autophosphorylation.20,21 We found that phosphorylation of FcRγ-associated immunoreceptor tyrosine-based activation motifs was reduced in DUSP3-deficient compared with WT platelets in response to CRP (Figure 4H and Figure IVJ in the online-only Data Supplement). In agreement with this observation, recruitment of Syk to FcRγ was impaired in DUSP3-deficient platelets (Figure 4H and Figure IVK in the online-only Data Supplement). Additionally, inducible tyrosine phosphorylation in phospholipase Cγ2, a key signaling molecule downstream of Syk, was reduced in both CRP- and rhodocytin-stimulated DUSP3-deficient compared with WT platelets (Figures 4I and 4J and Figure IVL and IVM in the online-only Data Supplement). In contrast, activation of SFKs, including Lyn, Fyn, and Src, was not altered (Figure VA and VB in the online-only Data Supplement), indicating that the reduced activation and recruitment of Syk in DUSP3-deficient platelets was not attributable to aberrant activation of SFKs.

Collagen-Induced Aggregation Under Flow, Calcium Fluxes, and Phosphatidylserine Exposure in DUSP3-Deficient Platelets

To further assess the role of DUSP3 in GPVI-dependent platelet responses, platelet aggregate formation and exposure of procoagulant phosphatidylserine on a collagen surface were analyzed in whole mouse blood under flow. The area covered by platelets was reduced by ≈40% for blood from Dusp3-KO compared with WT mice (Figure 5A and 5B), which was in agreement with reduced GPVI activation in DUSP3-deficient platelets.22 Accordingly, overall phosphatidylserine exposure on adhered platelets was also diminished (Figure 5C and 5D). Because phosphatidylserine exposure requires Ca2+ influx,23 we investigated whether Ca2+ flux was affected by DUSP3 deficiency. Convulxin-induced Ca2+ flux was greatly reduced (50%) in DUSP3-deficient compared with WT platelets (Figure 5E and 5F). Thapsigargin-induced Ca2+ increase occurred normally in DUSP3-deficient platelets (Figure VIA in the online-only Data Supplement). These data further support a positive role of DUSP3 in GPVI-mediated platelet activation under physiological flow conditions.

DUSP3 Deficiency and Thrombus Formation In Vivo

To evaluate the importance of DUSP3 in platelet function in vivo, we used a model of pulmonary thromboembolism induced by intravenous injection of a mixture of collagen and epinephrine. About 80% of DUSP3-deficient compared with 45% of WT mice survived (Figure 6A). Analyses of lung sections revealed significantly decreased numbers of occluded microvessels in DUSP3-deficient compared with WT mice (P<0.001; Figure 6B and 6C). We then examined thrombus formation in real time by intravital microscopy in a model of FeCl3-induced injury of the carotid arteries. In this model, collagen is exposed to circulating blood, and thrombus formation depends highly on GPVI. In DUSP3-deficient mice, blood vessels were never occluded as a result of failure to form stable thrombi, whereas full occlusion occurred at 8 to 10 minutes after FeCl3 application in WT vessels (Figure 6D and 6G). To test whether the defect in thrombus formation was due specifically to impaired platelet function, we generated chimeric mice by transferring Dusp3-KO bone marrow (KO>WT) or WT bone marrow (WT>WT) to lethally irradiated WT mice. Successful transplantation was evaluated by quantification of DUSP3 expression in peritoneal cell lysates from KO>WT and WT>WT mice (Figure 6F). Similar to Dusp3-KO, we found that thrombus formation was severely impaired in blood vessels of KO>WT mice (Figure 6E and 6G), confirming that the thrombosis defect in DUSP3-deficient animals was attributable to platelet dysfunction. Importantly, tail bleeding time, a measure of primary hemostasis in vivo, was identical for WT and DUSP3-deficient mice (Figure 6H).

Pharmacological Inhibition of DUSP3

To corroborate DUSP3 function in human platelets, we investigated the possibility of specifically inhibiting DUSP3 activity with small molecules. To identify DUSP3 inhibitors,
we used high-throughput screening, with a colorimetric phosphatase assay with p-nitrophenolphosphate as substrate, and screened 291,018 drug-like molecules. Of the 1524 primary high-throughput screening hits (≥50% inhibition), 1048 compounds were available from BioFocus DPI and ordered for confirmatory assays. The hits were tested in 2 reconfirmation single-dose screens in triplicate with the use of both the primary colorimetric assay and an orthogonal fluorescent assay with 3-O-methylfluorescein phosphate as substrate. Compounds with an average of ≥50% inhibition of DUSP3 activity were further tested in a 10-point dose-response assay in both colorimetric and fluorescent formats. IC_{50} values were determined, and 67 “cross-active” compounds were identified with IC_{50} values <20 μmol/L in both assays. On visual inspection of each molecule, 32 compounds were discarded from further consideration because of their known promiscuous PTP inhibitory activity. The remaining 35 compounds were taken into selectivity profiling studies for further prioritization. Compound selectivity for inhibiting DUSP3 over the related DUSP6 and 3 additional PTPs, HePTP, LYP, and STEP, was evaluated (Table II in the online-only Data Supplement).

On the basis of the selectivity and potency of compounds, 2 scaffolds were selected for structure-activity relationship studies: MLS-0103602 and MLS-0049585 (Table II in the online-only Data Supplement). MLS-0103602 (IC_{50}=0.37 μmol/L) was the most potent inhibitor with some degree of selectivity for DUSP3; MLS-0049585 (IC_{50}=2.68 μmol/L) exhibited the best selectivity for DUSP3. On the basis of the benzothioamide structure of MLS-0103602, 37 analogs were tested and counterscreened. All analogs were at least an order of magnitude less potent than the original hit, with no improvement in selectivity, leading to the termination of this series (data not shown). In contrast, several analogs containing the N-(benzo[d]thiazol-2-yl)-5-phenyl-1,3,4-oxadiazol-2-amine structure of MLS-0049585 with similar or even better potency could be identified (Table III in the online-only Data Supplement). The 4 most potent compounds were selected for testing in human platelets. Inhibition of platelet aggregation was assessed with platelets collected from 3 healthy donors. In these experiments, MLS-0437605 (Figure 7A) efficiently inhibited platelet aggregation in response to CRP and rhodocytin, but not after stimulation with thromboxane (Figure 7B and 7C). Tests on platelets from WT mice yielded similar results (Figure 7D). In contrast, MLS-0437605 only minimally affected the aggregation of DUSP3-deficient platelets (Figure 7D). Selectivity was further evaluated against 10 additional PTPs (Table). In these assays, MLS-0437605 showed excellent selectivity for DUSP3 over the vast majority of PTPs tested. Importantly, there was good selectivity of MLS-0437605 for DUSP3 over DUSP22 (7-fold), another dual-specificity phosphatase that is highly expressed in platelets (Figure 1A). We next examined the effect of MLS-0437605 on GPV1- and CLEC-2–induced signaling in human platelets. Global tyrosine phosphorylation was analyzed on total lysates from resting or activated platelets. MLS-0437605 caused a decrease in phosphotyrosine of an ~70-kDa band after stimulation with CRP or rhodocytin (Figure 7E and 7F). Tyrosine phosphorylation of immunoprecipitated Syk and PLCγ2 was also reduced by MLS-0437605 (Figure 7G and 7H). These data demonstrate that pharmacological inhibition of DUSP3 activity in human platelets affects platelet signaling in a manner similar to DUSP3 deficiency in Dusp3-KO platelets.

### Discussion

This is the first study implicating a member of the PTP subfamily of dual-specificity phosphatases in GPV1- and CLEC-2–induced signaling. Motivated by our finding that DUSP3 is highly expressed in human and mouse platelets, we used Dusp3-KO mice to study the role of this phosphatase in hemostasis and thrombosis. DUSP3-deficient mice were more resistant to pulmonary thromboembolism than their WT littermates. Thrombus formation was strongly impaired in the model of FeCl3-induced injury of carotid artery in a platelet-specific manner. Intriguingly, DUSP3-deficient mice did not bleed spontaneously and showed normal tail bleeding times. These findings suggest that DUSP3 plays a key role in arterial thrombosis but is dispensable for primary hemostasis.

Ex vivo, on platelet stimulation with a low concentration of collagen, convulxin, CRP, or rhodocytin, DUSP3 deficiency resulted in defective platelet aggregation, granule secretion, and integrin α_{IIb}β_{3} inside-out activation. In contrast, platelet activation mediated by G-protein–coupled receptor agonists was not affected. DUSP3 deficiency led to a reduction in thrombus formation on collagen-coated surfaces under arterial shear, as well as lower phosphatidylserine exposure at the surface of adhered platelets. These data indicate that GPV1- and CLEC-2–mediated platelet activation is impaired in DUSP3-deficient platelets. DUSP3 was dispensable for integrin α_{IIb}β_{3}, outside-in signaling, as indicated by unaltered fibrin clot retraction (data not shown). Dusp3-KO mice exhibited levels of thrombus formation comparable to the previously reported GPV1-KO/FcRγ-KO, CLEC-2–KO, CLEC-2–depleted, and CLEC-2/ GPV1-depleted mice. Similar to our findings in DUSP3-deficient mice, GPV1-KO and CLEC-2–KO mice do not exhibit prolonged bleeding time. DUSP3-deficient mice were also protected against pulmonary thromboembolism.
induced by a mixture of collagen and epinephrine, similar to GPV1-KO mice.33

At the molecular level, phosphorylation of the previously reported DUSP3 substrates ERK1/2 and JNK1/27 was not affected by DUSP3 deficiency, suggesting that signaling defects in DUSP3-deficient platelets are independent of the ERK1/2 and JNK1/2 pathways. However, we cannot exclude the possibility of functional or compensatory redundancies between DUSP3 and other phosphatases.

GPVI and CLEC-2 signaling pathways share many similarities, including the activation of Syk, phospholipase Cy2, and adapter proteins such as LAT and SLP-76.34 However, there is also a significant difference: in GPV1-stimulated platelets, SFKs initiate signaling through phosphorylation of the FeRγ-associated immunoreceptor tyrosine-based activation motifs, leading to binding and activation of Syk.20,21 In contrast, signaling through CLEC-2 depends on phosphorylation of CLEC-2 by Syk in an SFK-independent manner.22 Because DUSP3 deficiency limits platelet activation in response to both GPV1 and CLEC-2 stimulation, SFK function is likely not controlled by DUSP3, which is also supported by our data showing that phosphotyrosine in SFKs is not altered in Dusp3-KO platelets. On the contrary, Syk may be directly or indirectly targeted by DUSP3. Intriguingly, however, DUSP3 deficiency decreased phosphotyrosine levels in Syk. Furthermore, no hyperphosphorylated protein could be identified in phosphotyrosine blots of total lysates from DUSP3-deficient platelets. This raises the question of whether phosphoserine or phosphothreonine in Syk or other protein(s) may be targeted by DUSP3, a dual-specificity phosphatase able to dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine. Given the limited recognition sites of available phosphoserine/phosphothreonine antibodies, future studies using quantitative phosphoproteomics analysis are necessary to address this question.

Platelet binding to von Willebrand factor via GPIbα allows engagement of the collagen receptors GPVI and αIβ3, leading to platelet arrest and subsequent platelet thrombus formation. The von Willebrand factor–GPIb axis also induces GPVI dimerization, resulting in direct enhancement of GPVI interaction with collagen.35 However, platelets from Dusp3-KO mice exhibit normal binding to von Willebrand factor–coated surface under flow (Figure VII in the online-only Data Supplement), suggesting intact GPIb signaling in these animals. Interestingly, a recent study by Nieswandt’s group showed that combined depletion of GPVI and CLEC-2 was sufficient to abrogate arterial thrombosis in mice.27 Thus, the defects observed in DUSP3-deficient platelets on CLEC-2– and GPVI-induced signaling are sufficient to explain the impaired thrombus formation in Dusp3-KO mice.

Finally, platelets are anucleate cells that are not amenable to RNA interference or recombinant DNA technologies. Thus, to corroborate our findings in human cells, we used a chemical genomics approach. Specifically, a small-molecule inhibitor of DUSP3 was identified via high-throughput screening of a large chemical library and subsequent structure-activity relationship studies. Previously reported DUSP3 inhibitors suffer from poor selectivity, lack of efficacy, or both.37–41 or they cause immediate spontaneous aggregation of platelets (data not shown).42 Thus, we developed a novel, specific, and efficacious inhibitor that we used to inhibit DUSP3 function in human washed platelets. Similar to DUSP3 deficiency in murine cells, inhibition of DUSP3 activity in human platelets led to suppression of platelet aggregation, specifically in response to CRP and rhodocytin, but not in response to the G-protein–coupled receptor agonist thromboxane. MLS-0437605 is a drug-like compound43 and may serve as the basis for the development of potential therapeutics targeting DUSP3 for the treatment of arterial thrombosis.

Conclusions

We demonstrated that DUSP3 is a key signaling molecule for GPVI- and CLEC-2–induced platelet activation. We developed a specific small-molecule inhibitor of DUSP3 that efficiently inhibited human platelet activation in vitro. Given that Dusp3-KO mice remain healthy, do not exhibit any spontaneous phenotype, and do not suffer from increased bleeding events, our findings may lead to a novel antiplatelet therapy.

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Disclosures

None.

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CLINICAL PERSPECTIVE
A limitation of current antiplatelet therapies is their inability to separate thrombotic events from bleeding occurrences. The present study demonstrates that dual-specificity phosphatase 3 (DUSP3) phosphatase deficiency in mice does not cause bleeding but still protects against arterial thrombosis and collagen-induced thromboembolism. This protection is at least partially attributable to the selective inhibition of glycoprotein VI– and C-type lectin-like receptor 2–dependent signaling. Furthermore, ex vivo, a selective inhibitor of DUSP3 limits glycoprotein VI– and C-type lectin-like receptor 2–mediated aggregation of human platelets. Our findings pave the way for further preclinical studies in animal models and future validation in humans toward the development of a novel, DUSP3-based therapeutic strategy in arterial thrombosis.
Dual-Specificity Phosphatase 3 Deficiency or Inhibition Limits Platelet Activation and Arterial Thrombosis

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DUSP3 Phosphatase Deficiency or Inhibition Limit Platelet Activation and Arterial Thrombosis

Musumeci: DUSP3, a new player in arterial thrombosis

Supplementary material
Methods and reagents

Antibodies, reagents, and recombinant PTPs

Fluorescein isothiocyanate (FITC)-conjugated anti-P-selectin, and phycoerythrin (PE)-conjugated anti-active integrin αIβ3 (JON/A) antibodies were from Emfret Analytics (Würzburg, Germany). Anti-Fyn, anti-phosphotyrosine antibody (4G10) and anti-FcRγ subunit and mouse anti-rabbit light chain specific-HRP were from Millipore (Billerica, MA). Antibodies against Syk, Syk p-Tyr 525/526, Syk p-Tyr 323, Lyn p-Tyr-507, ERK (p44/42), p-ERK1/2 (Thr202/Tyr204), p38 MAPK, and p-p38 (Thr180/Tyr182) were from Cell Signaling (Danvers, MA). Anti-Src p-Tyr-416/418, anti-Src p-Tyr-529, and anti-Src pan antibodies were from Fisher Scientific (Erembodegem, Belgium). Anti-Fyn p-Tyr-530 was from Abcam (Cambridge, UK). Anti-Lyn and anti-DUSP3/VHR used for mice samples (sc-8889) were from Santa Cruz (Santa Cruz, CA). Anti-DUSP3/VHR antibody used for human samples (Clone 24/VHR), FITC-conjugated anti-CD3, PE-conjugated anti-B220, APC-Cy7-conjugated anti-Ly6G, PerCP-Cy5-conjugated anti-NK1.1 and Alexa-647 conjugated anti-rat antibodies were from BD Biosciences (Erembodegem, Belgium). Anti-CLEC2 antibody (clone 17D9) was from Serotec (Puchheim, Germany). Anti-vWF antibody was from Dako (Heverlee, Belgium). Goat anti-mouse kappa HRP-conjugated was from Southern Biotech (Birmingham, AL).

D-Phe-Pro-Ala-chloromethylketone (PPACK) was from Calbiochem (San Diego, CA). Fibrillar-type I equine tendon collagen was from Nycomed (Zurich, Switzerland). Bovine thrombin, ADP, and U46619 were from Sigma-Aldrich (Diegem, Belgium). Cross-linked collagen-related peptide (CRP) was provided by Prof. R.W. Farndale’s laboratory. Rhodocytin was purified from *C. rhodostoma* venom as described previously. [1] Annexin A5 labeled with Oregon Green OG488 and Fura-2 were from Molecular Probes (Leiden, the Netherlands). Convulxin was obtained from Kordia (Leiden, the Netherlands).
Para-nitrophenyl phosphate (pNPP), 3-O-methylfluorescein phosphate (OMFP), and dithiothreitol (DTT), and sodium orthovanadate (Na₃VO₄) were purchased from Sigma-Aldrich. Biomol Green reagent was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Compounds for follow-up studies were purchased from Specs or ChemBridge. All compounds had a purity of >95% (verified by LC/MS and 1H-NMR). Compounds chosen for cell-based assays were additionally repurified to >99% purity, and activity of the repurified substance was confirmed. All other chemicals and reagents were of the highest grade commercially available. Recombinant DUSP3, DUSP6, DUSP22, HePTP, LYP, PTP-SL, and STEP were expressed in *E. coli* and purified as described previously. [2-4] Recombinant CD45, TCPTP, LAR, and PTP1B were from Biomol Research Laboratories, Inc (Plymouth Meeting, PA, USA).

**Platelet RNA sampling and Microarray**

Platelet rich plasma (PRP) was prepared from citrate anticoagulated-blood. Depletion of CD45+ leukocytes was performed before total RNA extraction from freshly purified platelets using RNeasy Mini Kit on a QIAcube (Qiagen, Venlo, The Netherlands) and stored at -80°C until used. RNA was quantified by absorbance measurement, and 200 ng of RNA were engaged in reverse transcription with oligo-dT primers (Superscript III RT, Invitrogen), prior to biotin labeling and amplification using the TargetAmp Nano-g Biotin-aRNA Labeling Kit for the Illumina System (Epicentre). Biotin-labeled aRNA were purified using the RNeasy MinElute Cleanup Kit (Qiagen) and 400 ng were hybridized on Human HT-12 v4 arrays (Illumina) following the recommendations of the manufacturer. Arrays were scanned on an iScan microarray scanner (Illumina). Internal controls of the arrays were analyzed for quality control. Cell-specific expression markers were analyzed in all samples, ruling out contamination of platelet RNA with leukocyte RNA. Indeed, in this assay, comparison of the different DSPs mRNA levels is not possible. The raw fluorescence intensities for the probes
corresponding to the atypical DSPs have been corrected for the fluorescence background signal for each sample on the array by subtracting the fluorescence intensity of the negative control probes on the array. The data have then been normalized by dividing the intensity for each probe in each sample by the mean fluorescence intensity of 7 housekeeping genes (EEF1A1, UBC, ACTB, RPS9, GAPDH, TUBB2A and TXN) in the same sample. The data are presented as a ratio of the fluorescence intensity for the probe of interest and the mean fluorescence intensity for the housekeeping genes of each sample. A negative value corresponds to a lack of detection of the expression of the gene (expression level below the background).

**Chemical Library Screening for DUSP3 inhibitors**

DUSP3 HTS was performed within the MLPCN network, PubChem AID 1654. A total of 291,018 compounds (comprising the full MLPCN library at the time of screening) were screened at a concentration of 13.3 µM. A colorimetric phosphatase assay was set up in 1536-well format, using the general phosphatase substrate pNPP.[5, 6] The assay buffer contained 20 mM Bis-Tris (pH 6.0), 1 mM DTT, and 0.005% Tween-20. A detailed protocol of the HTS assay was published previously.[7]

**Single-concentration confirmatory assays for DUSP3 hits using OMFP.**

Phosphatase activity was measured in triplicate in a 1536-well format assay system, using the fluoresceine-based phosphatase substrate OMFP. The assay buffer contained 20 mM Bis-Tris (pH 6.0), 1 mM DTT, and 0.005% Tween-20. For a detailed protocol please see ref. 7.

**Selectivity profiling assays.**

Selectivity of compounds for inhibiting DUSP3 was tested against 10 additional PTPs using a 96-well format dose-response assay system with OMFP as substrate.[2] Enzyme concentrations were as follows: DUSP3, 2 nM; DUSP6, 10 nM; DUSP22, 10 nM; PTP-SL, 5 nM; HePTP, 5 nM; LYP, 5 nM; TCPTP, 2 nM; CD45, 2 nM; LAR, 1U/mL; STEP, 5 nM;
and PTP1B, 5 nM. OMFP was used at concentrations equal to the corresponding Km values: DUSP3, 13 μM; DUSP6, 50 μM; DUSP22, 2.2 μM; PTP-SL, 28 μM; HePTP, 117 μM; LYP, 185 μM; TCPTP, 56 μM; CD45, 347 μM; LAR, 78 μM; STEP, 32 μM; and PTP1B, 99 μM. The initial rate was determined using a FLx800 micro plate reader (Bio-Tek Instruments, Inc.), an excitation wave length of 485 nm and measuring the emission of the fluorescent reaction product 3-O-methylfluorescein at 525 nm. The nonenzymatic hydrolysis of the substrate was corrected by measuring the control without addition of enzyme. IC₅₀ values for each enzyme were determined as described previously.[2]
References


**Figure Legends**

**Figure S1.** *Surface expression of major platelet receptors on Dusp3-deficient platelets.* Resting WT and Dusp3-KO platelets were stained with (A) anti-GPVI-FITC, (B) anti-CD41-FITC, (C) anti-CD42C/GPib-FITC, and (D) anti-CD42D/GPV-FITC and analyzed by flow cytometry. Anti-mouse IgG-FITC (grey line) was used as a negative control antibody for the staining. Representative histograms with the mean fluorescence intensity (MFI) for WT (dark line) and Dusp3-KO (dashed line) are shown for each staining.

**Figure S2.** *CLEC-2 surface expression in platelets and mononucleated cells.* (A) Resting WP from WT and Dusp3-KO platelets were stained with anti-CLEC-2 antibody followed by a secondary staining using Alexa-647 conjugated anti-rat antibody and FITC-conjugated anti-CD41. A rat-anti-mouse antibody was used as a negative control (grey line). (B) Resting spleenocytes from WT and Dusp3-KO mice were stained using PE-conjugated anti-B220, FITC-conjugated anti-CD3, APC-Cy7-conjugated anti-Ly6G, PerCP-Cy5-conjugated anti-NK1.1 and anti-CLEC-2 followed by Alexa-647 conjugated anti-rat antibody. CD3⁺ (T lymphocytes), B220⁺ (B Lymphocytes), Ly6G⁺ (Neutrophils) and NK1.1⁺ (NK cells) were separately gated out of total live cells and analyzed for the expression of CLEC-2. Representative histograms with the % of Max of the mean fluorescence intensity for WT (dark line) and Dusp3-KO (grey line) are shown for each staining.

**Figure S3.** *MAPks activation in Dusp3-KO platelets.* Total cell lysates (TCLs) were prepared from CRP (0.3 µg/mL) activated WT or Dusp3-KO mouse platelets. Cells were non-activated or activated for 30, 90, and 300 s (for MAPks) or 30, 60, and 90 s (for SFKs) with CRP. Equal amounts of protein were resolved by SDS-PAGE, and western blot analysis was performed using: Anti-phospho-ERK1/2 (Thr202/Tyr204), anti-JNK1/2 (Thr183/Tyr185), and anti-phospho-p38 (Thr180/Tyr182). Anti-ERK1/2, anti-JNK1/2, and anti-p38 were used as loading controls.
Figure S4. Quantification of Syk, FcRγ, and PLCγ tyrosine phosphorylation and recruitment of Syk to FcRγ. Densitometric analysis of results presented in Figure 4 for tyrosine phosphorylation of immunoprecipitated Syk (Figure 4D and 4E) in CRP-activated conditions (A) and in rhodocytin stimulated conditions (B). Quantification of Syk phosphorylation on Tyr-323 and Tyr-525/526 (shown in Figure 4F and 4G) in CRP (C-D) or rhodocytin (E-F) activated platelets. Normalization was performed using total Syk. (G) Quantification of tyrosine phosphorylation (4G10) western blots on FcRγ immunoprecipitates. (H) Quantification of Syk recruitment to FcRγ. (I-J) Statistical analysis of tyrosine phosphorylation (4G10) of PLCγ2 immunoprecipitates from equal amounts of TCLs from CRP (I) or rhodocytin (J) activated platelets. Data were analyzed using Anova Bonteferroni multiple comparison test and are presented as mean ± SEM. Statistical analyses are shown for three independent experiments, each experiment was performed using pooled platelets from three mice.

Figure S5. SFK activation in Dusp3-KO platelets. Total cell lysates (TCLs) were prepared from CRP (0.3 µg/mL) or rhodocytin (10nM) activated WT or Dusp3-KO mouse platelets. Cells were non-activated or activated for 30, 60, and 90 s with CRP (A) or with rhodocytin (B). Equal amounts of protein were resolved by SDS-PAGE, and western blot analysis was performed using: Anti-phospho-Src (Tyr416), anti-phospho-Src (Tyr529), anti-phospho-Fyn (Tyr530), or anti-phospho-Lyn (Tyr507). Anti-Src and anti-Fyn were used as loading controls. Data were analyzed using Anova Bonteferroni multiple comparison test and are presented as mean ± SEM. Results are representative of three independent experiments.

Figure S6. Thapsigargin induced store mediated Ca^{2+} entry and GPCR agonist-triggered intracellular Ca^{2+} increase in Dusp3-KO platelets. (A) Fura-2 loaded platelets were stimulated with thapsigargin (200 nM) before adding 500 mM CaCl₂. (B-D) Platelets were stimulated with thrombin (IIA, 10 nM) (B), ADP (20 mM) (C), or TXA₂ mimetic U46619 (1
mM) (D). Traces are representative of three independent experiments.

**Figure S7. Platelet aggregate formation on whole blood on vWF coated surface.**

Anticoagulated blood from WT or Dusp3-KO mice was perfused over vWF-coated coverslip (1.4 µg) through a parallel-plate transparent flow chamber at a wall-shear rate of 1000 s⁻¹ for 4 min. Representative phase-contrast images of fixed platelets (A) and percentages of surface coverage by platelets (B) are shown. Results were analyzed using unpaired Student t-test. Data represent mean ± SEM of three independent experiments; ns=non significant.
Table S1. Hematological parameters of WT and DUSP3-KO mice.

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<th>Parameter</th>
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<th>DUSP3-KO (n=16)</th>
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<td>WBC (x10^3/mL)</td>
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<td>Monocytes (x10^3/mL)</td>
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<td>Eosinophils (x10^3/mL)</td>
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<td>Platelets (x10^3/mL)</td>
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<td>HCT (%)</td>
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<td>5.099±0.06</td>
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Table S2. Potency and selectivity of 35 selected DUSP3 inhibitors. IC$_{50}$ values are in µM; phosphatase substrates used are given in parentheses (OMFP or pNPP).

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<td>74.6</td>
<td>&lt;1.23</td>
<td>7.93</td>
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<td>MLS- 0216485</td>
<td>11.6</td>
<td>1.56</td>
<td>16.2</td>
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<td>n/d</td>
<td>n/d</td>
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<tr>
<td>MLS- 0304619</td>
<td>12.9</td>
<td>6.94</td>
<td>31</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
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<tr>
<td>MLS- 0202850</td>
<td>13.2</td>
<td>2.6</td>
<td>24</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
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<tr>
<td>MLS- 0284989</td>
<td>13.9</td>
<td>5.73</td>
<td>25.8</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
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<tr>
<td>MLS- 0308908</td>
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<td>2.99</td>
<td>78.7</td>
<td>n/d</td>
<td>9</td>
<td>3.97</td>
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MLS-0312434

19  7.42  >100  <1.23  n/d  n/d
Table S3. SAR studies for compound MLS-0049585.

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<th>Compound ID</th>
<th>Structure</th>
<th>Result Graph</th>
<th>IC₅₀, µM</th>
<th>Std Error</th>
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<td><img src="image1" alt="Graph" /></td>
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</table>
Figure S1

A. 

B. 

C. 

D.
Figure S2

A.

B.

Platelets

T lymphocytes

B lymphocytes

Neutrophils

NK cells

CLEC-2

CLEC-2

CLEC-2

CLEC-2

% of Max

% of Max

% of Max

% of Max

0 10^2 10^3 10^4 10^5

0 10^2 10^3 10^4 10^5

0 10^2 10^3 10^4 10^5

0 10^2 10^3 10^4 10^5
Figure S3

<table>
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<th>CRP (µg/ml)</th>
<th>WT</th>
<th>KO</th>
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<td>0</td>
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<td>0</td>
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<tr>
<td>300</td>
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CRP (s) 0.3 µg/ml

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<th>30</th>
<th>90</th>
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<td>p-ERK1/2</td>
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Figure S5

A. Total lysates

<table>
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<th>CRP (s)</th>
<th>WT</th>
<th>KO</th>
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<tbody>
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<td>0.3 μg/ml</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td></td>
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</tbody>
</table>

- pSrc-Y416
- pSFK-Y529
- pFyn-Y530
- pLyn-Y507
- Fyn
- Src

B. Total lysates

<table>
<thead>
<tr>
<th>Rhodocytin (s)</th>
<th>WT</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>nM</td>
<td>0</td>
<td>30</td>
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</tbody>
</table>

- pSrc-Y416
- pSFK-Y529
- pFyn-Y530
- pLyn-Y507
- Fyn
- Src
Figure S6

A. Thapsigargin and Calcium

B. IIA

C. ADP

D. TXA2
Figure S7

A. WT  

Dusp3-KO

vWF coated surface

B. Surface area coverage (%)  

ns

WT  KO

Surface area coverage (%)