Chapter 17

Functional Analysis of Protein Tyrosine Phosphatases in Thrombosis and Hemostasis

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Abstract

Platelets are small blood cells derived from cytoplasmic fragments of megakaryocytes and play an essential role in thrombosis and hemostasis. Platelet activation depends on the rapid phosphorylation and dephosphorylation of key signaling molecules, and a number of kinases and phosphatases have been identified as major regulators of platelet function. However, the investigation of novel signaling proteins has suffered from technical limitations due to the anucleate nature of platelets and their very limited levels of mRNA and de novo protein synthesis. In the past, experimental methods were restricted to the generation of genetically modified mice and the development of specific antibodies. More recently, novel (phospho) proteomic technologies and pharmacological approaches using specific small-molecule inhibitors have added additional capabilities to investigate specific platelet proteins.

In this chapter, we report methods for using genetic and pharmacological approaches to investigate the function of platelet signaling proteins. While the described experiments focus on the role of the dual-specificity phosphatase 3 (DUSP3) in platelet signaling, the presented methods are applicable to any signaling enzyme. Specifically, we describe a testing strategy that includes (1) aggregation and secretion experiments with mouse and human platelets, (2) immunoprecipitation and immunoblot assays to study platelet signaling events, (3) detailed protocols to use selected animal models in order to investigate thrombosis and hemostasis in vivo, and (4) strategies for utilizing pharmacological inhibitors on human platelets.

Key words Platelets, Aggregation, Secretion, Signaling, Flow cytometry, Aggregation under flow, Selectin, JON/A, Calcium, Bleeding time, Collagen, CLEC-2, GPVI, ADP, Thrombin, Intravital microscopy, Ferric chloride, Thromboembolism, DSPs, Dual-specificity phosphatases, PTPs, Protein tyrosine phosphatases, DUSP3, VHR, Small molecule inhibitors, Western blots, Immunoprecipitation

1 Introduction

Platelets are small anucleate cytoplasmic fragments of megakaryocytes produced in the bone marrow [1, 2]. Human platelets circulate for approximately 7–10 days in the blood stream before being cleared by macrophages in the spleen and liver. Only when the endothelial

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cell layer of blood vessels is damaged by injuries or pathological alterations, the adhesive potential of platelets becomes evident. Under these conditions, components of the subendothelial extracellular matrix (ECM) are exposed and trigger sudden platelet activation and adhesion [3]. The first step in the hemostatic cascade is the interaction of platelets with the exposed ECM, which contains a large number of adhesive macromolecules, such as laminin, fibronectin, collagen, and von Willebrand factor (vWF). The initial tethering of platelets to the ECM is mediated by the interaction between the platelet receptor glycoprotein (GP) Ib and vWF bound to collagen. The binding of GPIb to vWF has a fast off-rate and is therefore insufficient to mediate stable adhesion. 'Rolling' platelets establish contacts with the thrombogenic ECM protein collagen through their immunoglobulin superfamily receptor GPVI. This receptor triggers intracellular signals that shift platelet integrins to a high-affinity state and induce the release of the secondary mediators adenosine diphosphate (ADP) and thromboxane A_2 (TXA_2). These agonists, together with locally produced thrombin, contribute to cellular activation by stimulating G-protein coupled receptors, which induce various signaling events and act synergistically to induce full platelet activation [4, 5].

Platelet activation depends on the rapid phosphorylation and dephosphorylation of key signaling proteins, particularly on tyrosine residues. The repertoire of protein tyrosine kinases (PTKs) has been well described in platelet activation (reviewed in ref. 6). However, the expression, regulation, specificity, and function of the platelet-expressed protein tyrosine phosphatases (PTPs) are largely unknown. Quantitative proteomic analyses revealed that 14 out of 37 known classical, phosphotyrosine-specific PTPs are expressed in human platelets [7, 8]. However, only a few of these enzymes, including PTP1B, SHP1, SHP2, and CD148, have been investigated in platelet signaling [8, 9]. Expression and function of the dual-specificity phosphatases (DSPs), the largest subgroup of PTPs with (additional) activity towards phosphoserine and phosphothreonine or non-protein substrates, are largely unknown.

Because platelets have no nucleus and only very limited levels of mRNA and de novo protein synthesis, standard RNA interference or recombinant DNA techniques cannot be used to investigate novel regulatory platelet proteins. Instead, such studies depend on the generation of knockout animals and the development of specific antibodies, tool compounds, and new technologies such as (phospho)proteomics. Using both genetic knockout and pharmacological inhibition, we recently identified the dual-specificity phosphatase 3 (DUSP3) as a major player in platelet biology [10]. This was the first time a DSP had been implicated in platelet function. We showed that DUSP3 is essential for platelet activation and thrombus formation in vivo. We also demonstrated that signaling via the canonical GPVI and C-type lectin-like receptor (CLEC) 2-induced pathways is impaired in DUSP3-deficient platelets. To investigate DUSP3

function in human platelets, we developed a novel small-molecule inhibitor of DUSP3. We showed that inhibition of DUSP3 activity in human platelets phenocopies the effect of DUSP3 deficiency in murine cells, providing proof-of-principle for a DUSP3-based therapeutic approach in arterial thrombosis.

In this chapter we describe methods to investigate the role of DUSP3 in platelet signaling and activation. These methods are generally applicable also to other PTPs or signaling proteins. We provide detailed instructions on how to study platelet function both ex vivo (mouse and human platelets) and in vivo in mice.

2 Materials

All chemicals used should be of analytical grade. Distilled water (dH_2O) should be used for preparation of all buffers.

2.1 Anesthetic Reagents

1. Ketamine–xylazine mix: Ketamine 1000 and xylazine (Xyl-m; 2%). Stock concentrations: ketamine 100 mg/μL, xylazine 20 mg/μL. Prepare an anesthetic cocktail just before the anesthesia with a final concentration 100 mg/kg of ketamine and 10 mg/kg of xylazine. Add (body weight × 1 × 1.5 μL) of ketamine stock and (body weight × 0.5 × 1.5 μL) of xylazine stock. Complete with NaCl 0.9% to 300 μL. Inject to mice intraperitoneally (i.p.) 200 μL of the ketamine–xylazine mix using a 1 mL syringe with 26 G needle. Check for depth of anesthesia after 10–15 min by pinching the footpad of the mouse using forceps or fingertips. If the animal withdraws the foot or even "cries," anesthesia is not deep enough to proceed. If required, mice should be maintained under anesthesia by injecting Nembutal (*see* **Notes 1** and **2**).

2.2 Anticoagulants

Anticoagulant usage depends on the type of experiment as they can influence the results. For human and mouse platelet preparations, we recommend acid citrate dextrose (ACD) as anticoagulant when working with washed platelets, and citrate when working with whole blood or platelet-rich-plasma (PRP). Heparin should be avoided because of its platelet activating properties [11].

1. Acid-citrate dextrose (ACD) buffer: Dissolve 27.35 g sodium citrate (93 mM), 1.471 g citric acid (7 mM), and 0.252 g dextrose (14 mM) in a final volume of 1 L of dH_2O. This buffer can be kept at 4 °C for up to 6 months. Use 1 volume of anticoagulant for 6 volumes of blood. Just before the blood collection, add 1 U/mL of apyrase grade I. Once thawed, apyrase should not be frozen a second time.
2. Citrate buffers:
   - Sodium citrate tribasic dehydrate 3.2%: dissolve 40 g in 1 L dH_2O.

- Sodium citrate tribasic dehydrate 3.8%: dissolve 47.5 g in 1 L dH_2O.

Buffers can be kept at 4 °C for up to 6 months.

Use 1 volume of citrate 3.2% for 9 volumes of human blood, and 1 volume of citrate 3.8% for 9 volumes of mouse blood.

2.3 Blood Collection

1. 1 mL syringes with 26 G needles and 10 mL syringes with 18 G needles.
2. 1.5 mL polypropylene Eppendorf tubes.
3. Disposable Capillaries (10 μL).
4. 50 mL polypropylene conical tube.
5. Apyrase.

2.4 Platelet Preparation and Aggregation

1. Stock $CaCl_2$: 21.9 g (100 mM) $CaCl_2$ in 1 L dH_2O.
2. Stock solutions for Tyrode's buffer:
   - Stock solution 1: 8.01 g (137 mM) NaCl; 1.01 g (12 mM) $NaHCO_3$; 0.149 g (2 mM) KCl; 0.060 g (0.34 mM) Na_2HPO_4. Dissolve in 1 L of dH_2O.
   - Stock solution 2: Dissolve 119.2 g (5 mM) HEPES powder in 1 L of dH_2O.
   - Stock solution 3: Dissolve 203.3 g (1 mM) $MgCl_2$ in 1 L of dH_2O.

   Stock solutions can be kept at 4 °C for up to 6 months.
3. Tyrode's buffer: dissolve 10 mg of glucose and 35 mg of BSA in 10 mL of stock solution 1. Add 100 μL of stock solution 2 and 10 μL of stock solution 3. Bring to room temperature and use within the day of preparation.
4. Microcentrifuge.
5. Benchtop centrifuge with swinging bucket rotor.
6. 15 and 50 mL polypropylene conical tubes.
7. Hematology analyzer. We used Cell-Dyn 3700 from Abbott.
8. Cuvettes 450 μL (ChronoLog Corporation).
9. ChronoLog siliconized stir bars (ChronoLog Corporation).
10. Lumi-Aggregometer. We used ChronoLog Lumi-Aggregometer from ChronoLog Corporation.
11. 200 μL gel loading tips.

2.5 Platelet Secretion Using Flow Cytometry

1. FITC-conjugated anti-mouse CD62P antibody (clone RB40.34, BD Biosciences).
2. PE-conjugated anti-mouse active form of $\alpha_{IIb}\beta_3$ (clone JON/A, Emfret Analytics).

3. PE-conjugated anti-human CD62P antibody (clone AC 1.2, BD Biosciences).
4. FITC-conjugated anti-PAC1 antibody (clone PAC-1, BD Biosciences).
5. 5 mL polystyrene round bottom tube.
6. 1 % of Paraformaldehyde (PFA) (*see* **Note 3**).
7. BD FACS™ lysing solution (BD Biosciences); 10× concentrate. To be diluted in dH_2O.

2.6 Platelet Activation, Lysis, Immunoprecipitation (IP), and Immunoblot

1. Cuvettes 450 µL (ChronoLog Corporation).
2. Siliconized stir bars (ChronoLog Corporation).
3. 200 µL gel loading tips.
4. 96-well flat bottom plates.
5. Pierce Coomassie (Bradford) Protein Assay Kit (Thermo Scientific).
6. Spectrophotometer.
7. Complete EDTA-free protease inhibitor cocktail.
8. PhosphoSTOP phosphatase inhibitor cocktail.
9. Stock solutions for platelet 4× lysis buffer:
   - 5 M NaCl: dissolve 292.2 g NaCl in 1 L dH_2O.
   - 1 M Tris–HCl, pH 7.5: dissolve 60.55 g of Tris (hydroxymethyl aminomethane) in 460 mL dH_2O. Adjust the pH to 7.5 with conc. HCl. Bring to 500 mL with dH_2O.
   - 0.5 M EDTA pH 8: Dissolve 93.05 g EDTA in 460 mL dH_2O. Adjust the pH to 8.0 using 1 M NaOH. Bring to 500 mL with dH_2O.
   - 0.5 M EGTA pH 8: dissolve 95.1 g of EGTA in 460 mL dH_2O. Adjust the pH 8.0 with 1 M NaOH. Bring to 500 mL with dH_2O.
10. Platelet 4× lysis buffer: 60 mL of 5 M NaCl; 20 mL of 0.5 M Tris–HCl; 4 mL of 0.5 M EGTA; 4 mL of 0.5 M EDTA, and 20 mL of 100% NP-40. Bring to 500 mL with dH_2O. Mix well. Store the buffer at 4 °C for up to 6 months.

    When needed, prepare 2× platelet lysis buffer and add fresh complete EDTA-free protease inhibitor cocktail and PhosphoSTOP phosphatase inhibitor cocktail. Stocks of protease and phosphatase inhibitor cocktails can be prepared in advance (concentration 20×) and stored at –20 °C in small aliquots.
11. Stock solutions for Laemmli 2× sample buffer.
    - 20% SDS: dissolve 20 g SDS in 100 mL dH_2O.
    - 1 M Tris–HCl pH 6.8: dissolve 121.14 g Tris in 1 L dH_2O and adjust pH to 6.8 with HCl.

12. Laemmli 2× sample buffer: (4% SDS) 2 mL of 20% SDS; (20% glycerol) 2 mL of 100% glycerol; (50 mL Tris–HCl) 1 mL of 500 mM Tris–HCl; 1 mg of bromophenol blue (0.01%). Adjust to 9 mL with dH_2O. Stock in 800 µL aliquots at −20 °C. When needed, defreeze an aliquot and add 200 µL of 2-mercaptoethanol. This solution can be kept at room temperature (RT) under a fume hood and is stable for up to 3 months.
13. Immunoblot Assays:
    - Vertical acrylamide electrophoresis unit with power supply.
    - Electroblotting unit, fully submerged.
    - Nitrocellulose membrane (0.45 µm pore size).
    - Whatman #1 filter paper.
    - Methanol.
    - Precast 4–20% Tris–Glycine gradient gels.
    - SDS running buffer; 10× stock solution (2 L): 60.4 g Tris base, 288 g glycine, 20 g SDS. Make up the solution close to the desired volume using dH_2O. Stir for 10 min. Adjust the pH to be between 8.1 and 8.5. Adjust with dH_2O to the final desired volume.
    - TBST buffer; 10× stock solution (1 L): 24.2 g Tris base, 80 g NaCl, add 800 mL of cold dH_2O, adjust pH to 7.6 by adding ~15 mL conc. HCl, and add 10 mL Tween 20. Stir until Tween is completely dissolved and adjust the final volume to 1 L using cold dH_2O.
    - Blocking buffer: 3% BSA in 1× TBST; 5% fat-free milk in 1× TBST.
    - Molecular weight marker: Mix equal volumes of MagicMark (Life Technologies) and SeeBlue Plus2 Prestained Protein Standards (Life Technologies). This will allow visual control of migration of proteins on the gel (SeeBlue) and visualize the marker (MagicMark) together with proteins of interest after ECL.
    - Optional: phospho-specific antibodies and pan-antibodies directed against the signaling molecules involved in the platelet receptor pathway of interest.
    - Anti-phosphotyrosine antibody (4G10 clone); anti-actin or anti-GAPDH antibodies; HRP-conjugated anti-mouse and anti-rabbit secondary antibodies.
    - Chemiluminescent detection kit (ECL).

2.7 In Vivo Thrombosis Models

1. Carboxyfluorescein succinimidyl ester (CFSE). Add 50 µL of DMSO to one vial of 50 µg CFSE dye until fully dissolved. The stock solution is 1 µg/µL. Keep stocks at −20 °C protected from light.

2. Ferric chloride ($FeCl_3$). Prepare 10% solution of $FeCl_3$ in dH_2O. In 1.5 mL Eppendorf tube, dissolve 100 mg of $FeCl_3$ powder in 100 μL of dH_2O and vortex the mix. The solution should be freshly prepared.
3. NaCl 0.9% sterile: Mini Plasco NaCl 0.9.
4. Horm Collagen (Takeda Austria GmbH).
5. Epinephrine (1 mg/mL, Sterop laboratories).
6. Forceps: Dumont 7 and Dumont 7b.
7. Surgical scissor: Hardened Fine Iris Scissors.
8. Spring Scissor: Spring Scissors—8 mm Blades.
9. BD Intramedic™ Polyethylene Tubing (Non-Sterile). ID 0.28 mm, OD 0.6 mm.
10. Sponge: Sugi® points sterile (Kettenbach).
11. 0.5 × 0.5 × 0.5 cm triangle black plastic: from thin black plastic bag.
12. 4-0 silk suture.
13. 1 mm × 1 mm square Whatman filter paper.
14. 1.5 mL Eppendorf tubes.
15. 1 mL syringes with 26 G needles.
16. Balance.
17. Heating plate.
18. Binocular stereomicroscope equipped with a double-arm led illuminator.
19. Epifluorescence Microscope equipped with 10× dry objective.

3 Methods

Appropriate licensing and ethical permissions must be obtained from the researcher's local ethical committee for all animal procedures and experiments. Experiments using human samples should be approved by the researcher's institutional review board, and should be in accordance with the Helsinki Declaration.

Platelets easily become activated during preparation, and shape change, aggregation, and release of granule content may occur. The ideal preparation method should provide resting platelets that respond to ex vivo stimulation similarly as they would do in vivo. Platelets should also maintain their responsiveness to stimuli for few hours after blood collection to allow in vitro experiments. To prevent platelet activation during preparation, strong mechanical forces such as vigorous shaking, fast pipetting, use of needles with small gauge numbers (<21 G), or of vacuum blood collection system should be avoided.

3.1 Retro-orbital Mouse Blood Collection

This method allows the collection of large blood volumes (up to 1 mL) and therefore can only be used as a terminal procedure that should be performed by well-trained personnel. Cardiac puncture can also be used as a terminal procedure to allow the collection of 0.8–1 mL of blood. However, this method requires the use of a syringe/needle that may lead to the activation/desensitization of platelets. In contrast, the retro-orbital method allows for free-flow blood collection with lower risk of platelet disturbances.

1. Anesthetize mice by intraperitoneal (i.p.) injection of ketamine–xylazine mix as described in Subheading 2.1.
2. Prepare one Eppendorf tube (1.5 mL) for each mouse. Add 1 volume of ACD and 1 U/mL of apyrase as described in Subheading 2.2. Swirl the tubes to allow the anticoagulant mix to come into contact with tube walls.
3. Verify that the mouse is deeply anesthetized by pinching its footpad with forceps or fingertips. With your first finger and thumb, scruff the animal firmly enough to pull the skin around the eye (Fig. 1a).
4. Detach gently the eyeball from adjacent tissues with the help of the capillary (Fig. 1b).
5. Insert the glass capillary into the medial canthus of the eye under the nictitating membrane (Fig. 1c). Apply a rotation motion to the capillary to enter the slightly resistant sinus membrane (Fig. 1c). When the vein is broken, the blood enters the capillary and flows into the collection tube.
6. Stop collecting when the blood volume reaches 1 mL (*see* **Note 4**).
7. Mix immediately the blood with the anticoagulant with a gentle up and down movement (do no vortex).
8. Euthanize the mouse immediately after blood collection by cervical dislocation and make sure the animal is dead.

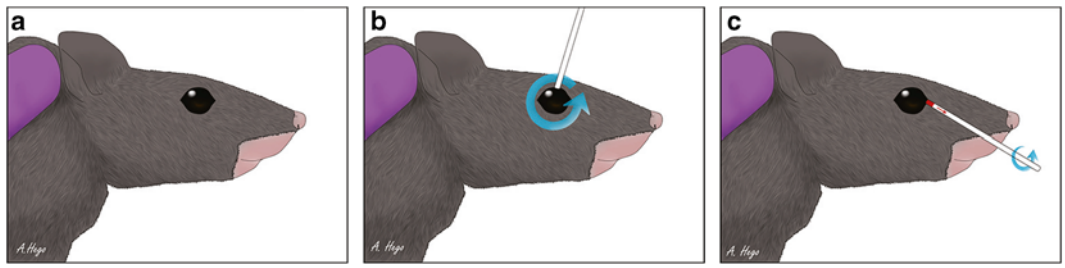

Fig. 1 Retro-orbital mouse blood collection. (**a**) With your index finger and thumb, scruff the animal firmly enough to pull the skin around the eye. (**b**) Detach gently the eyeball from adjacent tissues with the help of the capillary. (**c**) Insert the glass capillary into the medial canthus of the eye under the nictitating membrane and apply a rotation motion to the capillary to enter the sinus membrane

3.2 Isolation of Mouse Washed Platelets

1. Centrifuge the Eppendorf tube containing the collected mouse blood for 5 s at 800 × *g* followed by 5 min at 100 × *g* at room temperature.
2. Collect carefully the upper layer supernatant containing the platelet rich plasma (PRP) and transfer to a clean 15 mL conical tube. PRP from different mice of the same genotype can be pooled in one sample (*see* **Note 5**). Mix by gentle pipetting using a 1 mL tip.
3. To wash platelets, dilute the PRP three times in ACD containing 1 U/mL apyrase at room temperature.
4. Centrifuge at 800 × *g* 10 min at room temperature.
5. Discard supernatant and dry the 15 mL tube walls using Tork wiping paper.
6. Resuspend washed platelets pellet in 1 mL of 1× Tyrode's buffer containing 1 U/mL apyrase (room temperature). Mix gently by pipetting using 1 mL tips.
7. To count cells, take an aliquot of 20 μL of washed platelet suspension and add 180 μL of Tyrode's buffer. Mix gently by pipetting using a 1 mL tip and proceed with counting using a hematology analyzer. Set up the machine for mouse protocol.
8. Platelets should be resuspended in 1× Tyrode's buffer containing 1 U/mL apyrase to a concentration of 350×10^3 platelets/μL for aggregation and secretion experiments, and to 500×10^3 platelets/μL for immunoblot and immunoprecipitation (IP) experiments.

3.3 Mouse Platelet Aggregation Assay

Light transmission aggregometry is a widely used method to assess platelet responses to agonists, inhibitors, or after depletion of a specific protein [12]. Platelet aggregation can be monitored by measuring the transmission of light (expressed as percentage of light transmission) through a platelet suspension (PRP or washed platelets). Single platelets in suspension form a turbid solution that reduces the transmission of light. After addition of a platelet receptor agonist, platelets form aggregates that reduce the sample turbidity resulting in an increase of light transmission [13, 14]. Light transmission aggregometry is considered the 'gold standard' for testing platelet function and allows rapid collection of data for platelet responsiveness to a variety of platelet receptor agonists in small sample volumes [15]. Thus, we recommend this method as the first step to screen for platelet function defects in knockout animals. However, because this method is only semi-quantitative and performed under non-physiological conditions that do not fully mimic aggregate formation in vivo, it should be combined with additional experiments (as described below) to assess platelet function.

1. Aliquot 270 μL (350×10^3/μL mouse platelets) of washed platelets in 450 μL cuvettes containing one siliconized stir bar.
2. Let the platelets rest for 15 min at room temperature.
3. Meanwhile, set the aggregometer to optical mode at 37 °C and 1200 rpm.
4. Start the AGGRO/LINK8 program and select the test procedure within the Aggregometer window. Select the number of channels to be used (usually two or four, depending on the available equipment. We used the ChronoLog Lumi-Aggregometer).
5. Select "run patient" within the aggregometer window and indicate the test conditions when prompted.
6. Make sure to place the cuvette with 500 μL Tyrode's buffer in appropriate reference well.
7. Start the assay by clicking on "OK".
8. Press "set baselines" pushbutton for each test channel.
9. After baselines have stabilized, click on "Stop test" button and restart current test within the aggregometer window.
10. Using the 200 μL gel loading tips, add 2.7 μL of the 100 mM $CaCl_2$ stock solution to each cuvette.
11. About 30 s later, add the receptor agonist to be tested to each cuvette/sample. Start with the highest concentration agonist to be tested. For example, 0.5–1 μg/mL of collagen-related peptide (CRP) induces a full, irreversible aggregation of wild type (WT) mouse platelets 2 min after stimulation. Platelets from WT mice should always be used as a reference. Complete aggregation should be reached in the control conditions.
12. When a complete (80–100%) irreversible aggregation is achieved under control conditions, stop the assay and proceed with the next sample. If very low concentrations of platelet receptor agonist are used, aggregation may not be complete (usually lower than 50%). In these cases, the assay should be stopped when the aggregation is plateauing.
13. To calculate slope and amplitude, click on edit and set the start and stop time. Place the "start line" to where the agonist was added and the "stop line" to where the aggregation tracing was at full amplitude. Click on "done" and select "calculate result" within the Edit window. This command will allow calculation of the percentage of aggregation and the slope. Click the "OK" button to reveal the final data in the data box. This setting is specific to the ChronoLog Lumi-Aggregometer (ChronoLog Corporation).

3.4 Platelet Secretion Assay by Flow Cytometry

Flow cytometry is a quantitative, reliable, and sensitive method for the evaluation of platelet function. When activated, platelets undergo a shape change and release their granule content. As a result, several antigens are exposed on the platelet surface and can be utilized to discriminate between activated and resting platelets. P-selectin (also known as CD62P) resides within the alpha-granules and becomes exposed on the membrane surface after platelet degranulation [16]. It is probably the most widely used laboratory marker of platelet activation, either alone or in combination with other markers [17]. Monoclonal antibodies directed against P-selectin allow labeling and quantification of degranulated platelets. Additionally, during platelet activation, integrin $\alpha_{IIb}\beta_3$ (also known as GPIIb/IIIa) undergoes a conformational change by an inside-out activation phenomenon. The active form can be recognized using specific antibodies such as JON/A for mice and PAC-1 for humans [18]. Moreover, the conformational change allows fibrinogen to bind to its integrin receptor, and FITC-conjugated fibrinogen can be used to detect platelet activation.

Platelet secretion can be analyzed on washed platelets, PRP, or whole blood. We describe two methods using either washed platelets or whole blood. Specifically, we provide experimental details for the analysis of $\alpha_{IIb}\beta_3$ integrin activation and P-selectin exposure on CRP-activated mouse platelets. Similar methods can be used with other platelet agonists.

3.4.1 Mouse Platelet Secretion Assay in Whole Blood

Platelet secretion in whole blood is fast, requires a very small volume of blood, and does not require platelet isolation, thereby limiting the steps that may lead to accidental platelet activation. However, this method requires the use of higher concentrations of platelet receptor agonists as compared to washed platelet suspensions. It is most useful when the amount of available blood is a limiting factor.

1. Prepare three aliquots (25 μL each) of citrate-anticoagulated mouse blood in three tubes (1 volume of 3.8% citrate buffer for 9 volumes of mouse blood). Label the tubes as follows: no stimulation (NS), 1 and 3 μg/mL CRP.
2. To tube NS, add of 0.9% NaCl or Tyrode's buffer; to the other tubes add 1 or 3 μg/mL of CRP (final concentration), respectively.
3. Incubate for 15 min at room temperature.
4. Add 2.5 μL of FITC-CD62P and PE-JON/A antibodies. Mix gently and incubate for 15 min in the dark at room temperature.
5. Add 1 mL of 1× BD FACS lysing solution and incubate for 30 min.
6. Analyze cells by flow cytometry within 48 h.

3.4.2 Mouse Platelet Secretion Assay in Washed Platelets

1. Prepare five aliquots of 50 μL each (350×10^3/μL platelets) in five tubes. Label the tubes as follow: NS; 0.1, 0.3, 0.5, and 1 μg/mL CRP.
2. To tube NS, add of 0.9% NaCl or Tyrode's buffer, and to the other tubes add 0.1, 0.3, 0.5, and 1 μg/mL CRP (final concentrations).
3. Incubate for 15 min at RT.
4. Add 2.5 μL of FITC-CD62P and PE-JON/A antibodies. Mix gently and incubate for 15 min in the dark at room temperature.
5. Analyze cells by flow cytometry immediately. Otherwise, fix stained cells by adding 1% PFA and perform flow cytometry analysis within 48 h. In our experience, results do not significantly differ between non-fixed and PFA-fixed platelets.
6. Flow cytometry analysis: Use the logarithmic scale to visualize platelets in forward-scatter (FSC) and side-scatter (SSC) axis and gate on platelets (P1) as shown in Fig. 2a. On P1, analyze the expression of JON/A and CD62P under resting and CRP-activated conditions. Resting platelets should not result in any staining. An example is given in Fig. 2b. If the platelets are even slightly positive for Anti-JON/A and/or anti-CD62P, the sample should be discarded as this indicates that platelets were activated during the preparation procedure. An example is shown in Fig. 2c. Figure 2d shows an example of CRP-activated platelets.

3.5 Platelet Activation, Lysis, IP, and Immunoblot

Light transmission aggregometry and the platelet secretion assay by flow cytometry are powerful methods to rapidly and directly assess platelet responses to different receptor agonists. They allow the identification of receptor signaling pathways affected by a specific protein deletion or inhibition. After the identification of such a pathway, immunoblot assays and IP of target proteins are the methods of choice to dissect signaling defects and perhaps identify the direct targets of the PTP of interest. For instance, the tyrosine phosphorylation pattern of CRP-activated platelets is well known in the platelet signaling field. An example of an anti-phosphotyrosine immunoblot on CRP-activated WT and DUSP3$^{-/-}$ mouse platelets is shown in Fig. 3a. The major tyrosine phosphorylated bands are located between 40 and 80 kDa molecular weights. Because CRP activation of platelets leads to tyrosine phosphorylation of the Src family kinases (SFKs, 50–55 kDa) and the spleen tyrosine kinase (Syk, 70 kDa), anti-phosphotyrosine immunoblots can be very informative. Next, if phosphospecific antibodies are available (as is the case for most SFKs and Syk), immunoblot assays on total lysates can be performed. Otherwise, IP of the target protein followed by anti-phosphotyrosine immunoblot is required. As before, details

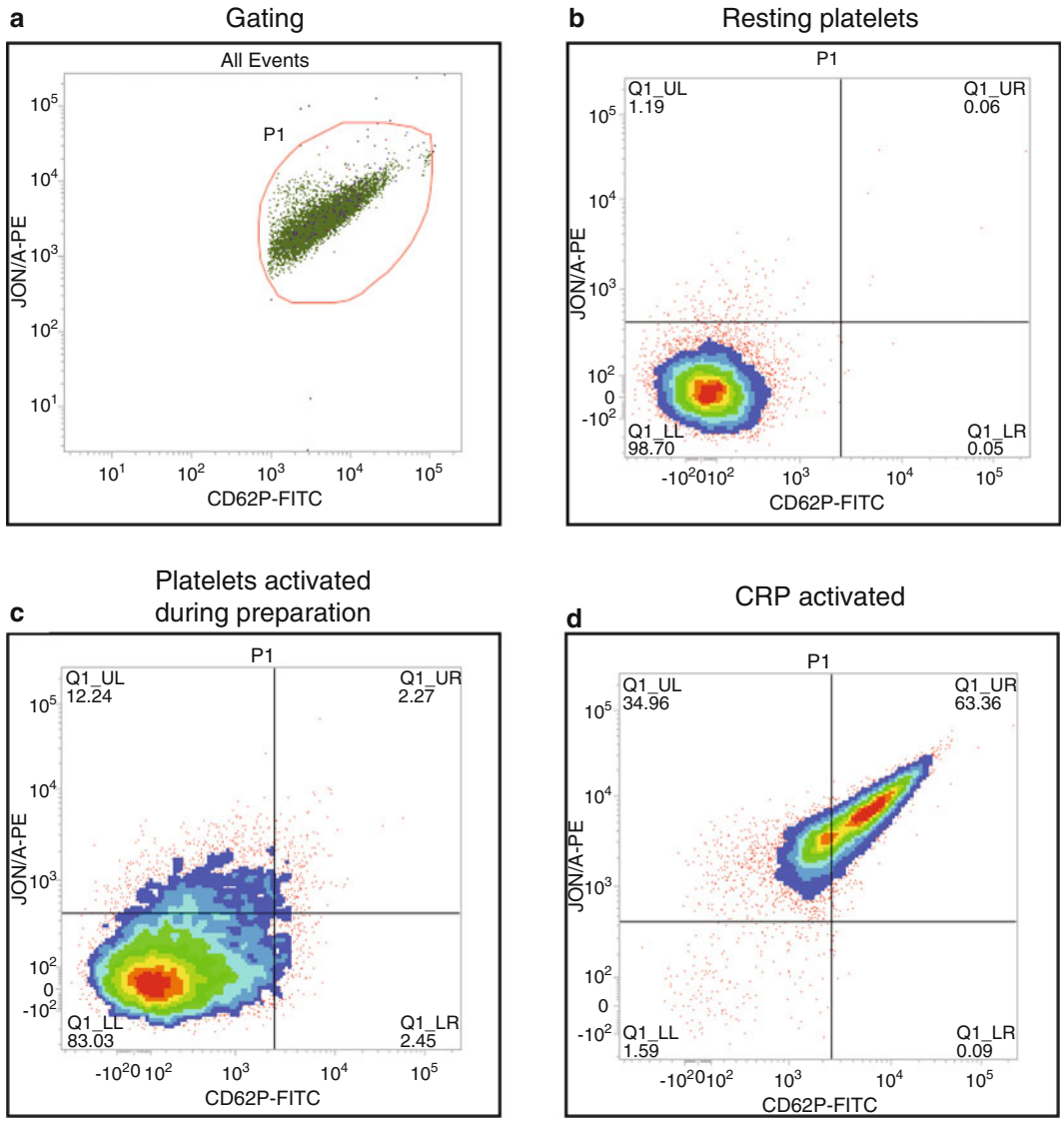

Fig. 2 Gating strategy to analyze platelet secretion by flow cytometry. (**a**) FSC and SSC axis (log scale) and gate on platelets (P1). (**b**) An example of resting platelets based on the analysis of JON/A and CD62P on P1 gated platelet population. (**c**) An example of platelets activated during preparation based on the analysis of JON/A and CD62P on P1 gate. (**d**) An example of CRP-activated platelets based on the expression of JON/A and CD62P

are provided for experiments using CRP to activate platelets through the GPVI receptor. However, the protocol can be easily adapted for experiments using other platelet receptor agonists.

3.5.1 Platelet Activation and Lysis

1. Adjust the concentration of washed platelets (WPs) (Subheading 3.2) to 500×10^3 platelets/μL in Tyrode's buffer without BSA. From the blood of two mice, up to 1 mL WPs can be obtained, which is enough to perform four IPs.

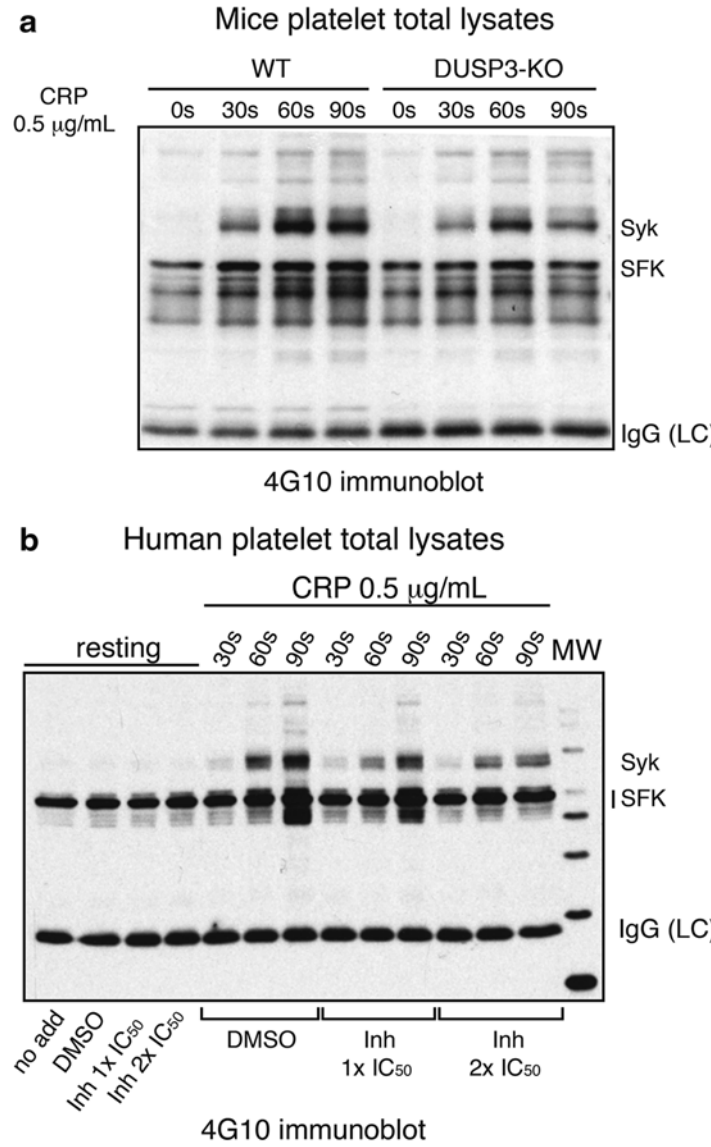

Fig. 3 Examples of an anti-phosphotyrosine immunoblot assay on total lysates from mouse and human platelets. (**a**) Lysates were prepared from non-activated or CRP (0.3 μg/mL)-activated WT and DUSP3-KO washed platelets for the indicated periods of time. Western blot was performed using anti-phosphotyrosine (4G10) antibodies. (**b**) Lysates were prepared from vehicle (DMSO) or DUSP3 inhibitor (MLS-0437605) pretreated washed human platelets. Cells were non-activated or CRP-activated (0.3 μg/mL) for the indicated periods of time, lysed, and immunoreacted using anti-phosphotyrosine antibody

2. Aliquot 270 μL WPs in a 450 μL cuvettes containing one siliconized stir bar. One cuvette of pooled WPs from each mice genotype should be kept with no stirring (as a control for resting conditions).

3. Perform pipetting carefully as rapid flows may activate platelets.
4. Let the platelets rest at room temperature for 25 min.
5. Meanwhile, set the aggregometer on optical mode at 37 °C and 400 rpm.
6. Prepare highly concentrated CRP working solutions in Tyrode's buffer for each CRP concentration to be tested. This will ensure the addition of equal volume of CRP in all cuvettes (2 μL of CRP working solution to 270 μL WPs). Keep working solutions on ice until needed. Equilibrate at room temperature for 5 min before use.
7. To analyze the tyrosine phosphorylation profile of platelets on western blots, titration and time course activation with CRP (or other platelet receptor agonists) should be performed.
8. Place the cuvettes in aggregometer set at 37 °C with stirring at 400 rpm and equilibrate WPs for 30 s.
9. Add 2 μL room temperature concentrated CRP solution using the long gel loading tips and stimulate for different time points (e.g., 30, 60, 90 s)
10. Stop platelet activation by adding an equal volume of 2× lysis buffer containing 2× concentrated protease and phosphatase inhibitors.
11. Transfer the cuvettes immediately on ice and transfer lysates into 1.5 mL Eppendorf tubes using long tips (Keep samples on ice or at 4 °C to preserve phosphorylated sites)
12. Lyse for 30 min on rotator at 4 °C (in cold room).
13. Centrifuge lysates at 7500 × *g* at 4 °C and transfer supernatants into fresh 1.5 mL Eppendorf tubes.
14. Determine the protein concentration of each sample using the Bradford assay in a 96-well plate.
15. Prepare samples by adding equal volumes of 2× SDS Sample Buffer (e.g., 50 μL lysate + 50 μL 2× SDS sample buffer).
16. Boil at 95 °C for 5 min to denature proteins. Use Eppendorf tubes that close tightly to avoid loss of samples by evaporation. Freeze the rest of lysates at −80 °C or proceed immediately with IP.
17. Load denatured samples directly on 4–20% Tris–Glycine gradient acrylamide gels for western blot assay or freeze at −20 or −80 °C for longer storage.
18. Perform western blot as previously described [19].

When performing immunoblot assays using anti-phosphotyrosine antibodies (4G10) on activated vs. non-activated platelets, a gradual increase (over time of stimulation) of

tyrosine-phosphorylated bands should be observed in activated platelets, especially at molecular weights between 45 and 80 kDa (Fig. 3a). The band corresponding to Syk (70 kDa) is of particular interest. Under non-activated conditions, no tyrosine-phosphorylated band should be observed at 70 kDa. When activated, Syk gets rapidly (30 s) phosphorylated on tyrosine and becomes visible on the total lysate blot. If Syk appears phosphorylated under resting conditions, WPs were likely activated during preparation, and results should be discarded (*see* also **Note 6**). The identity of tyrosine-phosphorylated proteins should be confirmed using phosphotyrosine-specific antibodies if available, or by IP of the candidate protein, followed by immunoblot assays using anti-phosphotyrosine antibodies.

3.5.2 Immunoprecipitation

1. Thaw lysates from non-activated and activated WPs on ice for 20 min.
2. Meanwhile, prepare Protein A-Sepharose (or Protein G) beads for preclearing lysates from platelet-bound murine IgGs. Use 20 µL slurry per sample and wash beads with 1 mL ice cold PBS three times by centrifuging beads at 800 × *g* for 1 min at 4 °C.
3. After centrifugation, let the beads rest for 2 min on ice before discarding supernatant.
4. Resuspend washed beads in 1 mL PBS and aliquot them in clean prelabeled 1.5 mL Eppendorf tubes for as many samples as needed. Centrifuge again at 800 × *g* for 1 min at 4 °C to eliminate extra PBS.
5. Add thawed lysates into the beads-containing tubes and incubate for 30 min at 4 °C with rotation.
6. Meanwhile, add 2 µg of selected antibody to a clean Eppendorf prelabeled tube and keep on ice.
7. As a negative control, perform an additional IP with rabbit-IgG or mouse-IgG, depending on the origin of the used antibody.

   For such a control, pooled lysates from WT and PTP-KO mouse WPs can be used to limit the number of mice needed for the experiment. When using human platelets, one negative IP for each donor should be performed.
8. Centrifuge the tubes containing WPs lysates and beads at 800 × *g* for 1 min at 4 °C and transfer precleared supernatants to the new tubes containing the antibody. Discard beads used for preclearing.
9. Incubate for 1 h at 4 °C with rotation to allow binding of the antibody to the target protein.
10. During this time, prepare protein A-Sepharose (or protein G) beads for all samples.
11. 40 µL beads slurry are needed per sample (40 µL × n (number of samples) = total volume of beads slurry to be prepared).

12. Add 1 mL of ice-cold-PBS to the required volume of beads slurry and mix by pipetting up and down.
13. Centrifuge for 1 min at 800 × *g* and carefully discard supernatant. Repeat the washing procedure two more times.
14. For the last wash, split the mixture of PBS/beads in as many clean 1.5 mL Eppendorf tubes as samples to be tested, plus one tube for the negative IP control.
15. Centrifuge at 4 °C for 1 min at 800 × *g* and carefully discard supernatants. Keep the beads on ice at all times.
16. Centrifuge the lysate/antibody mixtures at 5000 × *g* for 30 s and transfer them to the corresponding beads-containing tubes.
17. Incubate overnight at 4 °C with rotation (cold room).
18. Centrifuge samples at 4 °C for 1 min at 800 × *g*.
19. Save the flow through (FT) at 4 °C and proceed with beads.
20. Wash beads three times with 1 mL of ice-cold PBS or with 1× WPs lysis buffer.
21. After the last wash, aspirate the supernatant carefully without disturbing the beads. Add 30 μL of 2× SDS loading buffer to the beads, vortex, and boil at 95 °C for 5 min.
22. Vortex again and centrifuge at 15,000 × *g* for 1 min.
23. Load 10 μL of supernatants on acrylamide gel and proceed for western blotting as described in [19].

3.6 In Vivo Mouse Thrombosis Models

3.6.1 Ferric Chloride ($FeCl_3$) Carotid Injury Model

Intravital video-microscopy is a widely used technology to directly visualize, in real time, the initiation and progression of thrombus formation in live small animals. Importantly, this technique allows the investigation of thrombotic and hemostatic processes under physiological conditions. Thrombus formation in mice can be induced by different methods such as mechanical destruction of the endothelium, systemic infusion of Rose Bengal (a photoreactive chemical) followed by low-power laser excitation, activation of the endothelial cells by a high power nitrogen laser, or by chemical injury using topical application of $FeCl_3$ solution [5, 20]. Here, we provide a detailed protocol for the $FeCl_3$ injury model, which is a simple and well-established model known to be sensitive to both anticoagulants as well as antiplatelet drugs [5, 20].

$FeCl_3$ is an oxidative agent that can penetrate through the vessel, leading to the denudation of the endothelium and subendothelial matrix exposition. The severity of the damage induced depends on the $FeCl_3$ concentration used and the duration of the application [21]. $FeCl_3$ can be applied on small vessels like cremaster and mesenteric arterioles or on larger vessels like carotid artery [21]. In order to visualize and quantitate thrombus formation, platelets are labeled using CFSE and injected through the jugular vein before thrombus initiation. Quantification can be performed

by monitoring the time until full occlusion of the blood vessel or by comparing the fluorescence intensity between different conditions.

Staining of Washed Platelet with CFSE

1. Prepare PRP from one mouse as described in Subheading 3.2.
2. Transfer PRP (200 µL of 200×10^3/platelets per µL) to a 15 mL conical tube and add 3 mL of Tyrode's buffer and 200 µL of ACD containing Apyrase.
3. Add 15 µL of CFSE at 1 µg/µL and incubate 2 min at room temperature protected from light.
4. Centrifuge at $950 \times g$ for 10 min at room temperature.
5. Resuspend the pellets gently in 200 µL PBS and keep at room temperature protected from light (*see* **Note** 7).

Preparation of Material and Mouse Surgery

1. Cut 10 cm of an Intramedic™ Polyethylene catheter tube. Cut one end of the tube diagonally (preferably using a binocular loupe) in order to ease insertion of the tube into the blood vessel.
2. Few millimeters away from the diagonal cut, make three to four very slight diagonal incisions to create a kind of "self-retaining harpoon catheter". This will help maintaining the catheter inside the blood vessel.
3. Fill an insulin syringe with sterile 0.9% NaCl avoiding bubbles and insert it to the non-diagonally cut end of the catheter.
4. Using a binocular loupe, make sure that the liquid (NaCl) does not leak through the diagonal incisions/winglets.
5. Weigh the mice and inject i.p. the appropriate volume of the ketamine–xylazine mix. Overdosing may occur if animals are not properly weighed.
6. Following deep anesthesia, place the mouse in dorsal recumbency on a heating plate set to 37 °C.
7. Secure the front legs on the plate using rubber bands. Ensure that the mouse is positioned in a comfortable position to optimize breathing.
8. Gently scrub the surgical area with 70% ethanol solution.
9. Perform a midline skin incision in the mouse neck using a surgical scissor as indicated in Fig. 4, steps A and B.

Fig. 4 (continued) catheter tubing to the jugular vein with an additional suture on the cephalic end of the jugular vein. (**J**) Separate gently the sternomastoid and the sternohyoid to visualize the vagus nerve and the carotid artery. Place a black plastic triangle under the carotid artery vessel. (**K**) Place a $FeCl_3$-soaked filter on the carotid artery for 5 min. (*a*) submaxillary gland. (*b*) platysma. (*c*) jugular vein. (*d*) sternohyoid muscle. (*e*) sternomastoid muscle. (*f*) fat on the jugular vein. (*g*) jugular catheter with harpoon-like structure. (*h*) vagus nerve. (*i*) carotid artery. (*j*) black plastic triangle. (*k*) $FeCl_3$-soaked filter (site of injury)

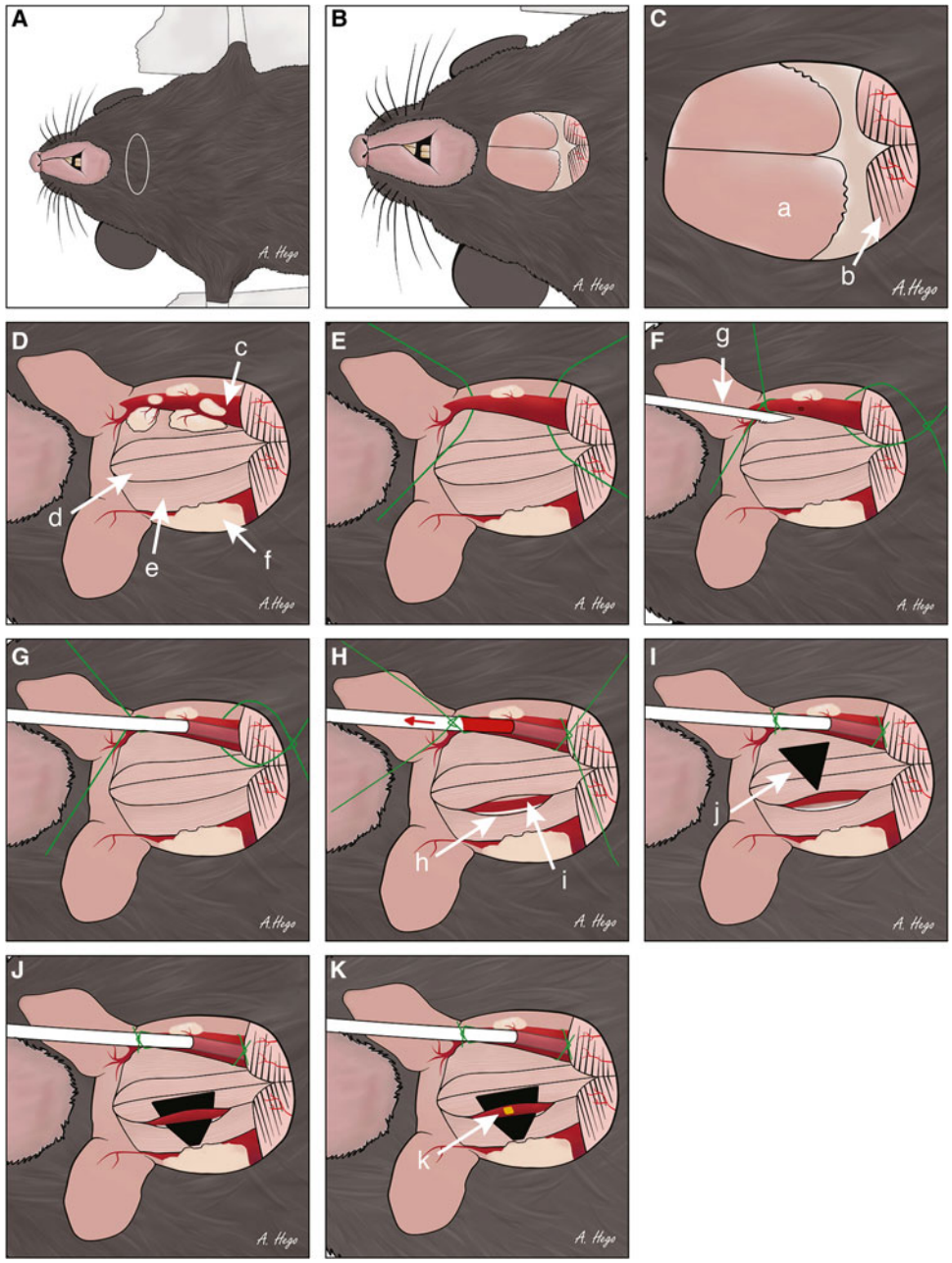

Fig. 4 Ferric chloride ($FeCl_3$) carotid injury model, surgical procedure. (**A**) Secure the front legs on the plate using rubber bands. (**B** and **C**) Perform a midline skin incision in the mouse neck and expose the submaxillary glands. (**D**) Separate the submaxillary glands and the muscle and place them near the mouse's face. (**E**) Isolate carefully the jugular vein on both sides and underneath for a distance of 1–1.5 cm and place a loose tie on both cranial and caudal ends of the vessel using suture silk. (**F**) Tie the cranial ligature around the vessel and make a very small incision in line with the vessel between the two ligatures. (**G**) Insert the free diagonally cut end of the catheter into the vessel incision toward the heart and push carefully the catheter until the entire cut end is inside the vessel. (**H**) Tie up the ligature at the caudal end to secure the catheter into the vessel and verify leaking by injecting 0.9 % NaCl in the jugular vein and aspirate blood. (**I**) Secure the position of the

10. Expose the submaxillary glands (Fig. 4, step C).
11. Gently separate the submaxillary glands and the muscle using surgical forceps and place them near the mouse's face (Fig. 4, step D).
12. The jugular veins and the sternohyoid muscle are now exposed.
13. Isolate carefully the jugular vein on both sides and underneath (with the help of the forceps) for a distance of 1–1.5 cm. Do not touch directly the jugular vein with the end of the forceps since this could damage the vein. Both left and right veins can be used.
14. Place a loose tie on both cranial and caudal ends of the vessel using suture silk (Fig. 4, step E). This will maximize the exposure of the vessel.
15. Tie the cranial ligature around the vessel (Fig. 4, step F).
16. Make a very small incision using microsurgical scissors in line with the vessel between the two ligatures (Fig. 4, step F). No blood should flow out of the vessel. If hemorrhage occurs, it may be controlled by gently pulling the cranial ligature end.
17. Insert the free, diagonally cut end of the catheter tubing into the vessel incision toward the heart with the help of the forceps (Fig. 4, step G). Push carefully the catheter until the entire end is inside the vessel.
18. Use the ligature at the caudal end to secure the catheter into the vessel. Verify licking by injecting 0.9% NaCl in the jugular vein and aspirate blood (Fig. 4, step H) (*see* **Note 8**).
19. Secure further the position of the catheter tubing to the jugular vein with additional suture on the cephalic end of the jugular vein (Fig. 4, step I).
20. Gently separate the sternomastoid and the sternohyoid muscles using the forceps; the carotid artery is located underneath (Fig. 4, step H). The vagus nerve, white and easily visible, resides also near the carotid artery.
21. Carefully separate the vagus nerve from the carotid artery by opening and closing the forceps placed between the nerve and artery.
22. Place the black plastic triangle under the vessel as shown in Fig. 4, steps I and J. The black plastic triangle will elevate the vessel and prevent autofluorescence from the adjacent tissue.
23. Prepare an insulin syringe with 120 μL of the CFSE loaded platelets.
24. Inject i.v. 100 μL of stained platelets through the jugular vein catheter.
25. Incubate a 2 × 2 mm square of Whatman paper in 10% $FeCl_3$ buffer.

26. Dry completely the carotid artery using a Sugi® sponge. Dilution of the $FeCl_3$ in residual liquid can induce significant variations between experiments.
27. Basal level fluorescence (488 nm band pass filter) in the vessels should be acquired before adding $FeCl_3$.
28. Take at least 50 snapshots using the Slidebook software (or equivalent) following the instructions of the manufacturer. Heart beating of the artery may influence the quality of images (mainly blurring).
29. Put the $FeCl_3$-soaked filter on the carotid artery and leave it for 5 min (Fig. 4, step K).
30. Remove the $FeCl_3$-soaked filter and clean the carotid artery using a pipette filled with 1× PBS.
31. Dry completely the vessel using the Sugi® sponge in order to prevent diffraction during image acquisition.
32. Start recording the thrombus formation with a 488 nm band pass filter. Take 50 snapshots every 2 min for 30 min or up to total occlusion of the blood vessel.
33. Stop the recording and euthanize the mouse either by injecting 50 µL of Nembutal or by cervical dislocation.
34. Analyze of the acquired images. Slidebook software, for example, can determine and compare the mean fluorescence intensities (MFI) between basal conditions and after $FeCl_3$-induced injury.

3.6.2 Collagen and Epinephrine-Induced Pulmonary Embolism Model

Collagen and epinephrine-induced pulmonary embolism is a very simple and reliable in vivo model to study the effectiveness of antithrombotic drugs or to investigate the role of a signaling molecule in thrombosis. The method was described for the first time by DiMinno and Silver in 1983 [22]. In this model, thromboembolism in mice is induced by injecting a combination of a platelet-aggregating agent such as collagen together with epinephrine. The time to death and the rate of survival is recorded, and the number and size of thrombi in lung sections can be analyzed. The protocol provided here is specific to C57BL/6 male mice. The dose of collagen and epinephrine should be adjusted if other mouse genetic backgrounds or female mice are used.

1. Weigh and label 8–12 weeks old male mice (WT and target PTP-KO, ten mice in each group). Labeling can be performed on tails using permanent marker.
2. Anesthetize the mice with an i.p. injection of ketamine–xylazine mix as described in Subheading 2.1.
3. Place the mice in lateral recumbency on the heating plate set at 37 °C.

4. While waiting for the mice to sleep, label Eppendorf tubes and prepare the mixture of epinephrine (170 μg/kg) and collagen (60 μg/kg) for every mouse (one Eppendorf tube per mouse).
5. Load 1 mL syringes/30 G needles with the appropriate volume of collagen/epinephrine mix for each mouse.
6. When mice are deeply anesthetized, inject each mouse into the plexus retro-orbital vein with the appropriate volume of collagen/epinephrine mix. Plexus retro-orbital vein injection can be achieved by inserting the needle into the medial canthus of the eye under the nictitating membrane (Fig. 2c).
7. Mice need to be kept on the heating plate during the entire procedure.
8. Monitor mice for vital signs such as breathing and heart beating. Under the conditions described here, time to death in WT mice was recorded between 10 and 20 min. The animals are considered as dead when they stop breathing/heart beating.
9. Animals that do not die after 40 min should be sacrificed by cervical dislocation.
10. Perfuse the lungs with 4% formaldehyde and collect them for histological analysis.
11. Results obtained can be presented as the percentage of survival and time to death. It is also possible to analyze the number and size of thrombi in lung sections from different groups (WT and target PTP-KO) using a simple hematoxylin eosin staining method.

3.7 Pharmacological Inhibition of Platelet PTPs

Platelets are anucleate cells that are not amenable to RNA interference or recombinant DNA technologies. Thus, chemical probes to pharmacologically inhibit (or activate) the function of a specific PTP (or other signaling molecule) in human platelets are invaluable tools to corroborate findings that originate from animal models in human cells.

3.7.1 General Considerations

All PTPs share a common catalytic mechanism based on a nucleophilic cysteine that is part of the active-site signature motif C(X)5R [23]. Sodium orthovanadate, which binds to the active site and functions as transition state analog, is a standard reagent to inhibit PTP activity in cells including platelets [24]. More potent general PTP inhibitors with proven activity in cells include peroxovanadium compounds such as potassium bisperoxo(1,10-phenanthroline) oxovanadate [bpV(phen)] [25]. The number of reported specific PTP inhibitors with selectivity for a particular target and efficacy in cells is very limited. This is due to the fact that the vast majority of small-molecule PTP inhibitors target the highly conserved active site, and thus lack sufficient selectivity for the PTP of interest. Nonetheless, several promising compounds have been

reported for PTPs known to be important for platelet signaling including PTP1B and SHP2 [8]. However, there is a paucity of data demonstrating efficacy of these compounds in platelets. Moreover, we found that our previously identified DUSP3 inhibitors, which showed excellent specificity and efficacy in HeLa cells [26], caused spontaneous aggregation of human platelets [10]. Thus, there appears to be an even more rigorous requirement for compound specificity when working with platelets. Several new strategies to generate PTP inhibitors with increased specificity are currently being investigated. For further reading on this topic we refer to our recently published review articles [8, 23].

3.7.2 *Inhibition of DUSP3 in Human Platelets*

Using a chemical genomics approach, we developed a novel inhibitor of DUSP3 (MLS-0437605; Fig. 5) [10]. MLS-0437605 specifically inhibited human platelet aggregation in response to stimulation of the GPVI and CLEC-2 receptors, analogous to the effect of DUSP3 deficiency in murine cells. Tests on platelets from WT mice yielded similar results, while MLS-0437605 only minimally affected aggregation of DUSP3-deficient platelets [10]. Inhibition of DUSP3 by MLS-0437605 in human platelets reduced tyrosine phosphorylation of immunoprecipitated Syk and PLCγ2 in response to GPVI and CLEC-2 stimulation, while global tyrosine phosphorylation was not affected. These results demonstrated that pharmacological inhibition of DUSP3 in human platelets phenocopies the effect of DUSP3 deficiency in murine platelets. Here, we provide detailed protocols for testing the effects of small-molecule inhibitors on human platelet activation and aggregation. Methods related to the development of specific PTP inhibitors

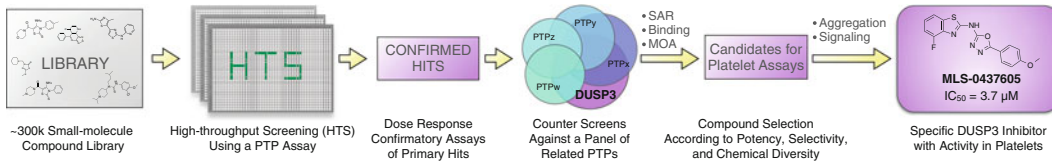

Fig. 5 Development of a DUSP3-specific inhibitor. High-throughput screening (HTS) of a drug-like compound collection was used in the search for novel DUSP3 inhibitors. Primary hits, i.e., compounds that inhibited DUSP3 activity by at least 50 %, were taken into dose response hit confirmation assays using two orthogonal assay formats. Cross-active hits with IC_{50} values <20 μM in both assays were subjected to counter screens against four additional PTPs. Based on potency and selectivity, several hits were selected for structure–activity relationship (SAR), binding, and mechanism-of-action (MOA) studies, resulting in several candidate compounds for tests on human platelets. These tests included platelet aggregation assays as well as biochemical assays assessing the tyrosine-phosphorylation levels of various signaling proteins involved in DUSP3-regulated pathways. Compound MLS-0437605 effectively inhibited mouse and human platelet aggregation, specifically in response to GPVI and CLEC-2 platelet stimulation, thereby phenocopying the effect of DUSP3 deficiency on mouse platelets. In contrast, aggregation of DUSP3-deficient platelets was only marginally affected by MLS-0437605. MLS-0437605 also decreased tyrosine phosphorylation of Syk and PLCγ2 in human platelets in response to GPVI and CLEC-2 stimulation, similar to the effect of DUSP3 deficiency on mouse platelets. (*see* ref. 10 to review the actual data.)

have been previously described [27, 28]. The major steps for the identification of the DUSP3 inhibitor MLS-0437605 are summarized in Fig. 5.

3.7.3 Human Blood Collection

For blood collection, make sure to not include donors that have taken aspirin or any other antiplatelet medication during the 15 days prior to blood donation.

1. Collect blood from a forearm vein using an 18 G needle (*see* **Note 9**). Blood collected should be free-flowing.
2. Discard the first 5 mL of blood. (The first 5 mL of blood should be discarded because it contains activated platelets that have been in contact with collagen released from the wound.)
3. Use a conical tube containing ACD and 1 U/mL of apyrase grade I (1 volume ACD + 1 U/mL of apyrase for 6 volumes of blood) to collect ~30 mL of blood.
4. Immediately after collection, close the tube and mix the blood gently with the anticoagulant.
5. Platelet assays should be performed within 3 h after blood collection.

3.7.4 Isolation of Human Platelets

1. Centrifuge blood for 15 min at 100 × *g* at room temperature to obtain PRP.
2. Carefully transfer PRP into a new 15 mL conical tube.
3. Wash platelets by adding 2 volumes of ACD containing 1 U/mL of apyrase grade I at RT and centrifuge at 800 × *g* 10 min and room temperature.
4. Proceed as described in Subheading 3.2, **steps 5–8**. Select human protocol for counting platelets when using the hematology analyzer.
5. Resuspend washed platelet pellets in Tyrode's buffer containing 1 U/mL apyrase to a concentration of 250×10^3 platelets/μL for aggregation and secretion experiments, and to 500×10^3 platelets/μL for immunoblot and IP experiments.

3.7.5 Effects of Small-Molecule Inhibitors on Human Platelet Aggregation

1. Prepare 20 mM inhibitor stock solutions in DMSO, which will allow testing of inhibitors in platelets at up to 40 μM final concentration at the nontoxic concentration of 0.2% DMSO (*see* **Note 10**). Store stock solutions as small aliquots as needed at −20 or −80 °C and avoid repeated thawing and freezing. For inhibitor treatment, platelets are resuspended in Tyrode's buffer with glucose and BSA.
2. Prepare 500× concentrated inhibitor working solutions in DMSO for each inhibitor concentration to be tested. This will ensure equal amounts of DMSO in inhibitor dose–response assays. (A 500× working solution results in 0.2% DMSO final

concentration.) For dose–response studies prepare 1/3-log steps serial dilutions (highest final concentration 5- or 10-times inhibitor IC_{50} value, but not exceeding 40 μM). We recommend four to five inhibitor dilutions (e.g., 30–10–3–1–0.3–0 μM final inhibitor concentration), so that the lowest final concentration is well below the inhibitor IC_{50} value.

3. Prepare human WPs as described in Subheading 3.7.4.
4. Aliquot 270 μL (250×10^3 human platelets/μL) of WPs in 450 μL cuvettes containing one siliconized stir bar.
5. Let the platelets rest for 25 min at room temperature.
6. Make sure the inhibitor does not spontaneously cause platelet aggregation (*see* **Note 11**).
7. Add DMSO or inhibitor working solution to WPs and incubate at room temperature for 30 min (to allow inhibitor uptake). Since the ChronoLog aggregometer can analyze only two cuvettes in parallel, prepare only two cuvettes at a time and allow an interval of at least 10 min between additional inhibitor/DMSO incubations. Some aggregometers allow the analysis of four cuvettes at a time. Adjust the number of cuvettes depending on the available equipment.
8. Proceed with the aggregation assay as described in Subheading 3.3. Determine the percentage of aggregation inhibition by comparing to the vehicle (DMSO) control. Human platelet responses to receptor agonists are highly variable between individuals. We recommend determining the optimal concentration of a platelet receptor agonist (e.g., CRP) for every donor before proceeding with the inhibitor dose–response aggregation assay.
9. Repeat the aggregation assays with more fine-tuned, narrower range of inhibitor concentrations. The goal is to find the lowest concentration of inhibitor that yields maximal inhibition of platelet aggregation. MLS-0437605 inhibited CRP- and rhodocytin-induced platelet aggregation at a concentration of $1\times$ IC_{50}.

3.7.6 Effects of Small-Molecule Inhibitors on Human Platelet Secretion

Platelet secretion analysis using flow cytometry is a quantitative and highly sensitive method for the evaluation of platelet function. This method allows for further characterization of the effects of selected inhibitors. Since secretion can be performed on small aliquots of WPs, several conditions can be tested simultaneously for the same donor. Thus, dose response data for both the inhibitor and the agonist can be obtained. Typically, for WPs from a particular donor, the highest concentration of platelet receptor agonist used in the secretion experiment corresponds to the lowest concentration of the same agonist used in the platelet aggregation assay (Subheading 3.7.5). We here describe a method analyzing

JON/A and p-Selectin exposure on CRP-activated human washed platelets using three CRP and two inhibitor concentrations plus vehicle control. The same method can be used with other platelet receptor agonists.

1. Prepare 12 aliquots of 100 µL (250×10^3/µL) each of washed human platelet. Label the tubes as shown in the table:

NS—DMSO	0.1 µg/mL CRP DMSO	0.3 µg/mL CRP DMSO	0.5 µg/mL CRP DMSO
NS—inhibitor conc. 1	0.1 µg/mL CRP inhibitor conc. 1	0.3 µg/mL CRP inhibitor conc. 1	0.5 µg/mL CRP inhibitor conc. 1
NS—inhibitor conc. 2	0.1 µg/mL CRP inhibitor conc. 2	0.3 µg/mL CRP inhibitor conc. 2	0.5 µg/mL CRP inhibitor conc. 2

2. Add the appropriate amounts of DMSO or inhibitor working solution to the tubes. Mix gently by pipetting twice up and down using 1 mL pipette tips and incubate at room temperature for 30 min.
3. Add CRP at the indicated concentrations and mix gently. Add the equivalent volume of 0.9% NaCl or Tyrode's buffer to the NS samples. Incubate for 15 min at room temperature.
4. Add 10 µL of FITC-conjugated anti-human CD62P and 10 µL of PE-PAC1 antibodies. Mix gently and incubate for 15 min in the dark at room temperature.
5. Fix cells using 1 mL of 1% PFA. Proceed with flow cytometry analysis within 48 h (*see* **Note 12**).

3.7.7 Effects of Small-Molecule Inhibitors on Human Platelets Using Immunoblot Assays

The aggregation assay is a relatively simple method that can be used as screening assay to prioritize compounds. However, aggregation assays only measure the final effect of a compound on platelet activation. More direct effects of PTP inhibitors can be evaluated by determining the overall and specific change in tyrosine phosphorylation using immunoblot analyses with anti-phosphotyrosine (4G10) or phospho-specific antibodies on total lysates or IPs from activated vs. non-activated platelets. A specific PTP inhibitor is not expected to increase global tyrosine phosphorylation, but should phenocopy the effect of the PTP deficiency in mouse platelets. If the inhibitor induces a dramatic change in global tyrosine phosphorylation, the compound is likely acting unspecifically. An example of a phosphotyrosine immunoblot using the specific DUSP3 inhibitor MLS-0437605 on human platelets is shown in Fig. 3b. Further, if the direct substrate(s) of the target PTP is known, and phospho-specific antibodies for this/these substrate(s) are available, immunoblot assays probing directly the phosphorylation site of the substrate(s) can be performed on total lysates. If phospho-specific antibodies are not available, IP of the substrate protein using a

pan-antibody, followed by an anti-phosphotyrosine (4G10) western blot can be performed. For dose–response experiments with inhibitors, platelets are prepared and treated similarly as described above for aggregation assays. However, platelet activation should be performed at a lower stirring speed of 400 rpm. This is because stirring speeds higher than 400 rpm lead to the formation of tight platelet aggregates and highly variable lysis efficiency. We recommend repeating the dose–response aggregation assay (as described in the previous section) for the selected inhibitor and vehicle control (DMSO) at 400 rpm and determine optimal concentrations of CRP (or other agonist) and inhibitor. Once optimal conditions are found, a time course stimulation of inhibitor- and DMSO-treated platelets should be performed at 400 rpm stirring condition. For lysis and immunoblotting, follow the steps described in Subheading 3.6. Because platelet activation is highly variable between donors, experiments should be performed on platelets from at least three donors.

4 Notes

1. Nembutal stock solution: 6 mg/mL in 9% NaCl. Prepare 1 mL. If the experiment lasts more than 30 min, inject mice with Nembutal (10 μg/g) to maintain anesthesia. Repeat the injection every 30 min if required.
2. Many factors such as genotype, age, and stress levels can result in variation in anesthetic depth and time to recover. Therefore, the anesthesia protocol should be adjusted accordingly. We provide the conditions that worked well for DUSP3$^{-/-}$ and WT littermates in C57BL/6 mice background.
3. Preparation of paraformaldehyde (PFA): Weigh 4 g of PFA in beaker. Add 80 mL of 1× PBS. Cover with Parafilm. Place the beaker on magnetic stirrer/heater (52 °C). Mix with moderate stirring until the solution becomes clear. Heating and a slight alkaline pH are necessary to dissolve PFA. Since the pH of PBS is around 7.4, PAF dissolves easily in the PBS. Adjust the final volume to 100 mL with PBS. Store the solution in small aliquots, depending on the usage, at −20 °C. PFA solution can also be stored at room temperature up to 1 week; longer storage at room temperature will lead to formation of formic acid.
4. Take a small aliquot of collected blood and make sure (using the hematology analyzer (Cell-Dyn 3700 in our case)) that platelet counts are higher than 8×10^5 platelets/μL. Lower platelet concentrations indicate that the blood contains small platelet aggregates, and those samples should be discarded.
5. Collect 0.8–1 mL of blood from one mouse. Less blood volume usually means that the quality of the collected blood is not good enough to proceed, and the sample should be discarded.

About ten aggregation cuvettes can be prepared out of a pool of PRP from three mice. Secretion assays using flow cytometry can be performed on non-pooled PRP because smaller platelet numbers are required.

6. Care should be taken when the primary antibody against the target protein to be detected in western blot is raised in mouse and the target protein is at the same size of immunoglobulins (IgGs) heavy or light chains. Indeed, murine IgGs bind to platelet surface and contaminate PRP lysates. They are visible on immunoblots when secondary HRP-conjugated anti-mouse antibodies are used. One way to get partially rid of these contaminants is to preclean WP lysates with protein G or protein A sepharose beads. This is particularly useful in IPs.
7. CFSE loaded platelets should be used within 2 h after staining, as the staining intensity decreases rapidly.
8. Vessels surrounding tissues should not swell when injecting NaCl. If this happens, the catheter is likely not well inserted.
9. To minimize platelet activation, use <21 G needles to avoid shear stress. Further, discard the first few milliliters of blood, which can be in contact with collagen. Finally, gently mix the collected blood with the anticoagulant immediately, minimize the time from sample collection to analysis, and avoid sudden manipulation.
10. DMSO concentrations higher than 0.2% significantly inhibit both human and mouse platelet aggregation.
11. The inhibitor should be highly pure, and we strongly recommend repurification of commercial screening compounds using preparative HPLC. Platelets are a highly reactive cell type and may get activated by nonspecific binding of contaminants. Therefore, a platelet aggregation assay in response to the compound alone (with no platelet receptor agonist added) should be performed to test for unspecific, spontaneous platelet aggregation.
12. When dealing with a large number of blood donors, an alternative method can be used. Platelets can be fixed in 1% PFA after activation and stored for up to 5 days at 4 °C. However, anti-PAC1 antibodies do not work on fixed platelets. Anti-fibrinogen antibodies can be used in this case. For analysis, centrifuge the fixed cells at 800 × *g* for 5 min at 4 °C. Discard the supernatant and add 1 mL of PBS to the washed platelets. Centrifuge again at 800 × *g* for 5 min at 4 °C. Discard supernatant and add 150 μL of PBS. Add 10 μL of anti-CD62P antibody 2 μL of anti-Fibrinogen antibody (Dako #F0111; diluted 5× in PBS). Incubate for 15 min at RT in the dark. Add 1 mL of 1% PFA and proceed with flow cytometry analysis.

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