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Perspective: Tyrosine Phosphatases As Novel Targets For Antiplatelet Therapy

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Abstract

Arterial thrombosis is the primary cause of most cases of myocardial infarction and stroke, the leading causes of death in the developed world. Platelets, highly specialized cells of the circulatory system, are key contributors to thrombotic events. Antiplatelet drugs, which prevent platelets from aggregating, have been very effective in reducing the mortality and morbidity of these conditions. However, approved antiplatelet therapies have adverse side effects, most notably the increased risk of bleeding. Moreover, there remains a considerable incidence of arterial thrombosis in a subset of patients receiving currently available drugs. Thus, there is a pressing medical need for novel antiplatelet agents with a more favorable safety profile and less patient resistance. The discovery of novel antiplatelet targets is the matter of intense ongoing research. Recent findings demonstrate the potential of targeting key signaling molecules, including kinases and phosphatases, to prevent platelet activation and aggregation. Here, we offer perspectives to targeting members of the protein tyrosine phosphatase (PTP) superfamily, a major class of enzymes in signal transduction. We give an overview of previously identified PTPs in platelet signaling, and discuss their potential as antiplatelet drug targets. We also introduce VHR (DUSP3), a PTP that we recently identified as a major player in platelet biology and thrombosis. We review our data on genetic deletion as well as pharmacological inhibition of VHR, providing proof-of-principle for a novel and potentially safer VHR-based antiplatelet therapy.

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Keywords

Protein tyrosine phosphatases; PTP; Inhibitor; Thrombosis; Antithrombotic; Platelets; Antiplatelet; Therapy; Drug; GPVI; CLEC-2; DUSP3; VHR; CD148; PTP1B; SHP1; SHP2

1. Introduction

Circulating platelets play a crucial role in hemostasis, acting to maintain the integrity of a closed circulatory system after blood vessel injury.[2] They are recruited to the site of vascular damage, where they adhere to the exposed subendothelial matrix and become a major component of the developing thrombus that seals the breach. Under normal conditions, regulatory mechanisms restrain thrombus formation both temporally and spatially. When pathologic processes overwhelm the regulatory mechanisms of hemostasis, excessive quantities of thrombin are formed, initiating thrombosis.[1, 2] Thrombosis can occur in the arterial or the venous circulation and has a major medical impact.[3] Venous thromboembolism is the third leading cause of cardiovascular-associated death. Acute arterial thrombosis is the primary cause of most cases of myocardial infarction and stroke, collectively the most common causes of death in the developed world.[4]

Available antiplatelet therapies are used for both the prevention and the acute treatment of arterial thrombosis. These drugs target either one of the three major processes leading to platelet activation and thrombus formation: *1)* the initial phase of platelet recruitment and adhesion to the vessel wall, *2)* the platelet aggregation phase, and *3)* the stabilization of platelet aggregates during the amplification phase (**Fig. 1**). Aspirin has been used clinically for more than 40 years and is the most commonly used antiplatelet drug.[5, 6] It inhibits cyclooxygenase-1, which is required for the synthesis of thromboxane A_2 (TXA_2), a secondary mediator of platelet aggregation. Thienopyridines, including clopidogrel, ticlopidine, and prasugrel, are irreversible inhibitors of the $P2Y_{12}$ ADP receptor and also widely used as antiplatelet drugs.[7, 8] In fact, clopidogrel (*Plavix*) is one of the best selling drugs of all time, with peak sales of $9.3 billion in 2011 (source: *FirstWord Pharma*). Both, aspirin and clopidogrel inhibit the formation of stable platelet aggregates. More recently developed amplification phase inhibitors include ticagrelor,[9] a $P2Y_{12}$ receptor antagonist, vorapaxar[10, 11] and atopaxar,[12] which are antagonists of the thrombin-activated G-protein coupled receptor (GPCR) PAR-1, and dipyridamole and cilostazol,[13] both phosphodiesterase 3 (PDE3) selective inhibitors that increase cAMP levels and block platelet responses to ADP. Investigational drugs include the TXA_2 receptor antagonists terutroban and EV-077.[14, 15] Platelet aggregation phase inhibitors in clinical use include the monoclonal antibody abciximab, the cyclic heptapeptide eptifibatide, and the small molecule tirofiban.[16] These drugs all inhibit the interaction between glycoprotein IIb/IIIa (also known as integrin $\alpha_{IIb}\beta_3$) and fibrinogen, thereby potently inhibiting platelet aggregation. At present, there are no drugs in clinical use that block the initial recruitment and binding of platelets to adhesive macromolecules of the subendothelial matrix, such as collagen or von Willebrand factor (vWF). Molecules in clinical studies that inhibit the interaction between vWF and glycoprotein (GP) Ib on the surface of platelets include the aptamer ARC-1779,[17] the monoclonal antibody caplacizumab,[18] and the snake venom-derived C-type lectin-like protein anfibatide.[19] Collagen receptor GPVI inhibitors in development include compounds that induce depletion of GPVI, antibodies that bind to GPVI, and soluble GPVI mimetics that bind to collagen.[20]

Antiplatelet therapy has been very effective in reducing the mortality and morbidity of acute myocardial infarction and stroke.[4] However, approved antiplatelet agents have adverse side effects, including gastrointestinal toxicity, neutropenia, thrombocytopenia, and bleeding.[4] Moreover, there remains a considerable incidence of arterial thrombosis in patients resistant to currently available antiplatelet therapy. For example, the delayed onset of action and the occurrence of poor platelet inhibition responders with clopidogrel is well documented.[4] Thus, despite the great success of antiplatelet therapy, there is an unmet medical need for novel antiplatelet agents with a more favorable safety profile, better efficacy in certain patient groups, and rapid onset and offset of action in acute events. Efforts in the pharmaceutical industry have focused on developing new-generation drugs of well-established targets, including the $P2Y_{12}$ ADP receptor, the PAR-1 thrombin receptor, and the TXA_2 receptor.[4] In addition, the discovery of novel targets is the matter of intense ongoing research. Recently, various kinases have emerged as promising antiplatelet targets. Proof-of-principle studies have been reported for phosphatidylinositol 3-kinase (PI3K) isoform p110β,[21, 22] the non-receptor spleen tyrosine kinase (Syk),[23, 24] and Src family kinases (SFKs).[25, 26] These findings demonstrate the possibility of targeting key regulators of platelet receptor signaling pathways as novel antiplatelet strategies.

Tyrosine phosphorylation is an important mechanism of signal transduction,[27, 28] including in platelets,[29, 30] as it crucially activates or inhibits the function of signaling molecules. For instance, tyrosine phosphorylation can result in conformational changes of the tertiary or quaternary structure of proteins, thereby rapidly amplifying or attenuating the catalytic activity of enzymes.[31] It also promotes crucial protein-protein interactions, e.g., the recruitment and activation of signaling molecules to the cytoplasmatic tail of cell-surface receptors. Tyrosine phosphorylation is a rapidly reversible post-translational modification, controlled by the opposing activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs).[27, 28] Historically, research had long been focused on the role PTKs in signaling and disease, as it was generally believed that PTPs functioned merely as indiscriminate ‘housekeeping’ enzymes with broad specificities. However, we now appreciate that PTPs are not only as numerous and diverse as PTKs, we have also started to understand how highly specific these enzymes indeed act on their substrates *in vivo*, and how tightly regulated they are to maintain homeostasis.[32-36] Indeed, the rapidly increasing number of disorders associated with PTP abnormalities has begun to elicit a growing interest in these enzymes as drug targets, including in major diseases such as cancer,[37, 38] diabetes,[39] autoimmunity,[40, 41] and Alzheimer’s.[42] While PTPs have been well studied in numerous cell types, in particular in cells of the immune system,[40, 43, 44] the expression, regulation, specificity, and function of PTPs in platelets are largely unknown. Recent proteomic analyses found that 20 out of the 37 classical, phosphotyrosine (pTyr)-specific PTPs are expressed in human platelets.[45] Only four of them had been previously identified as critical regulators of platelet function. Platelet expression and function of the 63 dual-specificity phosphatases (DSPs),[32, 34] the largest subgroup of PTPs, had been entirely unexplored. A summary of PTPs reported in platelet function is presented in **Table 1**.

Our laboratories recently published work investigating the role of DSPs in human platelets, implicating the *Vaccinia* H1-related (VHR) phosphatase (also known as DUSP3) as a key positive regulator of platelet signaling through the GPVI collagen receptor and the C-type lectin-like 2 (CLEC-2) podoplanin receptor.[46] More importantly, we found that VHR-deficient mice were more resistant to collagen- and epinephrine-induced thromboembolism, compared to wild-type (WT) mice, and showed severely impaired thrombus formation upon $FeCl_3$-induced carotid artery injury.[46] Intriguingly, bleeding times were not altered in VHR-deficient mice. To investigate VHR function in human platelets, we developed a specific small-molecule inhibitor of VHR. This compound specifically inhibited GPVI- and CLEC-2-induced human platelet aggregation, thereby phenocopying the effect of VHR deficiency in murine cells. This was the first time a specific platelet PTP had been targeted with a small-molecule drug. Our findings, which we discuss in more detail in Section 3 of this perspective article, may lead to a novel, effective, and safer antiplatelet therapy.

2. Classical PTPs in platelet signaling

Four classical PTPs have been identified as critical regulators of platelet function, namely: the transmembrane receptor-like PTP CD148 and the intracellular non-receptor like PTPs PTP1B, SHP1, and SHP2. Below we discuss the main functions of these PTPs in platelet reactivity, as well as their potential as antithrombotic drug targets.

2.1. CD148 (PTPRJ, DEP-1): Master regulator of platelet reactivity

CD148 is a fundamental regulator of platelet reactivity (**Fig. 2**).[47] Platelets rely heavily on CD148 function to regulate SFK activity and signaling from immunoreceptor tyrosine-based activation motif (ITAM)-containing receptors and integrins.[48, 49] CD148 consists of a large, highly glycosylated ectodomain (containing eight fibronectin type III domains), a single transmembrane domain, and a single PTP domain in its cytoplasmic tail. Physiologically relevant ligands of CD148 remain ambiguous, although syndecan-2 and thrombospondin-1 were recently reported to bind CD148.[50, 51] The C-terminal inhibitory tyrosine residue of SFKs is the most well established substrate of CD148. However, CD148 also attenuates SFK activity by dephosphorylating the activation loop tyrosine residue, thus acting as a molecular rheostat, optimizing SFK activity under resting and activated conditions.[49]

Targeted deletion of CD148 in mice has profound consequences on platelet reactivity and thrombosis due to a dramatic reduction in SFK activity both in resting and activated platelets.[47] The capacity of CD148 to attenuate SFK activity is masked in CD148-deficient mice, where the net effect is a reduction in platelet reactivity. CD148-deficient platelets exhibit impaired responses to collagen and fibrinogen.[47] However, responses to thrombin and TXA_2 are only marginally impaired, while responses to ADP are normal. Thrombus formation is markedly reduced in CD148 constitutive knockout mice, following laser- and $FeCl_3$-induced injury of arterioles, with a minor prolongation of bleeding. CD148-deficient platelets also exhibit a concomitant 50% reduction in GPVI expression, which contributes to the reduced reactivity to collagen.[47] These findings clearly establish CD148 as a critical positive regulator of platelet activation and aggregation.

CD148 expression is tightly regulated in human platelets, in which little inter-individual variability is observed.[47] Specific polymorphisms identified in the extracellular region of CD148 (Q276R and R326Q) have a protective effect in the development of heparin-induced thrombocytopenia by reducing platelet reactivity to antibody-platelet factor 4/heparin complexes, which activate platelets via clustering of the ITAM-containing Fc-γ receptor IIA (FcγRIIA).[52] It is proposed that these mutations introduce torsional stress and a loss of positive charge in the second fibronectin type III domain of CD148 that may affect ligand binding or membrane compartmentalization of CD148.[52] Collectively, these findings suggest CD148 as a potential antiplatelet drug target.

Because CD148 not only regulates the basal level of SFK activity, but also the threshold of signaling from a number of platelet receptors (**Fig. 2**), we anticipate that inhibiting CD148 is likely to have dramatic effects on thrombus formation. Thus, we believe it would be an appropriate target in the most severe thrombotic cases, such as disseminated intravascular coagulation or post-operatively, when thrombus formation is a high risk. As with all antiplatelet therapies, bleeding side effects are a distinct possibility, which may be minimized by titrating the dose of a reversible inhibitor. The ectodomain of CD148 is an obvious target, owing to the ease of access. Blocking ligand binding would presumably alter the threshold of platelet activation. Indeed, a monoclonal antibody targeting the extracellular domain of CD148 has been shown to inhibit CD148-dependent endothelial cell growth and angiogenesis in mouse cornea, providing proof-of-principle of this strategy.[53] The cytoplasmatic juxtamembrane region, PTP domain, and C-terminal tail region are also potential targets, with the added challenge of the drug needing to cross the plasma membrane. Currently, no CD148-specific inhibitors are available.

2.2. PTP1B (PTPN1): Positive regulator of outside-in integrin signaling

PTP1B is highly expressed in platelets and is a positive regulator of outside-in signaling from the integrin $\alpha_{IIb}\beta_3$.[54, 55] Specifically, PTP1B dephosphorylates the C-terminal inhibitory tyrosine of β3-associated SFKs, initiating downstream signaling (**Fig. 3**).[55, 56] Outside-in signaling regulates cytoskeletal remodeling and is essential for platelet spreading on fibrinogen, as well as clot retraction, two essential processes for stable thrombus formation and cessation of bleeding. Interestingly, mice lacking PTP1B have normal platelet counts and show no apparent bleeding tendency, suggesting that PTP1B is dispensable for primary hemostasis.[55] However, they exhibit a dramatic reduction in thrombus formation following laser-induced injury of arterioles. Whether or not the reduced thrombus formation is due exclusively to the absence of PTP1B in platelets has yet to be elucidated. PTP1B-deficient platelets exhibit reduced spreading on fibrinogen,[55, 57] yet they bind soluble fibrinogen and aggregate normally in response to the GPVI agonists collagen-related peptide (CRP) and convulxin, the $P2Y_{12}$ and $P2Y_1$ agonist ADP, and the PAR-4 agonist thrombin.[55, 57] In line with these observations, PTP1B-deficient platelets aggregate normally on collagen under intermediate shear conditions, but aggregate volume is dramatically reduced at high shear conditions (3,000 s^{-1}), typically found in stenotic arteries prone to occlusion.[57] This provides further compelling evidence for PTP1B as a potential anti-thrombotic target. The highly specific role of PTP1B in platelets may in fact make it a better target than the more globally acting CD148, predicting fewer bleeding side effects compared to inhibiting CD148.

PTP1B has been considered a potential drug target in the treatment of type 2 diabetes since its role as a negative regulator of insulin receptor signaling was published some 15 years ago.[58] More recently, PTP1B has also been implicated in the development of breast[59, 60] and prostate[61] cancer. Despite a tremendous effort put into the development of specific PTP1B inhibitors, both in academia and industry, only a few compounds progressed to clinical trials, and none have advanced beyond phase II (**Table 2**).[62-65] This is largely due to the conventional approach taken to target the highly conserved PTP1B active site.[32] However, novel strategies of inhibiting PTP1B have emerged, including the use of cell-permeable antisense oligonucleotides (ISIS-PTP1B$_{RX}$, ISIS Pharmaceuticals), as well as the development of inhibitors that target less conserved, allosteric sites in PTP1B (e.g., trodusquemine, **Table 2**).[66, 67] These novel and promising approaches may result in the first PTP1B inhibitors with clinical potential. Importantly, targeting PTP1B may have secondary health benefits in conditions such as diabetes and cancer where thrombosis is a known consequence.

2.3. SHP1 (PTPN6) and SHP2 (PTPN11): Critical regulators of platelet homeostasis

The closely related Src homology 2 (SH2) domain-containing phosphatases SHP1 and SHP2 use their tandem N-terminal SH2 domains to bind to phosphorylated immunoreceptor tyrosine-based inhibition motifs (ITIMs) associate with the platelet endothelial cell adhesion molecule 1 (PECAM-1), which functions as a negative regulator of platelet reactivity and thrombosis,[68] but has also been implicated as a positive regulator of the outside-in signaling properties of $\alpha_{IIb}\beta_3$.[69] PECAM-1-mediated membrane localization of SHP1 and SHP2 is thought to allow them to access and dephosphorylate key signaling and cytoskeletal molecules. Work done using SHP1 and SHP2 conditional knockout mice revealed complex phenotypes that are not compatible with a simple model of SHP1 and SHP2 solely acting as negative regulators of platelet signaling.[70] Table 1 and Figure 4 highlight putative functions attributed to SHP1 and SHP2 in platelets and megakaryocytes. Mice lacking either SHP1 or SHP2 in the megakaryocyte lineage (i.e., in platelets and their progenitors megakaryocytes) exhibited distinct phenotypes that were exacerbated in double knockout mice, demonstrating non-redundant functions that collectively regulate platelet production and homeostasis. Deletion of both SHP1 and SHP2 resulted in a critical block in megakaryocyte development and survival and almost a complete absence of platelets in the circulation. As a result, SHP1/2 double knockout mice had a severe bleeding diathesis. Interestingly, a similar phenotype was seen in mice lacking G6b-B,[70] an ITIM-containing receptor that was previously shown to be associated with SHP1 and SHP2.[71]

Based on these findings, targeting SHP2 may be beneficial in treating conditions characterized by overproduction of platelets such as thrombocythemia or polycythemia vera, both myeloproliferative disorders that are frequently caused by elevated signaling via the thrombopoietin receptor MPL (also known as myeloproliferative leukemia protein). Indeed, SHP2 inhibitors (**Table 2**) are currently being developed for the treatment of various leukemias and other forms of cancer,[72, 73] where elevated SHP2 activity contributes to oncogenesis.[74, 75] In contrast, inhibition of SHP1, for which no specific inhibitors have been reported, may result in undesirable side effects, given its important negative regulatory role in many survival and growth signaling pathways in hematopoietic cells,[43] and the fact that mice deficient in SHP1 display a severe autoimmune and immunodeficiency syndrome (*motheaten* mouse).[76, 77] Thus, one of the main challenges in targeting SHP2 is to generate inhibitors with sufficient selectivity for SHP2 over its closely related cousin SHP1.

3. VHR (DUSP3): Major positive regulator of platelet signaling and thrombosis

VHR, first described by Ishibashi et al.,[78] and named for its sequence similarity with the VH1 gene in *Vaccinia* virus, is a member of the dual-specificity phosphatases. The DSP subfamily of PTPs is the most diverse group in terms of substrate specificity, ranging from pTyr-specific to phosphothreonine (pThr)/pTyr-specific, to phosphoserine (pSer)-specific, to pSer/pThr-specific DSPs. Additionally, some DSPs can also dephosphorylate mRNA or phosphatidylinositol phosphates.[32, 34] VHR is a small DSP (185 amino acids; 20.5 kDa), containing only a catalytic PTP domain, with a preference for dephosphorylating pTyr over pSer/pThr.[79] Reported substrates of VHR *in vitro* are the receptor tyrosine kinases EGFR, KGFR, PDGFR, and the insulin receptor,[78] as well as the MAP kinases ERK1/2 and JNK1/2.[80, 81] However, dephosphorylation of MAP kinases by VHR remains controversial.[46, 82, 83] Several reports implicated VHR in cell cycle regulation and cancer.[84-87] For instance, RNA interference-mediated knockdown of VHR in HeLa cells caused cell cycle arrest at the G1-S and G2-M transitions and also induced cell senescence.[84] In addition, VHR was found upregulated in prostate and cervix carcinomas,[86, 87] and pharmacological inhibition of VHR decreased the proliferation of HeLa and other cervix cancer cell lines.[85] Finally, in the LNCaP prostate cancer cell line, androgen-induced VHR expression reduced 12-*O*-tetradecanoylphorbol-13-acetate- and thapsigargin-induced apoptosis.[87] Based on these reports, VHR behaves like a prooncogene.[88] However, VHR also acts as a tumor-suppressor in breast cancer[89] and non-small cell lung cancers.[82, 90] Regardless, the actual biological functions of VHR are still poorly understood. (A more detailed survey of the literature can be found in a recent review by Pavic et al.[91])

3.1. VHR is a key positive regulator of the GPVI and CLEC-2 pathways in platelets

We recently found that VHR is highly expressed in human and mouse platelets.[46] We previously generated VHR-knockout (VHR-KO) mice[92] and utilized these animals to study the role of VHR in hemostasis and thrombosis.[46] We found that VHR-KO mice were more resistant to pulmonary thromboembolism than their WT littermates. Moreover, thrombus formation was strongly impaired after $FeCl_3$-induced injury of carotid arteries in a platelet-specific manner. Intriguingly, bleeding was not affected by VHR deficiency, suggesting that VHR plays a key role in arterial thrombosis, but is dispensable for primary hemostasis.[46] *Ex vivo*, VHR deficiency resulted in defective platelet aggregation, granule secretion, and integrin $\alpha_{IIb}\beta_3$ inside-out activation, specifically in response to GPVI and CLEC-2 stimulation (**Fig. 5**). Platelet activation mediated by GPCRs (i.e., the $P2Y_{12}$, TXA_2, and thrombin receptors) was not affected. Impaired thrombus formation in VHR-KO mice was comparable to the previously reported GPVI-KO/FcRβ–KO,[93-95] CLEC-2-KO,[96] CLEC-2-depleted,[97] GPVI-depleted,[98] and CLEC-2/GPVI-depleted mice.[99] The finding that VHR-deficient mice show no apparent bleeding tendency was in line with what was observed previously in GPVI-KO and CLEC-2-KO mice.[93, 96, 100] VHR-KO mice were also protected against pulmonary thromboembolism, similar to GPVI-KO mice.[100]

At the molecular level, phosphorylation of the previously reported VHR substrates ERK1/2 and JNK1/2 was not affected by VHR deficiency in platelets. However, we found significantly reduced pTyr levels in the tyrosine kinase Syk, a key signaling molecule in GPVI- and CLEC-2-mediated platelet activation (**Fig. 5**). Upon stimulation, Syk phosphorylation was reduced on the activatory residues Tyr-525/526, while phosphorylation on Tyr-323, which is implicated in Syk ubiquitination/degradation,[101] was not affected. Previous data suggested that Syk is recruited to the GPVI/Fc receptor γ-chain (FcRγ) complex via phosphorylation of FcRγ-associated ITAMs by SFKs.[102, 103] We found that phosphorylation of FcRγ-associated ITAMs was reduced, and recruitment of Syk to FcRγ was impaired in VHR-KO compared to WT platelets. Additionally, inducible tyrosine phosphorylation in phospholipase-C (PLC) γ2, a key signaling molecule downstream of Syk, was also reduced in VHR-deficient compared to WT platelets. In contrast, phosphorylation of inhibitory or activatory tyrosine residues in SFKs, including Lyn, Fyn, and Src, was not altered, indicating that the reduced activation and recruitment of Syk in VHR-deficient platelets was not due to aberrant activation of SFKs. At this point we do not know the direct target(s) of VHR in platelets. Given that VHR deficiency led to a decrease of pTyr in Syk, and no proteins with an increase in pTyr could be identified, our data raise the question whether pSer/pThr in Syk, SFKs, or other protein(s) may be dephosphorylated by VHR. However, due to the limited usability of available pSer/pThr antibodies, future studies using quantitative phospho-proteomics will be necessary to address this question.

3.2. Pharmacological inhibition of VHR in human platelets phenocopies the effect of VHR deficiency

Platelets derive from cytoplasmatic fragments of megakaryocytes and hence are anucleate cells that are not amenable to RNA interference or recombinant DNA technologies. Tool compounds to pharmacologically inhibit (or activate) the function of a specific signaling molecule in human platelets are invaluable to corroborate findings that originate from animal models. In our study, we utilized a chemical genomics approach to develop a specific small-molecule inhibitor of VHR (**Fig. 6**).[46] High-throughput screening (HTS) of ~300,000 drug-like molecules (from the NIH Molecular Libraries Program) culminated in 67 hits with confirmed activities in two orthogonal PTP assays (colorimetric and fluorimetric),[104, 105] and dose-dependent responses with IC_{50} values <20 μM. Based on compound potency and selectivity (derived from subsequent counter-screens against four related PTPs), two scaffolds were selected for structure-activity relationship (SAR) studies: MLS-0103602 (IC_{50} = 0.37 μM) and MLS-0049585 (IC_{50} = 2.68 μM). Analogs of MLS-0103602 included 37 benzothioamide derivatives that were tested against VHR. All analogs were at least an order of magnitude less potent than the original hit with no improvement of selectivity, leading to the termination of this series. In contrast, several analogs of MLS-0049585 with similar or even better potency and selectivity could be identified. Testing of these inhibitors on human platelets collected from healthy donors revealed an efficient inhibition of platelet aggregation by compound MLS-0437605. This effect of MLS-0437605 was specific to platelets activated through the GPVI and CLEC-2 receptors, equivalent to the effect of VHR deficiency in murine cells. Tests on platelets from WT mice yielded similar results, while MLS-0437605 only minimally affected aggregation of VHR-deficient platelets. In selectivity profiling studies against a larger panel of PTPs, MLS-0437605 showed excellent selectivity for VHR over the vast majority of PTPs tested, including good selectivity over VHX (DUSP22), another DSP that we found highly expressed in platelets.[46] Similar to VHR deficiency, inhibition of VHR by MLS-0437605 in human platelets reduced tyrosine phosphorylation of immunoprecipitated Syk and PLCγ2 in response to GPVI and CLEC-2 stimulation; global tyrosine phosphorylation was not affected. Collectively, our data demonstrate that pharmacological inhibition of VHR activity in human platelets phenocopies the effect of VHR deficiency in murine platelets. Given that VHR-KO mice remain healthy and do not suffer from increased bleeding events, MLS-0437605 may serve as the basis for the development of a novel antiplatelet strategy in the treatment of arterial thrombosis.

4. Challenges in targeting PTPs

PTPs are the largest class of protein phosphatases with over 100 members in humans.[32-36] They are defined by their active-site signature motif $\mathbf{C}(X)_5\mathbf{R}$, which is part of the phosphate-binding loop (P-loop) that, together with additional conserved loops, forms the catalytic pocket.[32] PTPs share a common catalytic mechanism that is based on key amino acids within these loops, most importantly a nucleophilic cysteine and an invariant arginine that are part of the signature motif. The vast majority of reported small-molecule PTP inhibitors target the catalytic site. These compounds are usually potent, but poorly selective, due to the fact that they often carry a charged pTyr-mimicking group that provides most of the binding energy through interactions with the highly conserved P-loop.[32, 64, 105-110] High-affinity peptides carrying nonhydrolyzable (fluoro)phosphonate group(s) exhibit incredible potency and selectivity, but little to no cell-based activity, owing to their multi-charged nature and poor drug-likeness, resulting in the lack of cell membrane permeability.[111] Peptidic tool compounds that cross cell membranes were generated by the addition of long-chain fatty acids[112] or cell-penetrating peptides such as polyarginines.[113] Additionally, prodrug strategies to deliver charged compounds more easily into cells have been utilized.[114-116] However, generating small-molecule drug-like PTP inhibitors with significant selectivity for a particular PTP over related enzymes remains challenging. Selectivity of active site inhibitors can be increased by tailoring compounds to interact with unique amino acids and surface features peripheral of the catalytic pocket, an approach that is supported by large-scale structural analyses of PTPs.[117] Because such compounds are designed to interact with both the catalytic site and additional peripheral sites, the trade-off is usually expanded size and molecular weight of inhibitors, resulting in lower ligand efficiency,[118] and compounds that are less likely to enter cells or to be absorbed in the gut. Several new approaches to overcome the selectivity hurdle have been proposed. These include inhibitors that target a less conserved inactive-state PTP conformation, inhibitors that target allosteric sites in PTPs, as well as inhibitors that specifically bind a PTP in its oxidized state. (For more information on these new approaches please see our recent review article.[32]) Interestingly, Michaelis-Menten kinetic studies suggest a noncompetitive inhibition mode for our VHR inhibitor MLS-0437605, a compound that lacks any pTyr-mimicking group (unpublished data). Noncompetitive inhibition is typical for allosteric inhibitors; however, further studies are needed to conclusively determine the mode of action of MLS-0437605. Certainly, allosteric inhibition of PTPs has been gaining significant traction recently,[66, 67, 119, 120] and further success could result in a major paradigm shift in how we target PTPs in the future. Finally, targeting the extracellular domains of receptor-like PTPs such as CD148 has been under-exploited to date, but may provide unique opportunities in terms of drug specificity and target accessibility. In a recent study by Takahashi and co-workers, antibody-induced cross-linking of CD148 resulted in inhibition of endothelial cell growth and angiogenesis.[53] It is conceivable that a similar approach may inhibit activation of platelets.

5. Conclusion

Antiplatelet therapy has been very successful in the prevention and treatment of arterial thrombosis, the primary cause of most cases of myocardial infarction and stroke. However, approved antiplatelet drugs have adverse side effects and are not effective in a considerable subset of patients. In the search for novel antiplatelet agents with a more favorable safety profile and better efficacy, the discovery of new targets is the matter of intense research. Protein tyrosine phosphatases are important molecules for receptor signal transduction and platelet activation. Previous research implicated CD148, PTP1B, SHP1, and SHP2 as critical regulators in platelet signaling. Recent findings from our laboratories show that VHR, a dual-specificity phosphatase, plays a selective and essential role in GPVI- and CLEC-2-mediated platelet activation and thrombus formation *in vivo*. In contrast to current antiplatelet therapies, which completely inhibit a specific receptor or signaling pathway, findings from the CD148, PTP1B, and VHR knockout mouse studies suggest that inhibiting a specific PTP can reduce platelet function, but not completely abrogate it. Thus, platelets can still respond to all agonists, albeit less strongly, which, in turn, may result in fewer bleeding side effects. However, because CD148 is a global regulator of platelet reactivity, inhibition of CD148 is more likely to have bleeding consequences than, for instance, targeting PTP1B or VHR. Effective targeting of PTPs with drug-like molecules has been very difficult to achieve. Specifically, the generation of selective PTP inhibitors remains challenging. However, recent developments, in particular in regards to allosteric mechanisms, demonstrate the possibility to overcome the selectivity hurdle, which so far has impeded the success of PTP inhibitors in the clinic. Our novel VHR inhibitor efficiently inhibited human platelet activation *in vitro*. These data provide, for the first time, proof-of-principle for a PTP-based antiplatelet strategy using a specific small-molecule inhibitor.

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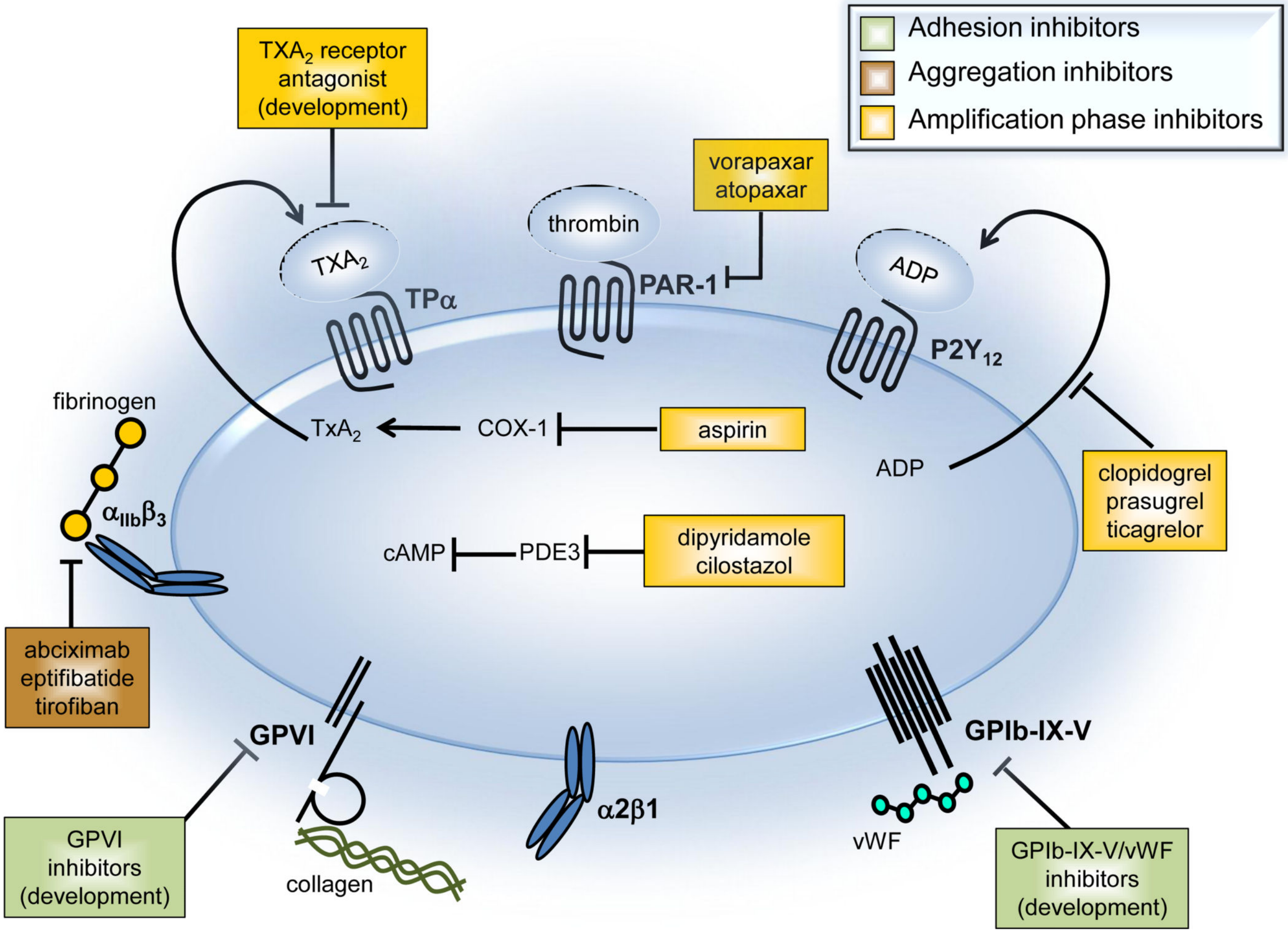


Adhesion inhibitors
Aggregation inhibitors
Amplification phase inhibitors
TXA2 receptor antagonist (development)
vorapaxar atopaxar
thrombin
TXA2
TPα
PAR-1
ADP
P2Y12
fibrinogen
TxA2
COX-1
aspirin
ADP
clopidogrel prasugrel ticagrelor
αIIbβ3
cAMP
PDE3
dipyridamole cilostazol
abciximab eptifibatide tirofiban
GPVI
GPIb-IX-V
α2β1
vWF
collagen
GPVI inhibitors (development)
GPIb-IX-V/vWF inhibitors (development)

Figure 1.

Platelet activation pathways targeted by current and novel antiplatelet agents.

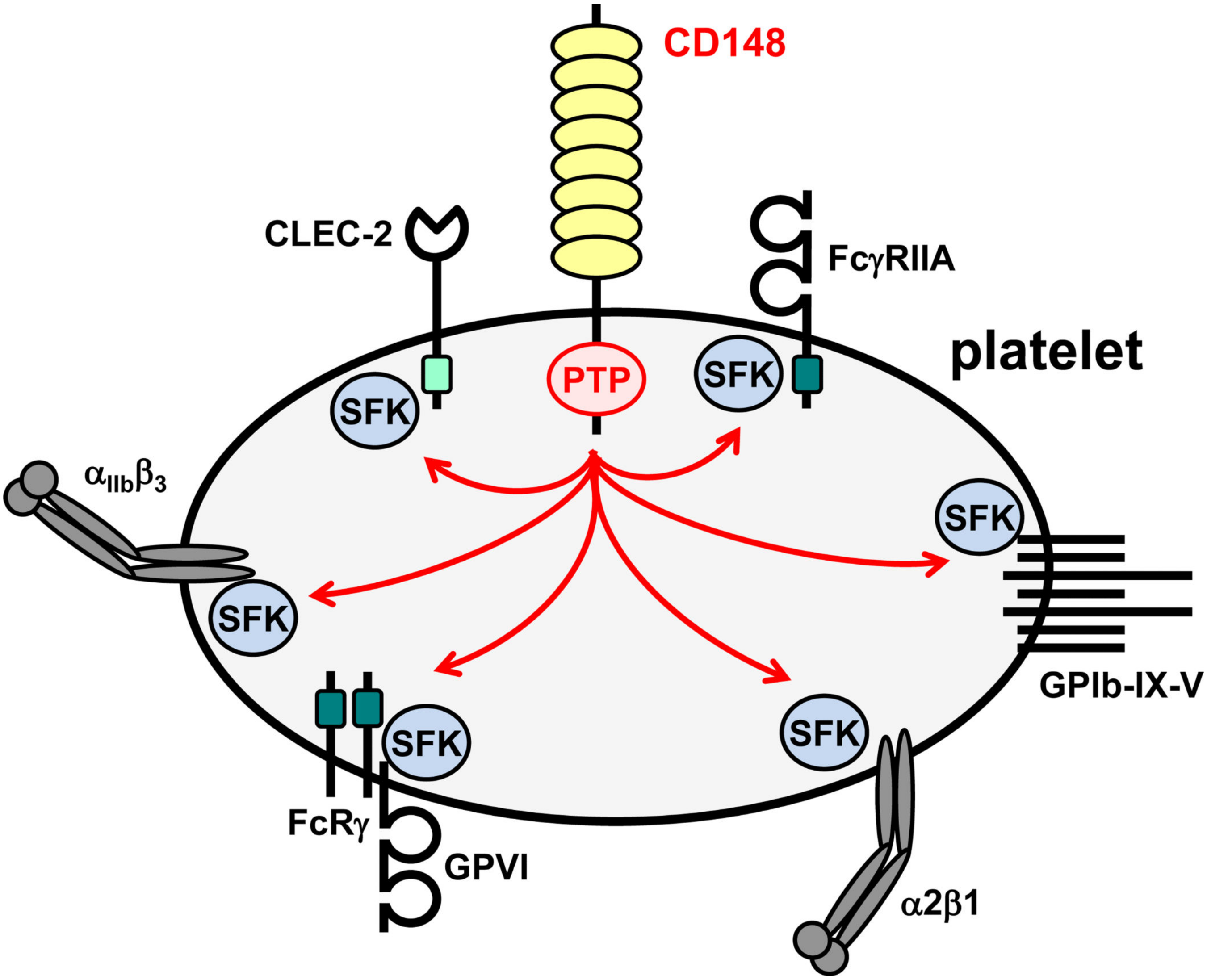


Figure 2. CD148 is a critical regulator of platelet Src family kinases and platelet reactivity

Src family kinases (SFKs) are essential for initiating and propagating signals from a variety of platelet receptors, including: the low affinity immunoglobulin receptor FcγRIIA; the von Willebrand factor (vWF) receptor complex GPIb-IX-V; the collagen integrin α2β1; the collagen activation receptor complex GPVI-FcRγ; the integrin $\alpha_{IIb}\beta_3$, which binds a variety of extracellular matrix and plasma proteins including fibrinogen and vWF; and the podoplanin receptor CLEC-2. Dark green boxes in the cytoplasmic regions of FcγRIIA and the FcRγ denote immunoreceptor tyrosine-based activation motifs (ITAMs); the light green box in the cytoplasmic tail of CLEC-2 denotes a hemi-ITAM.

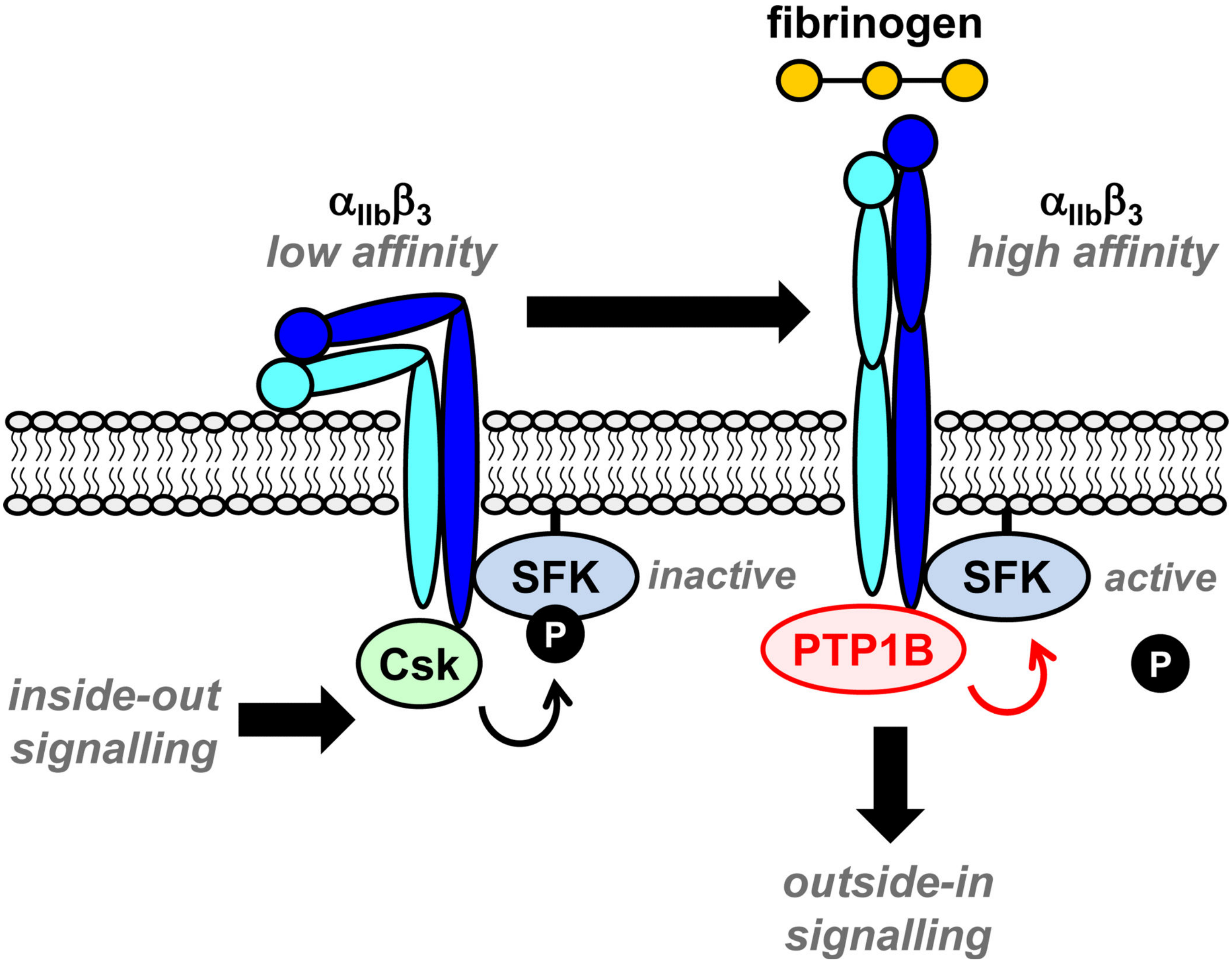
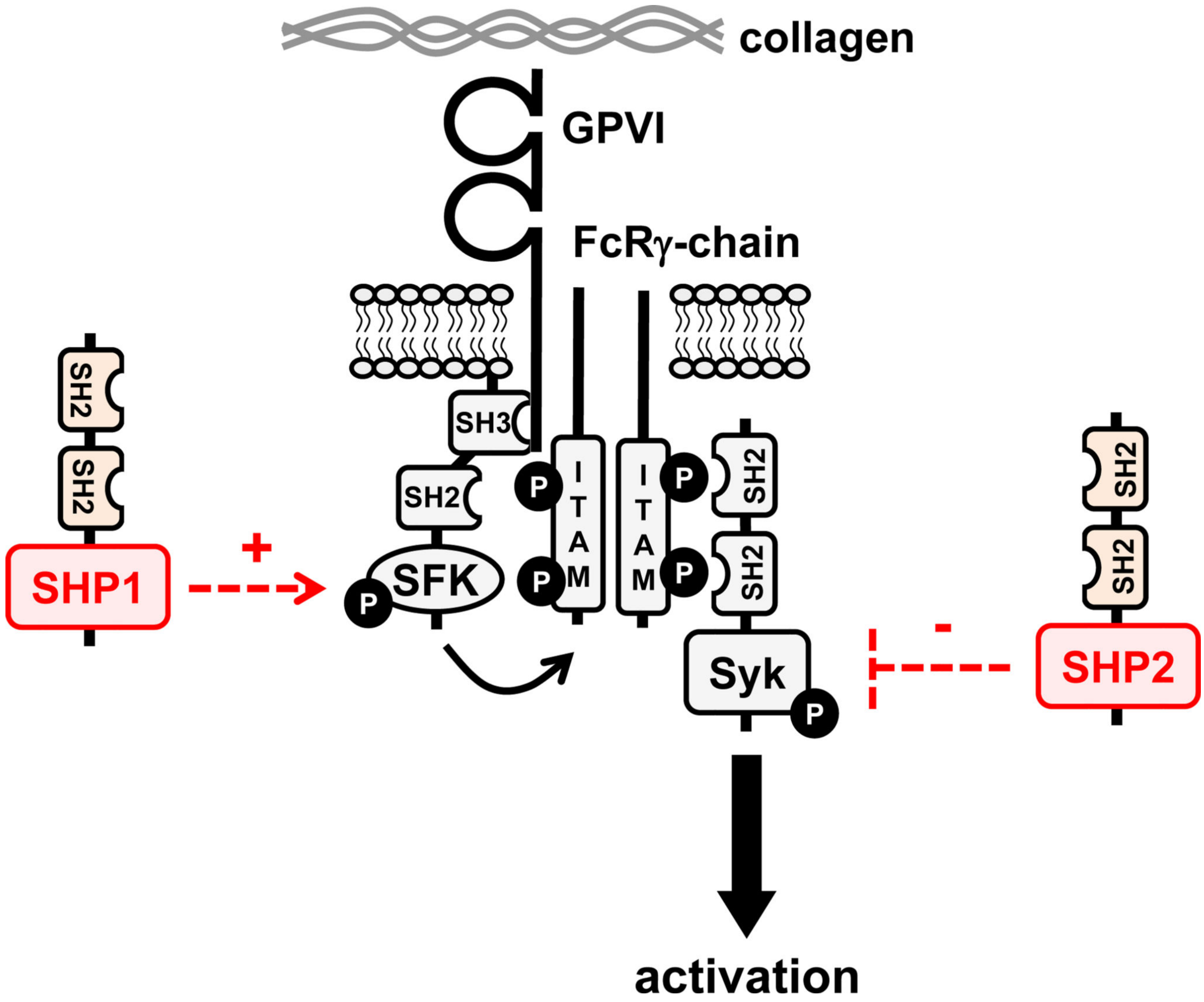
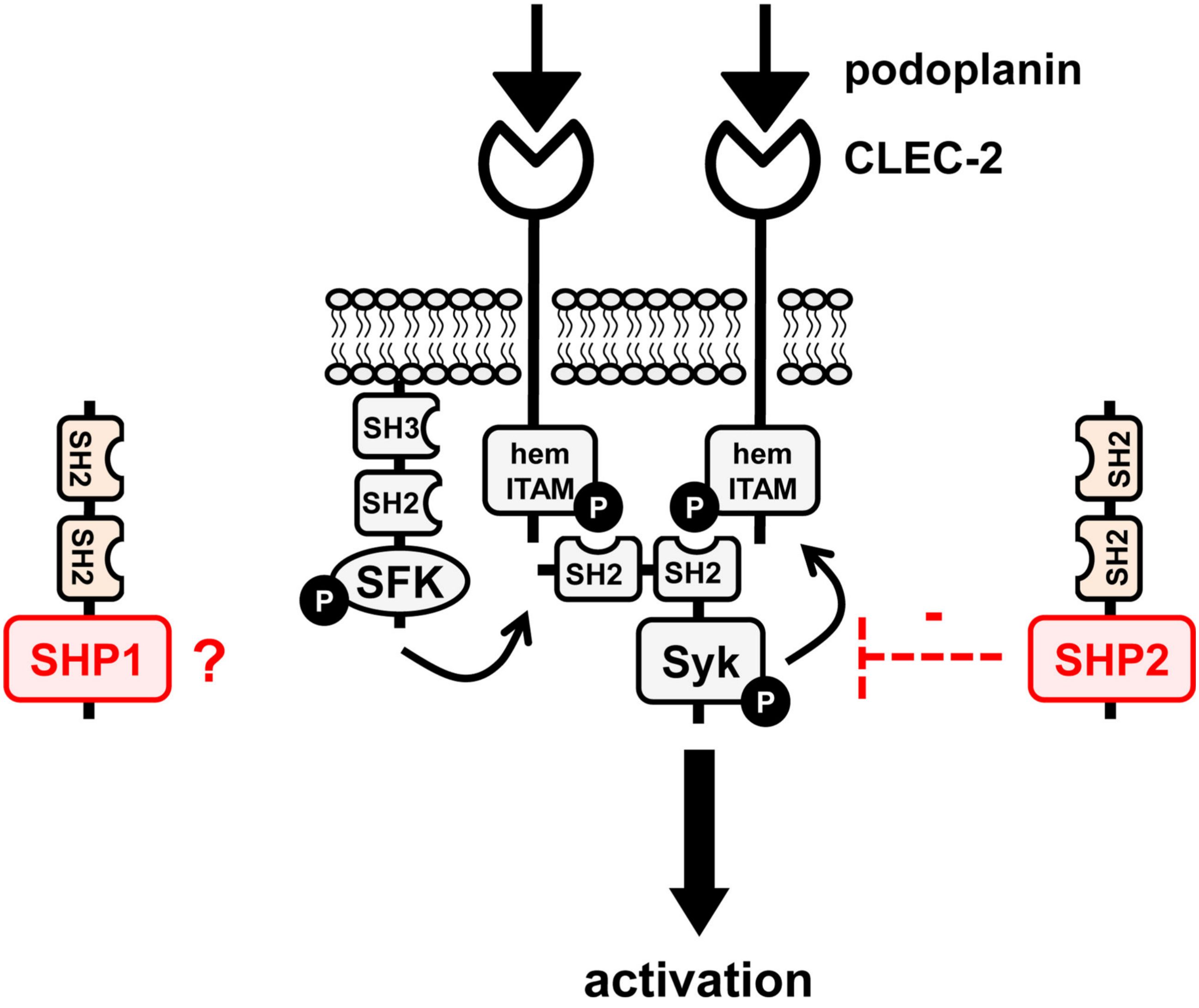
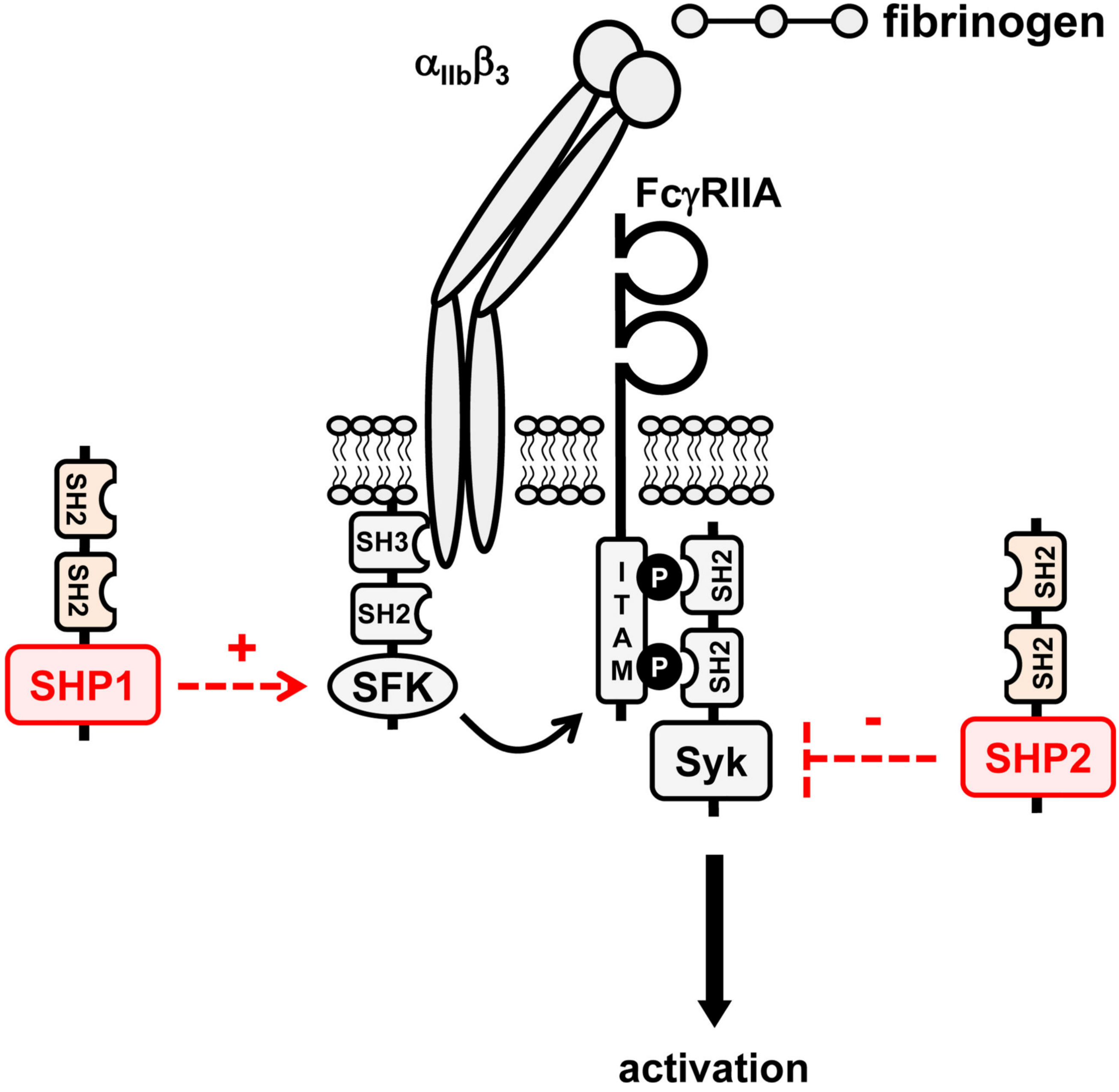

fibrinogen
αIIbβ3
low affinity
αIIbβ3
high affinity
SFK
inactive
P
Csk
inside-out
signalling
PTP1B
SFK
active
P
outside-in
signalling

Figure 3. PTP1B is a positive regulator of outside-in signaling from the integrin $\alpha_{IIb}\beta_3$

Inside-out signals from various activation receptors induce the integrin $\alpha_{IIb}\beta_3$ to undergo a conformational change from a low-affinity to a high-affinity state. C-terminal Src kinase (Csk), associated with the β3 subunit, maintains SFKs in an inactive state. Fibrinogen-mediated clustering of the high-affinity form of the intregrin triggers dissociation of Csk and association with PTP1B, which dephosphorylates the C-terminal inhibitory tyrosine residue of SFKs and initiates outside-in signaling.


collagen
GPVI
FcRγ-chain
SH3
SH2
SFK
P
ITAM
ITAM
SH2
SH2
Syk
SH2
SH2
SHP1
+
SH2
SH2
SHP2
-
activation


podoplanin
CLEC-2
SH3
SH2
SFK
P
hem ITAM
P
hem ITAM
P
SH2
SH2
Syk
P
SH2
SH2
SHP1
?
SH2
SH2
SHP2
activation


fibrinogen
$\alpha_{IIb}\beta_3$
FcγRIIA
SH2
SH2
SHP1
+
SH3
SH2
SFK
I
T
A
M
P
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SH2
SH2
SHP2
activation

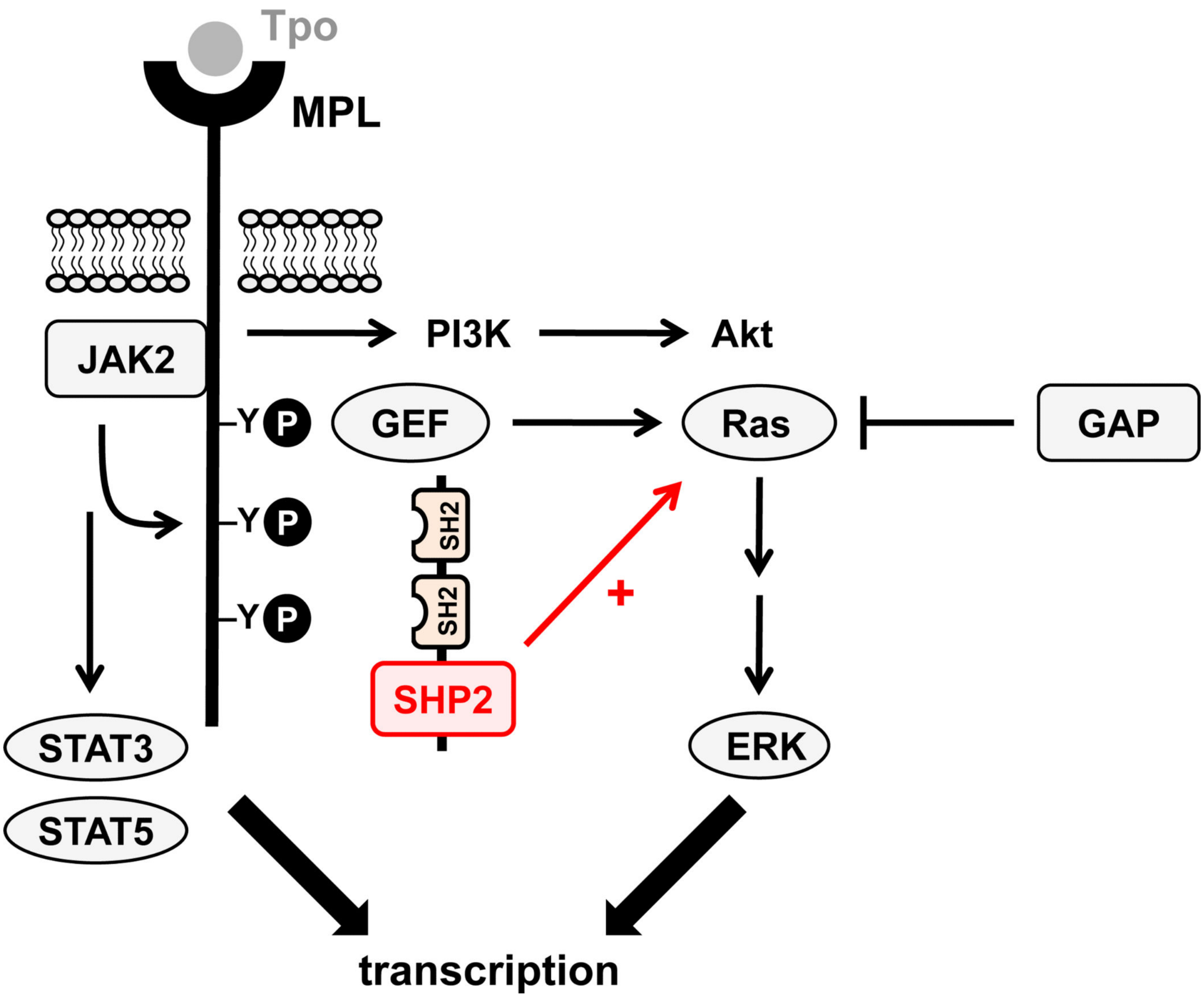
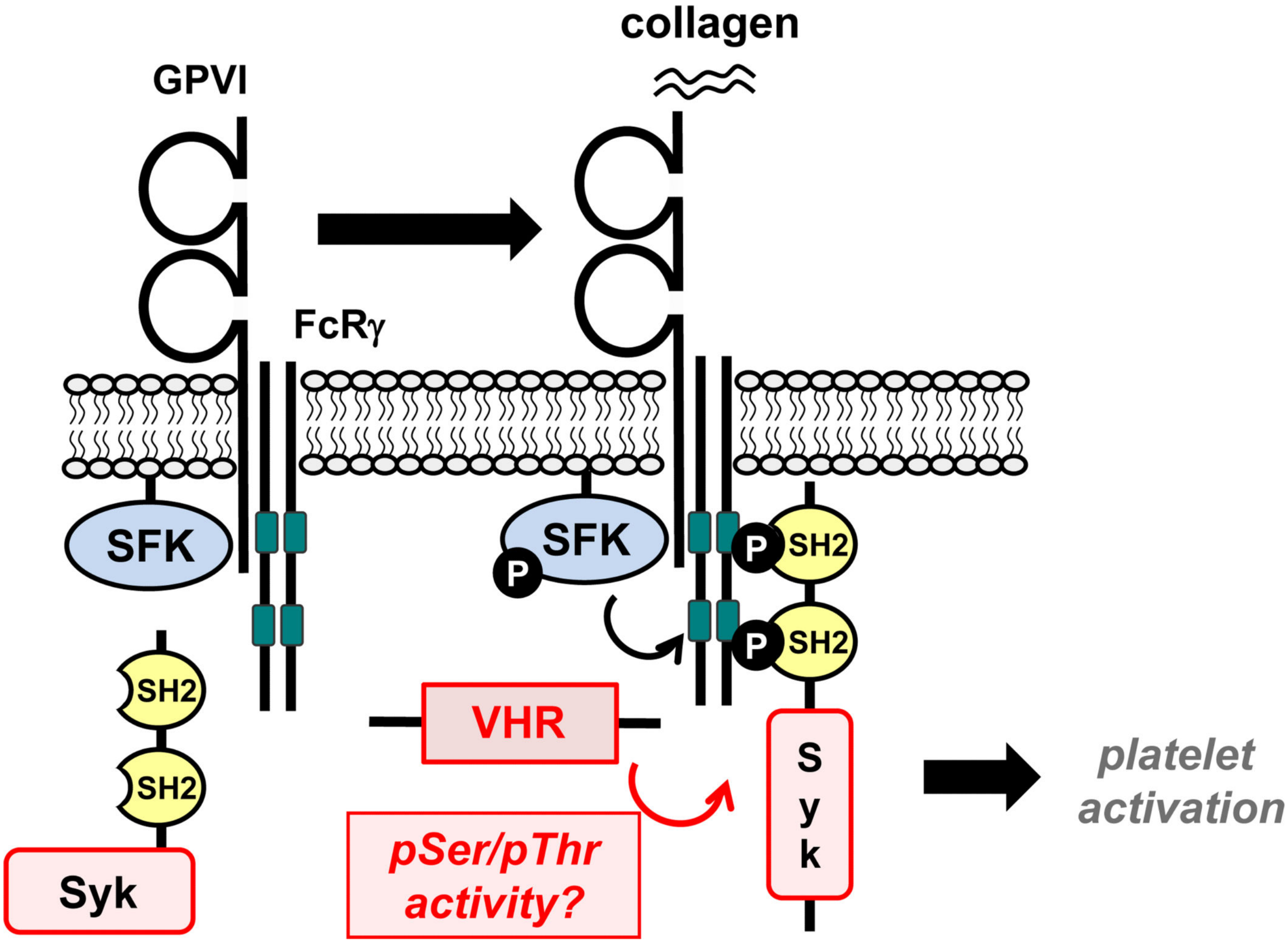


Figure 4. Putative functions of SHP1 and SHP2 in platelets and megakaryocytes

(A/B) SHP1 and SHP2 have been implicated in regulating proximal signaling events downstream of the immunoreceptor tyrosine-based activation motif (ITAM)-containing collagen receptor complex GPVI-FcRγ, and the hemi-ITAM-containing podoplanin receptor CLEC-2. **(C)** SHP2 has also been implicated as a negative regulator of the integrin $\alpha_{IIb}\beta_3$ in mice. It should be noted that the ITAM-containing low affinity immunoglobulin receptor FcγRIIA acts as a docking site for Syk downsream of $\alpha_{IIb}\beta_3$ in human platelets, but not in mouse platelets, which do not express FcγRIIA. **(D)** The best-characterized function of SHP2 in the megakaryocyte lineage is its positive regulatory role of the Ras-ERK1/2 pathway, downstream of the thrombopoietin (Tpo) receptor MPL. However, the exact mechanism remains ambiguous. Functions illustrated in this figure are mainly based on findings from SHP1 and SHP2 conditional knockout mice and need to be validated in mouse and human platelets through the use of SHP1- and SHP2-specific inhibitors.


collagen
GPVI
FcRγ
SFK
SFK
P
P
SH2
P
SH2
SH2
SH2
Syk
VHR
pSer/pThr activity?
S y k
platelet activation

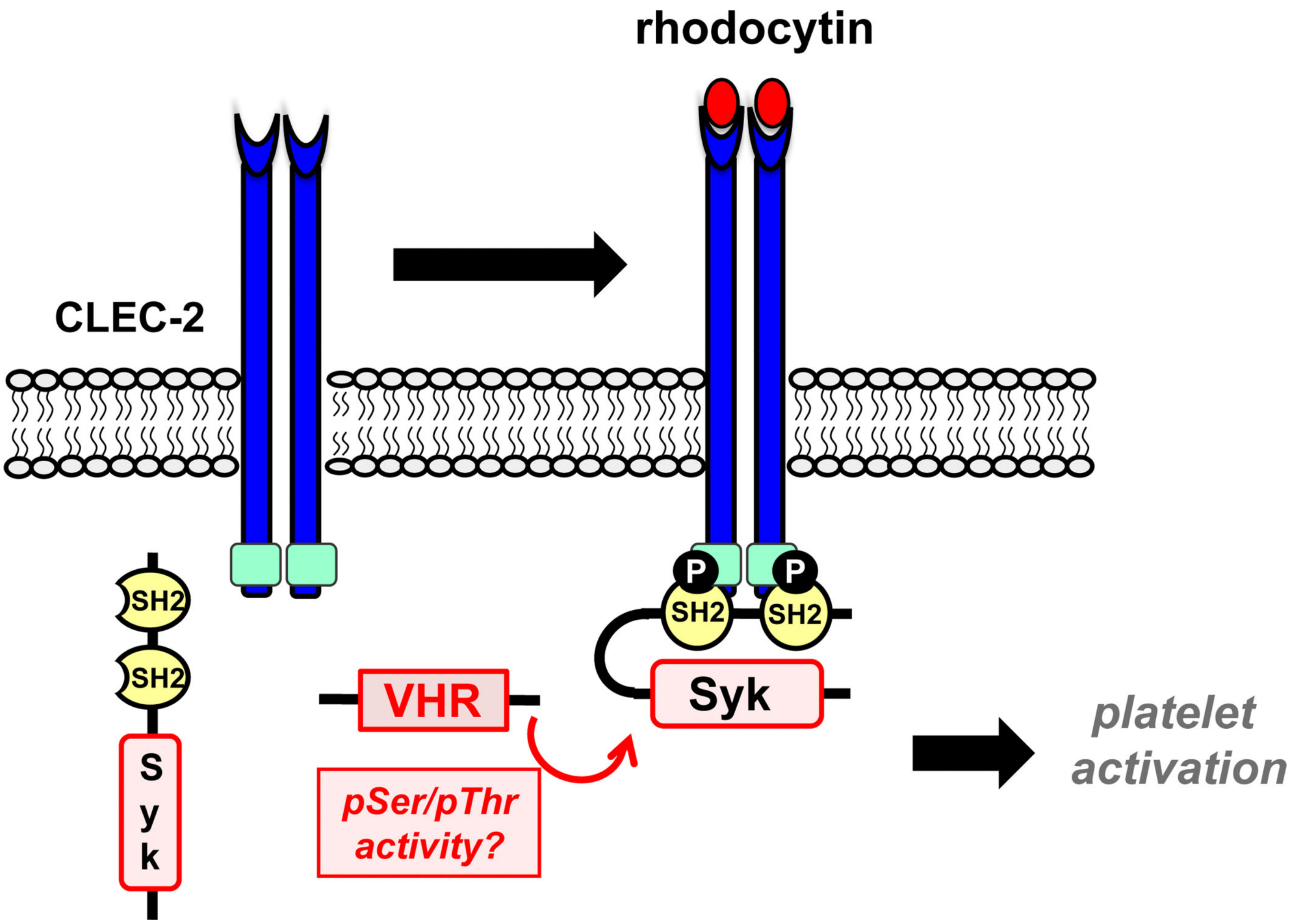
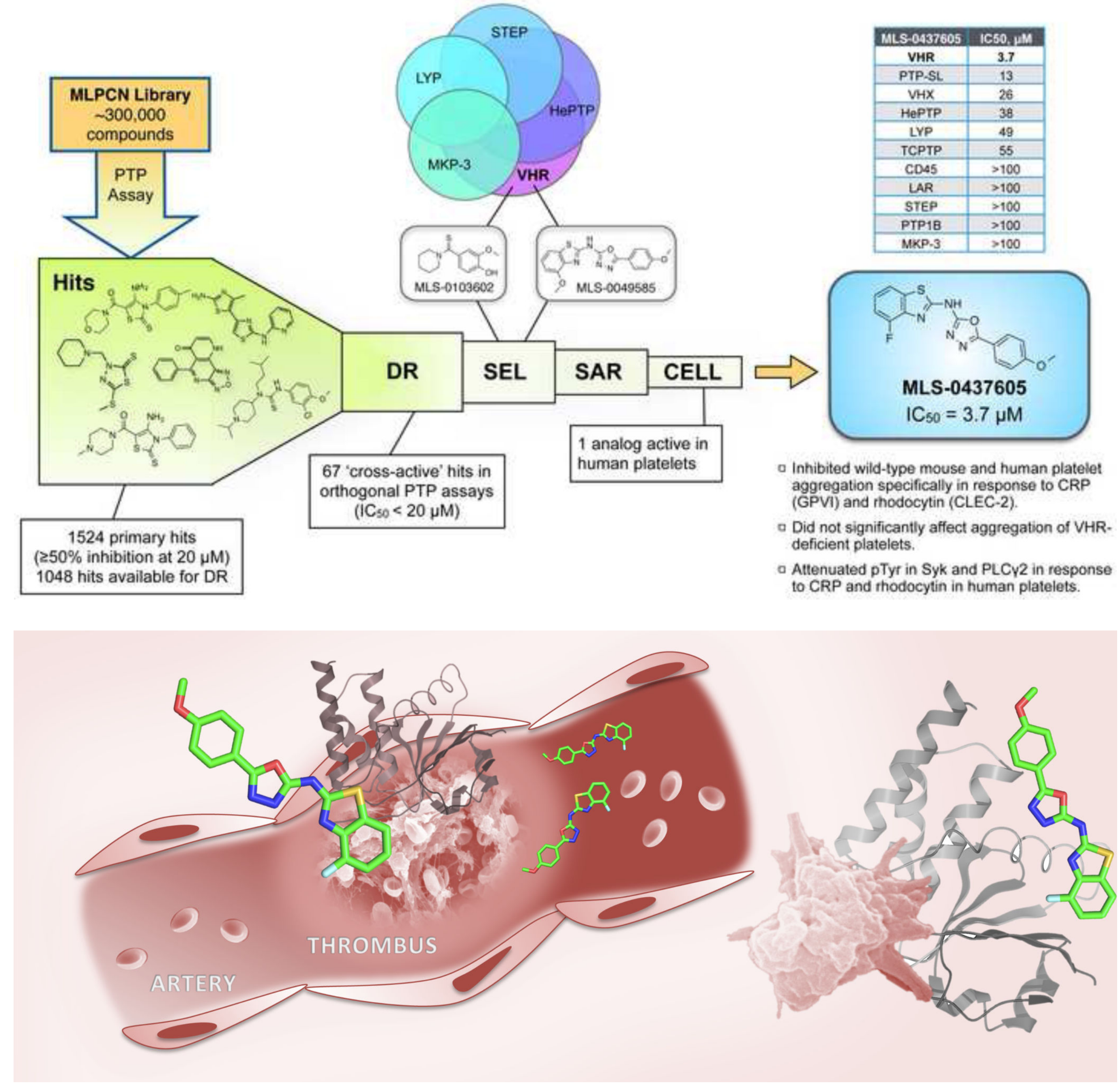

rhodocytin
CLEC-2
SH2
SH2
Syk
VHR
pSer/pThr activity?
P
P
SH2
SH2
Syk
platelet activation

Figure 5. VHR is a positive regulator of platelet activation in response to GPVI and CLEC-2 stimulation

Upon GPVI **(A)** and CLEC-2 **(B)** receptor stimulation, VHR positively regulates the phosphorylation of GPVI-FcRγ-associated ITAMs (A, dark green) and CLEC-2-associated hemi-ITAMs (B, light green), resulting in the recruitment of Syk to these receptors, and subsequent platelets activation.


MLPCN Library
~300,000
compounds
PTP
Assay
Hits
DR
SEL
SAR
CELL
1524 primary hits
(≥50% inhibition at 20 μM)
1048 hits available for DR
67 'cross-active' hits in
orthogonal PTP assays
(IC50 < 20 μM)
STEP
LYP
HePTP
MKP-3
VHR
MLS-0103602
MLS-0049585
1 analog active in
human platelets
MLS-0437605
IC50 = 3.7 μM
THROMBUS
ARTERY

MLS-0437605	IC50, μM
VHR	**3.7**
PTP-SL	13
VHX	26
HePTP	38
LYP	49
TCPTP	55
CD45	>100
LAR	>100
STEP	>100
PTP1B	>100
MKP-3	>100

- ▫ Inhibited wild-type mouse and human platelet aggregation specifically in response to CRP (GPVI) and rhodocytin (CLEC-2).
- ▫ Did not significantly affect aggregation of VHR-deficient platelets.
- ▫ Attenuated pTyr in Syk and PLCγ2 in response to CRP and rhodocytin in human platelets.

Figure 6. Development of a VHR-specific inhibitor

High-throughput screening (HTS) of the Molecular Libraries Probe Production Centers Network (MLPCN) compound collection, using a colorimetric phosphatase assay with *p*-nitrophenyl phosphate (pNPP) as substrate, resulted in 1524 primary hits. Of these hits, 1048 compounds were available for dose-response (DR) hit confirmation assays using two orthogonal assay formats, the colorimetric pNPP assay, as well as a fluorimetric assay using *O*-methylfluorescein phosphate (OMFP) as substrate. 67 cross-active hits with IC_{50} values <20 μM in both assays were taken into selectivity profiling experiments (SEL) against four additional PTPs (HePTP, STEP, LYP, and MKP-3). Based on potency and selectivity, two scaffolds (MLS-0103602 and MLS-0049585) were selected for structure-activity relationship (SAR) studies. Several analogs with similar or improved potency/selectivity could be identified for MLS-0049585. The four most active analogs were chosen for cell-based assays (CELL) and tested on human platelets freshly isolated from healthy donors. Compound MLS-0437605, which inhibited recombinant VHR with an IC_{50} of 3.7 μM, effectively inhibited platelet aggregation at IC_{50} concentration, specifically in response to platelet stimulation with collagen-related peptide (CRP; GPVI stimulation) and rhodocytin (CLEC-2 stimulation), but not after stimulation with the TXA_2 analog U46619, thereby phenocopying the effect of VHR deficiency on mouse platelets. Selectivity of MLS-0437605 for VHR was evaluated against a total of 10 recombinant PTPs, as well as under physiological conditions on VHR-deficient platelets, whose aggregation was only minimally affected by MLS-0437605. Finally, MLS-0437605 (3.7 μM) decreased tyrosine phosphorylation of Syk and PLCγ2 in human platelets in response to CRP and rhodocytin, similar to the effect of VHR deficiency on mouse platelets. (Please see Musumeci et al.[46] to review the actual data.)

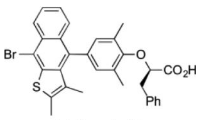
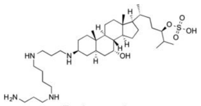
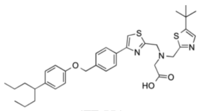
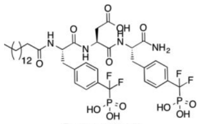
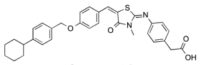
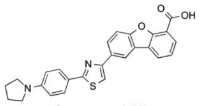
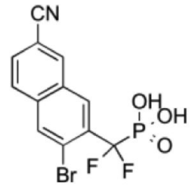
Table 1

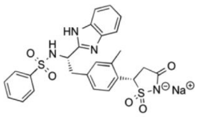
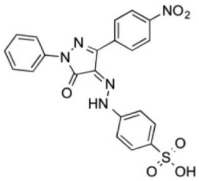
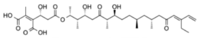
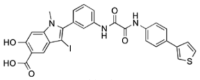
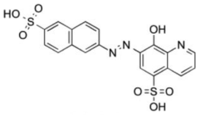
Protein tyrosine phosphatases (PTPs) implicated in platelet function

PTP	Functions	Evidence	References
CD148 (PTPRJ)	-positive regulator of GPVI- and CLEC-2-mediated platelet activation, aggregation and $\alpha_{IIb}\beta_3$-mediated spreading -positive and negative regulator of SFKs	-CD148-deficient mouse model -transiently transfected cell line -*in vitro* biochemical	Senis Y et al. *Blood.* 2009, 113:4942-54; Ellison S et al. *J Thromb Haemost.* 2010, 8:1575-83; Mori J et al. *Arterioscl Thromb Vasc Biol.* 2012, 32:2956-65
PTP1B (PTPN1)	-positive regulator of late stage platelet activation and aggregation -positive regulator of SFKs -implicated in dephosphorylating LAT	-PTP1B-deficient mouse models -transiently transfected cell line -*in vitro* biochemical	Frangioni JV et al. *EMBO J.* 1993, 12:4843-56; Ragab A et al. *J Biol Chem.* 2003, 278:40923-32; Arias-Salgado EG et al. *J Cell Biol.* 2005, 170:837-45; Kuchay SM et al. *Mol Cell Biol.* 2007, 27:6038-52
SHP1 (PTPN6)	-positive regulator of GPVI-mediated platelet aggregation and $\alpha_{IIb}\beta_3$-mediated spreading -positive regulator of SFKs -negative regulator of GPCR signaling -mediates release of RGS10 and RGS18 from sphinophilin -dephosphorylates actinin downstream of $\alpha_{IIb}\beta_3$	-SHP1-deficient mouse models -transiently transfected cell line -pharmacological (inhibitors: NSC87877, PTPI-1) -*in vitro* biochemical	Pasquet JM et al. *J Biol Chem.* 2000, 275:28526-31; Lin SY et al. *J Biol Chem.* 2004, 279:25755-64; Tadokoro S et al. *Blood.* 2011, 117:250-8; Ma P et al. *Blood.* 2012, 119:1935-45; Mazharian A et al. *Blood.* 2013, 121:4205-20
SHP2 (PTPN11)	- negative regulator of platelet GPVI- and CLEC-2-mediated activation, aggregation and $\alpha_{IIb}\beta_3$-mediated spreading	-SHP2-deficient mouse model -transiently transfected cell line -*in vitro* biochemical	Jackson DE et al. *J Biol Chem.* 1997, 272:6986-93; Newman DK et al. *Blood.* 2001, 97:2351-7; Mazharian A et al. *Blood.* 2013, 121:4205-20
PTP-MEG2 (PTPN9)	- biogenesis and fusion of vesicle membranes with the plasma membrane	- PTP-MEG2-deficient mouse model	Wang Y et al. *J Exp Med.* 2005, 202:1587-97
VHR (DUSP3)	- positive regulator of GPVI- and CLEC-2-mediated platelet activation and aggregation	-VHR-deficient mouse model -pharmacological (inhibitor: MLS-0437605)	Musumeci L et al. *Circulation.* 2015, 131:656-68
PTEN	- negative regulator of GPVI-mediated platelet activation and aggregation	- PTEN-deficient mouse model	Weng Z et al. *Blood.* 2010, 116:2579-81
LMPTP (ACP1)	- implicated in down-regulating FcγRIIA-mediated platelet activation	-transiently transfected cell line -*in vitro* biochemical	Mancini F et al. Blood. 2007, 110:1871-8

Table 2

Selected pharmacological inhibitors of PTP1B and SHP2 with demonstrated activity in cells and/or *in vivo*.

Structure/Name	Potency	Activity in cells	Activity *in vivo*	Notes and references
PTP1B Inhibitors				
 Ertiprotafib	IC_{50} = 384 nM	Increased phosphorylation of insulin receptor (IR) and IR substrate (IRS) 1 in 3T3-L1 adipocytes.	Lowered glucose, insulin, and triglycerides in rodent diabetes models.	Progressed to phase II clinical trials for treatment of type 2 diabetes (*Wyeth Research*). Erbe D et al. *Mol Pharmacol.* 2005, 67:69-77
 Trodusquemine	IC_{50} = 1,000 nM	Increased insulin-stimulated phosphorylation of IR in HepG2 cells	Caused obesity-dependent body weight loss.	Lantz KA et al. *Obesity.* 2010, 18:1516-23. Trodusquemine is an allosteric PTP1B inhibitor (Krishnan N et al. *Nat Chem Biol.* 2014, 10:558-66). It was tested in phase I clinical trials for treatment of type 2 diabetes (*Genaera Corp.*). A Phase I clinical trial in HER2-positive breast cancer patients is planned (*DepYmed, Inc.*).
 JTT-551	K_i = 220 nM	Increased insulin-stimulated glucose uptake in L6 cells.	Improved glucose metabolism in rodents by enhancing insulin signaling.	Fukuda S et al. *Diabetes Obes Metab.* 2010, 12:299-306
 Compound II	K_i = 26 nM	Increased phosphorylation of IR and IRS-1, enhanced glucose uptake, Elk1 phosphorylation, and cell proliferation, and activated Akt and ERK1/2 in CHO/HIRc cells.	Restored leptin sensitivity in mature, leptin-resistant rats.	Xie L et al. *Biochemistry.* 2003, 42:12792-804 Morrison C et al. *Endocrinology.* 2007, 148:433-40
 Compound 2	IC_{50} = 240 nM		Improved oral glucose tolerance and decreased plasma glucose and triglyceride levels in diet-induced obese mice.	Arora SK et al. PCT Int Appl, WO 2009/109998
 Compound 5E	IC_{50} = 82 nM		Caused reduction in body weight, fed-state whole blood glucose (WBG), fasting WBG, plasma glucose, and plasma cholesterol levels in *ob/ob* mice.	Lakshminarayana N et al. *Eur J Med Chem.* 2010, 45:3709-18
 Compound 3g	IC_{50} = 120 nM		Inhibited glucose excursion in DIO mice. Caused a delay in the onset of tumor development in NDL2 *Ptpn1* transgenic mice.	Han Y et al. *Bioorg Med Chem Lett.* 2008, 18:3200-5

Structure/Name	Potency	Activity in cells	Activity *in vivo*	Notes and references
 Compound 79a	IC_{50} = 35 nM	Increased phosphorylation of IR in HEK293 cells transfected with plasmids encoding human IR.		Combs AP et al. *J Med Chem.* 2006, 49:3774-89
SHP2 Inhibitors				
 PHPS1	IC_{50} = 2,100 nM	Inhibited hepatocyte growth factor/scatter factor (HGF/SF)-induced epithelial cell scattering and branching morphogenesis. Blocked HGF/SF-induced sustained phosphorylation of ERK1/2 and dephosphorylation of paxillin. Blocked anchorage-independent growth of tumor cell lines.		Hellmuth K et al. *Proc Natl Acad Sci U S A.* 2008, 105:7275-80.
 Tautomycetin (TTN)	IC_{50} = 2,900 nM	Blocked T cell receptor–mediated ERK1/2 activation and SHP2 gain-of-function mutant-induced hematopoietic progenitor hyperproliferation and monocytic differentiation.		Liu S et al. *Chem Biol.* 2011, 18:101-10.
 11a-1	IC_{50} = 200 nM	Reduced lung cancer cell (H1975) proliferation and blocked SHP2-dependent signaling. Inhibited ERK1/2 and Akt activity and ErbB2+ breast cancer cell growth (SKBR3).		Zheng LF et al. *J Med Chem.* 2014, 57:6594-609.
 NSC-87877	IC_{50} = 318 nM	Inhibited EGF-induced activation of SHP2, Ras, and ERK1/2 in HEK293 cells.		Chen L et al. *Mol Pharmacol.* 2006, 70:562-70 NSC-87877 does inhibit SHP1 with a similar IC_{50} value.