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REVIEW ARTICLE

Protein-tyrosine phosphatases: a new frontier in platelet signal transduction

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Summary. Platelet activation must be tightly controlled in order to allow platelets to respond rapidly to vascular injury and prevent thrombosis from occurring. Protein-tyrosine phosphorylation is one of the main ways in which activation signals are transmitted in platelets. Although much is known about the protein-tyrosine kinases (PTKs) that initiate and propagate activation signals, relatively little is known about the protein-tyrosine phosphatases (PTPs) that modulate these signals in platelets. PTPs are a family of enzymes that dephosphorylate tyrosine residues in proteins and regulate signals transmitted within cells. PTPs have been implicated in a variety of pathological conditions, including cancer, diabetes and autoimmunity, but their functions in hemostasis and thrombosis remain largely undefined. Exciting new findings from a number of groups have revealed that PTPs are in fact critical regulators of platelet activation and thrombosis. The primary aim of this review is to highlight the unique and important functions of PTPs in regulating platelet activity. Establishing the functions of PTPs in platelets is essential to better understand the molecular basis of thrombosis and may lead to the development of improved antithrombotic therapies.

Tyrosine phosphorylation is essential for platelet activation

Platelet activation is a rapid process that causes platelets to transform from a resting, non-adherent state to an adherent, pro-thrombotic state. The rate of activation is especially important in the high shear environment of the arterial system, where platelets must quickly adhere to sites of vascular injury and form thrombi that prevent excessive blood loss. Aberrant platelet activation can have life-threatening consequences. A slow or weak response can result in excessive bleeding, whereas platelet hyperactivity can causes thrombosis, myocardial infarction and stroke. A better understanding of the molecular basis of platelet activation has important clinical implications and can lead to the identification of novel antithrombotic drug targets.

Protein-tyrosine phosphorylation is one of the main ways in which activation signals are transmitted in platelets. Phosphorylation of key tyrosine residues in signaling proteins by protein-tyrosine kinases (PTKs) provides docking sites for Src homology 2 (SH2) domain-containing proteins and alters the catalytic activity of enzymes (Fig. 1). Platelet contact with exposed extracellular matrix proteins induces a rapid burst in PTK activity and a dramatic increase in intracellular tyrosine phosphorylation. This is primarily mediated by Src family kinases (SFKs) and Syk kinase activated downstream of the immunoreceptor tyrosine-based activation motif (ITAM)-containing collagen receptor complex GPVI-FcR γ-chain [1]. Collagen-induced clustering of GPVI causes an increase in GPVI-associated SFK activity, phosphorylation of tyrosine residues in the FcR γ-chain ITAM and recruitment of the tyrosine kinase Syk [2]. SFKs and Syk propagate the signal, which leads to the activation of the lipid hydrolase phospholipase (PLC)γ2, generation of inositol triphosphate and diacylglycerol, an increase in PKC activity and a rise in intracellular calcium [3]. The SFK-Syk-PLCγ2 signaling pathway is utilized by many platelet activation receptors, including the hemi-ITAM-containing podoplanin receptor CLEC-2 [4], the integrin αIIbβ3 [2] and the VWF receptor GPIb-IX-V [5]. However, the kinetics and strength of the signal differ for each receptor, depending upon the ligand and downstream effectors [6–8].

Although much is known about the PTKs that transmit activation signals in platelets, comparatively little is

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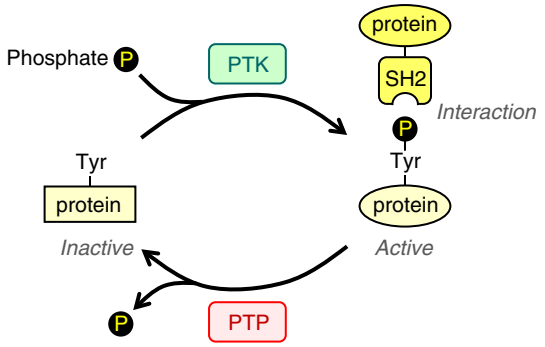

Fig. 1. Reciprocal regulation of protein-tyrosine (Tyr) phosphorylation by protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs). Tyrosine phosphorylation regulates protein-protein interactions by providing docking sites for Src homology 2 (SH2) domain-containing proteins and alters the catalytic activity of enzymes.

known about the protein-tyrosine phosphatases (PTPs) that dephosphorylate tyrosine residues and modulate these signals. PTPs work in conjunction with PTKs to modulate activation signals in all cells (Fig. 1). The primary aim of this review is to introduce the wider platelet community to PTPs and highlight their critical and unique functions as both positive and negative regulators of platelet activation and thrombosis.

The classical protein-tyrosine phosphatases

Protein phosphatases are divided into separate families that are structurally and mechanistically distinct [9]. These include the serine/threonine-specific phosphoprotein phosphatases (PPPs) and the PTPs [10]. The PTP family is further divided into the tyrosine-specific classical PTPs and the dual-specificity phosphatases (DSPs) that have the capacity to dephosphorylate serine, threonine and tyrosine residues, and in some instances, lipids and mRNA [11]. The classical PTPs are further divided into the receptor-like PTPs and the non-transmembrane PTPs, depending on whether they contain a transmembrane domain (Fig. 2) [11]. Humans have 21 genes that code for receptor-like PTPs and 16 genes that code for non-transmembrane PTPs (Table 1) [9]. Mouse orthologues exist for all of the classical PTPs. All classical PTPs have a ~ 280 amino acid PTP catalytic domain that contains the active-site signature motif HC-(X_5)-R found in all PTPs and is essential for catalysis [12]. Most receptor-like PTPs contain tandem PTP domains in their cytoplasmic tails, of which the membrane-proximal domain (D1) is catalytically active and the membrane-distal domain (D2) is catalytically inactive (Fig. 2). In contrast, non-transmembrane PTPs have a single PTP domain flanked by motifs and domains that regulate compartmentalization and activity (Fig. 2) [12]. The ectodomains of the receptor-like PTPs come in various shapes and sizes and typically contain domains found in adhesion proteins, including immunoglobulin-like, fibronectin type III and meprin/A5/μ (MAM) domains (Fig. 2) [12]. The ligands of most receptor-like PTPs are not known [13]. It should be noted that most PTPs have multiple names that complicate the literature. However, this has now been standardized to PTPRA-Z for receptor-like PTPs and PTPN1-23 for non-transmembrane PTPs (Table 1).

A long-standing misconception was that PTPs were simply housekeeping enzymes that inhibited cellular responses and lacked substrate specificity. In fact, PTPs are tightly controlled, highly specific enzymes that can both activate and inhibit cellular responses, depending on their subcellular localization and downstream targets [12]. PTPs regulate a variety of important cellular functions, including differentiation, proliferation, adhesion, migration and secretion. Many have also been implicated in major pathological conditions, including cancer, diabetes and autoimmunity [14]. This begs the question, what are PTPs doing in platelets?

Classical protein-tyrosine phosphatases in platelets

To date, 10 receptor-like and 10 non-transmembrane PTPs have been identified in human platelets using proteomics-based approaches (Table 1) [15,16]. Transcripts of most of these PTPs have also been identified in human megakaryocytes (Table 1) [17–19]. Differences between the two datasets may reflect differential expression during megakaryocyte development and platelet production or shortcomings of the techniques employed. Only a few PTPs have been shown to be expressed in platelets and megakaryocytes by antibody-based techniques and the functional roles of most remain undefined.

Early studies of protein phosphatases in platelets relied on the use of non-specific inhibitors, including the PPP inhibitor okadaic acid and the broad spectrum PTP inhibitor pervanadate [20,21]. These studies provided the first evidence of the importance of protein phosphatases in regulating platelet function; however, they failed to provide insights into the functions of specific protein phosphatases. Indeed, the lack of specific PTP inhibitors has hindered our progress in elucidating the functions of PTPs in platelets. More recently, genetically modified mouse models have been used to address the question of the physiological functions of individual PTPs in platelets and thrombosis. Tables 2 and 3 summarize key findings of some of these studies and highlight the unique functions played by receptor-like and non-transmembrane PTPs in platelets.

CD148: master regulator of Src family kinases in platelets

CD148 (also referred to as DEP-1, PTPRJ and RPTPη) was the first receptor-like PTP identified in platelets [22]. We first became interested in CD148 when we repeatedly

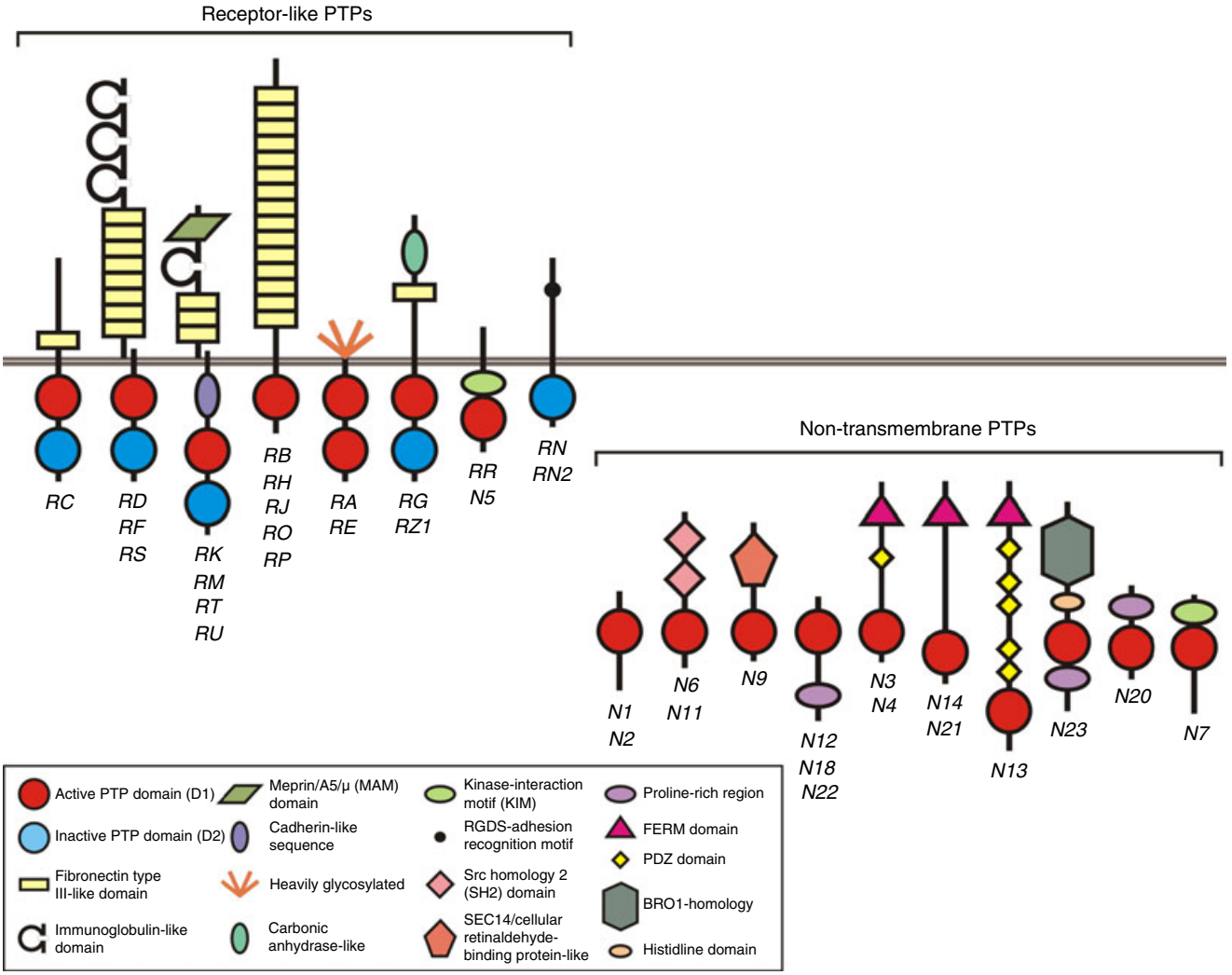

Fig. 2. The classical protein-tyrosine phosphatase (PTP) family. Classical PTPs specifically dephosphorylate tyrosine residues. Humans have 21 receptor-like PTPs that contain a transmembrane domain and ectodomain, and 16 non-transmembrane PTPs that lack a transmembrane domain. Mouse orthologs exist for all of the classical PTPs. Domains and motifs are indicated in the legend. The standardized nomenclature is PTPRA-Z for receptor-like PTPs, and PTPN1-23 for non-transmembrane PTPs [11]. Figure reproduced with permission from the Nature Publishing Group.

identified it in human platelets using a proteomics-based approach, suggesting it was highly expressed [23]. We hypothesized that CD148 was a CD45-like PTP that initiated GPVI-FcR γ-chain signaling. This was based on the well-established role of CD45 as an activator of SFKs downstream of the ITAM-containing B and T cell receptors, which signal in a similar way to the GPVI-FcR γ-chain complex [24]. CD45 is highly expressed in all hematopoietic cells, except platelets and erythrocytes. To test our hypothesis we studied platelets from CD148-deficient mice. Platelet counts were normal in these mice, but CD148-deficient platelets responded less well than control platelets to the GPVI-specific agonist collagen-related peptide (CRP) [25]. Responses were normal to intermediate concentrations of agonists that signal through G protein-coupled receptors, including thrombin, U46619 (thromboxane A_2 analogue) and ADP, suggesting a defect in tyrosine kinase-linked signaling [25]. The reduced CRP response was partially due to an approximately 50% reduction in GPVI-FcR γ-chain expression and a marked reduction in SFK activity, indirectly measured as a reduction in auto-phosphorylation of the activation loop tyrosine residues of all SFKs [25,26].

Interestingly, SFK activity was also reduced under non-stimulated conditions, suggesting CD148 is essential for maintaining a pool of active SFKs in resting platelets [25]. The C-terminal inhibitory tyrosine residues of Lyn, Fyn and Src were concomitantly hyper-phosphorylated, suggesting these sites are substrates of CD148, and supporting the notion that SFKs were predominantly in an inactive conformation in CD148-deficient platelets [25,27]. Thus, it appeared as though CD148 constitutively antagonizes the action of C-terminal Src kinase (Csk) that phosphorylates the inhibitory tyrosine residues of SFKs and

Table 1 Human platelet and megakaryocyte PTPomes

Gene	Protein names	Platelet PTP proteome [15,16]	Megakaryocyte PTP transcriptome [17–19]
Receptor-like PTPs			
Ptpra	PTPRA, RPTPα	■	■
Ptprb	PTPRB, RPTPβ	■	
Ptprc	PTPRC, CD45, LCA	■	■
Ptprd	PTPRD, RPTPδ		
Ptpre	PTPRE, RPTPε	■	■
Ptprf	PTPRF, LAR		
Ptprg	PTPRG, RPTPγ	■	
Ptprh	PTPRH, SAP1		
Ptprj	PTPRJ, DEP1, CD148, RPTPη	■	■
Ptprk	PTPRK, RPTPκ	■	
Ptprm	PTPRM, RPTPμ		
Ptprn	PTPRN, IA-2, Islet cell antigen 512		
Ptprn2	PTPRN2, PTPRP, RPTPπ, IA-2β, phogrin		
Ptpro	PTPRO, PTPROτ isoforms A, B, C, GLEPP1, PTP-U2	■	
Ptprq	PTPRQ, PTPS31		
Ptprr	PTPRR, PTP-SL, PCPTP, PTPBR7, PC12-PTP1	■	■
Ptprs	PTPRS, RPTPσ		
Ptprt	PTPRT, RPTPρ	■	
Ptpru	PTPRU, PTPJ, PTP-U1, PTPRo isoforms 1, 2, 3		
Ptprv	PTPRV, OST-PTP		
Ptprz	PTPRZ, RPTPζ		
Non-transmembrane PTPs			
Ptpn1	PTPN1, PTP-1B	■	■
Ptpn2	PTPN2, TC-PTP, MPTP, PTP-S	■	■
Ptpn3	PTPN3, PTPH1		
Ptpn4	PTPN4, PTP-MEG1, TEP	■	■
Ptpn5	PTPN5, STEP		
Ptpn6	PTPN6, Shp1, PTP1C, SH-PTP1, HCP	■	■
Ptpn7	PTPN7, HePTP, LCPTP		
Ptpn9	PTPN9, PTP-MEG2		
Ptpn11	PTPN11, Shp2, SH-PTP2, Syp, PTP1D, PTP2C, SH-PTP3	■	■
Ptpn12	PTPN12, PTP-PEST, PTP-P19, PTPG1	■	■
Ptpn13	PTPN13, PTP-BAS, FAP-1, PTP1E, RIP, PTPL1, PTP-BL	■	■
Ptpn14	PTPN14, PTP36, PEZ, PTPD2		
Ptpn18	PTPN18, PTP-HSCF, PTP20, BDP	■	■
Ptpn20	PTPN20, TypPTP		
Ptpn21	PTPN21, PTPD1, PTP2E, PTP-RL10		
Ptpn22	PTPN22, LYP, PEP		■
Ptpn23	PTPN23, HD-PTP, PTP-TD14	■	■

PTPomes, protein-tyrosine phosphatase-omes; gray denotes expression.

reduces their activity [28] (Fig. 3). CD148-deficient platelets also failed to spread normally on fibrinogen, which is dependent on outside-in signaling via the integrin αIIbβ3 and SFK-Syk-PLCγ2 pathway [25] (Fig. 3). Indeed, CD148-deficient platelets responded less well to all agonists tested that signal predominantly via SFKs, including CLEC-2 [29], establishing CD148 as a master positive regulator of SFKs in platelets. Collectively, the platelet defects resulted in reduced thrombus formation following laser-induced injury of arterioles and a mild bleeding diathesis in CD148-deficient mice (Table 3) [25].

However, the CD148 story is not quite that simple. There is another level of complexity that was not immediately apparent from the knockout mouse studies. In a follow-up study, we found that CD148 also has the capacity to attenuate SFK activity by directly dephosphorylating the activation loop [26] (Fig. 3). The same function was reported in T cells by Brdicka *et al.* [30]. Similarly, Alexander and Weiss independently demonstrated that CD45, which performs similar functions to CD148, can reciprocally regulate SFK activity in immune cells by dephosphorylating either the inhibitory or activation loop tyrosine residues [31,32]. This was explained by the level of CD45 expression. A high level of CD45 predominantly inhibits SFK activity by dephosphorylating the activation loop tyrosine, whereas a low level of expression favors SFK activation by dephosphorylating the inhibitory tyrosine [31,32]. Thus, CD45 and CD148 act as molecular switches that modulate optimal levels of SFK activity in resting and activated conditions (Fig. 3). However, the net effect of deleting CD148 in platelets was to reduce SFK and platelet activity.

Table 2 Functions of classical PTPs in platelets and megakaryocytes

PTP	Localization	Functions
Receptor-like PTP		
CD148	Plasma membrane	Master positive regulator of SFKs [25,29]
		Implicated as a negative regulator of SFKs [26]
Non-transmembrane PTPs		
PTP-1B	Cytosolic surface of ER	Positive regulator of late-stage platelet activation and aggregation [45,50]
	Calpain-mediated shedding from ER	Positive regulator of SFKs downstream of αIIbβ3 [51]
	Translocation to PM	Implicated in dephosphorylating LAT downstream of FcγRIIA [54]
Shp1	Cytosolic	Positive regulator of SFKs downstream of GPVI and αIIbβ3 in platelets [83]
	Associates with phosphorylated	Negative regulator of GPCR signaling in platelets [79]
	ITIM-containing receptors and adapters	Mediates release of RGS10 and RGS18 from sphinophilin [79]
		Dephosphorylates actinin downstream of αIIbβ3 [80,81]
		Required for megakaryocyte polyploidization [83,101]
Shp2	Cytosolic	Inhibits GPVI signaling by complexing with PECAM-1 [73,76]
	Associates with phosphorylated	Negative regulator of CLEC-2- and αIIbβ3-mediated platelet responses [83]
	ITIM-containing receptors and adapters	Positive regulator of Mpl signaling in megakaryocytes [83,85,86]
		Positive regulator of αIIbβ3-mediated response in megakaryocytes [83]
		Positive regulator of proplatelet formation [83]
MEG2	Cytosolic	Biogenesis and fusion of vesicle membranes with the plasma membrane [88]
	Associates with PIP_2 and PIP_3 in the PM	

PTP, protein-tyrosine phosphatase; PM, plasma membrane; ER, endoplasmic reticulum; SFKs, Src family kinases; ITIM, immunoreceptor tyrosine-based inhibition motif; GPCR, G protein-coupled receptor; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate.

Consistent with CD148 being an important regulator of platelet activation, we found very little inter-individual variation in CD148 expression in human platelets taken from different individuals [25]. Recent findings by Gruel *et al.* [33] revealed that specific polymorphisms in the extracellular region of CD148 (Q276R and R326Q) have a protective effect in the development of heparin-induced thrombocytopenia by reducing platelet activity in patients with antibody-platelet factor 4 (PF4)/heparin complexes. Antibody-PF4/heparin complexes mediate platelet activation via clustering of the ITAM-containing FcγRIIA receptor [33]. It is proposed that the Q276R and R326Q substitutions introduce torsional stress and a loss of positive charge in the second fibronectin type III domain of CD148, which may affect ligand binding or membrane compartmentalization of CD148 [33].

Several important questions remain regarding CD148 in platelets, including how it is regulated and whether it has a ligand. The PTP domain of CD148 lacks an inhibitory wedge and does not form dimeric structures *in vitro* under physiological buffer conditions, suggesting it is not auto-inhibited by dimerization [24,34], as is the case for other receptor-like PTPs, including CD45 and RPTPα [35,36]. Matrigel, a mixture of extracellular matrix proteins that includes large amounts of collagen, has been shown to increase CD148 activity in transfected porcine aortic endothelial cells [37]. More recently, the ectodomain of CD148 was shown to bind to the extracellular region of the transmembrane heparin sulphate proteoglycan syndecan-2 and the secreted glycoprotein thrombospondin-1, suggesting they are ligands of CD148 [38,39]. The kinetic-segregation model provides an alternative mechanism of how CD148 may be regulated that does not involve a ligand. According to this model, CD45 and CD148 are excluded from tight immunological synapses that form between T cells and antigen-presenting cells by their large ectodomains [40]. Thus, they cannot access components of the T-cell receptor signaling pathway in tight cell-cell contacts. This raises the possibility that CD148 is also excluded from platelet-platelet contacts.

It would be naïve to think that SFKs are the only substrates of CD148 in platelets. Putative substrates identified in other cell types include LAT, PLCγ1, Gab-1 and phosphatidylinositol 3-kinase (PI3-kinase), all of which are expressed in platelets [41–43]. However, demonstrating that they are hyper-phosphorylated and *bona fide* physiological substrates of CD148 in CD148-deficient platelets is complicated by the fact that they all lie downstream of SFKs and SFK activity is markedly reduced in these platelets. Over-expression of substrate-trapping mutants routinely used to identify PTP substrates in nucleated cells cannot be used in platelets [44], which lack a nucleus and cannot be manipulated using common molecular biology-based techniques. Thus, alternative approaches must be employed to identify substrates of CD148 and other PTPs in platelets.

PTP-1B: selective positive regulator of Src family kinases

PTP-1B is a ubiquitously expressed non-transmembrane PTP that consists of an N-terminal PTP domain and a short C-terminal tail containing a sequence of hydrophobic residues that anchors it to the cytosolic surface of the ER (Fig. 4) [45]. Much interest has surrounded PTP-1B for many years, because it inhibits insulin and leptin receptor signaling, and has been implicated in metabolic

Table 3 Phenotypes of classical PTP knockout mouse models

Mouse model	Platelets	Megakaryocytes
CD148 KO [25,26,29]	Normal count and size Reduced rate of recovery Decreased spreading Decreased GPVI expression Decreased SFK-Syk-PLCγ2 signaling Decreased GPVI, αIIbβ3 and CLEC-2 responses Normal hemostasis Decreased thrombosis	Reduced spreading Reduced migration
PTP-1B KO [29,50,51]	Normal count and size Decreased spreading Decreased SFK activation downstream of αIIbβ3 Normal GPVI and CLEC-2 signaling Reduced aggregation on collagen under high shear Normal hemostasis Decreased thrombosis	Not analysed
CD148/PTP-1B DKO [29]	Normal count and size Decreased spreading Decreased GPVI expression Decreased SFK-Syk-PLCγ2 signaling Decreased GPVI, αIIbβ3 and CLEC-2 responses	Not analysed
MP-Shp1 KO [83]	Normal count and size Decreased spreading Decreased GPVI expression Decreased SFK-Syk-PLCγ2 signaling Normal hemostasis Marginally decreased thrombosis	Decreased ploidy Normal spreading Normal proplatelets
MP-Shp2 KO [83]	Mild macrothrombocytopenia Increased clearance Reduced rate of recovery Increased spreading Increased CLEC-2 response Normal hemostasis and thrombosis	Decreased ploidy Decreased spreading Decreased proplatelets Decreased integrin and Mpl signaling
MP-Shp1/2 DKO [83]	Severe macrothrombocytopenia Increased clearance Aberrant receptor expression Decreased response to agonists Increased bleeding	Severely decreased ploidy
MEG2 KO [88]	Hemorrhaging and cerebral infarction during embryogenesis Decreased thrombin response	Not analysed

Megakaryocyte/platelet (MP)-specific Shp1 and Shp2 knockout (KO) mouse models were generated by crossing *Shp1flox/flox* and *Shp2flox/flox* mice with *PF4-Cre+* transgenic mice (76); DKO, double-knockout.

disorders, including diabetes and obesity [46,47]. More recently, PTP-1B has also been shown to regulate Jak-STAT signaling and PTP-1B-deficiency has a protective effect in mouse models of cancer [48,49].

PTP-1B is highly expressed in platelets and was initially implicated in regulating outside-in integrin αIIbβ3 signaling [45]. Neel *et al*. [45] demonstrated that calpain-mediated cleavage within the C-terminal tail of PTP-1B releases it from the cytosolic surface of the ER and increases its catalytic activity following platelet activation (Fig. 4). This was subsequently confirmed by Chishti *et al*. [50] using PTP-1B- and calpain-1-deficient mouse models. The molecular mechanism underlying how PTP-1B initiates outside-in αIIbβ3 signaling was elucidated by Shattil *et al*. [51–53] using a combination of cell line-based assays and knockout mouse model. They showed that PTP-1B displaces Csk from a complex with the β3 subunit and Src, and subsequently activates Src by dephosphorylating its C-terminal inhibitory tyrosine (Fig. 4). Consistent with this model, PTP-1B-deficient mouse platelets did not respond as well as control platelets to fibrinogen, and thrombus formation was dramatically reduced in PTP-1B knockout mice following laser-induced injury of arterioles (Table 3) [51]. PTP-1B-deficient mice had only a minor bleeding diathesis, highlighting PTP-1B as a potential antithrombotic drug target [51]. Interestingly, PTP-1B-deficient platelets responded normally to other platelet agonists, including the GPVI agonist convulxin, ADP and thrombin [51].

Based on these findings, it was concluded that PTP-1B plays a specialized role in regulating outside-in integrin αIIbβ3 signaling. However, in a separate study by Ragab *et al*. [54], PTP-1B was shown to interact with and possibly regulate the adapter protein LAT downstream of the

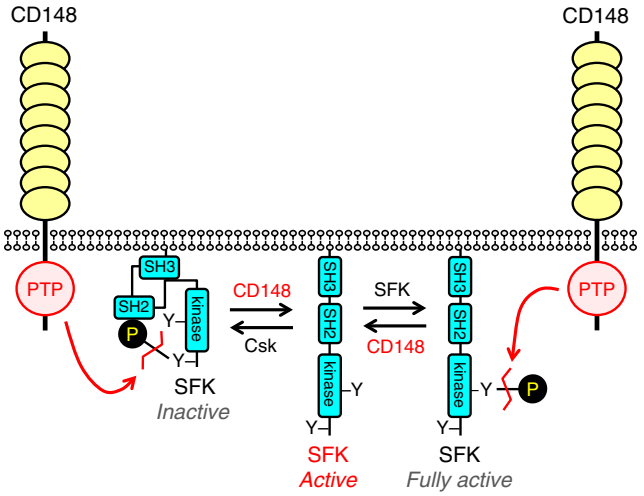

Fig. 3. CD148 is a master regulator of Src family kinases (SFKs) in platelets. CD148 both activates and attenuates SFK activity by dephosphorylating either the C-terminal inhibitory tyrosine residue or the activation loop tyrosine residue, respectively. C-terminal Src kinase (Csk) inhibits SFKs by phosphorylating the C-terminal inhibitory tyrosine residue. *Trans*-autophosphorylation of the activation loop tyrosine residue locks the SFK in a fully active conformation. This is based on the model proposed for the regulation of Lck by CD45 by McNeill *et al.* and Zamoyska [31,102]. Figure reproduced with permission from Elsevier.

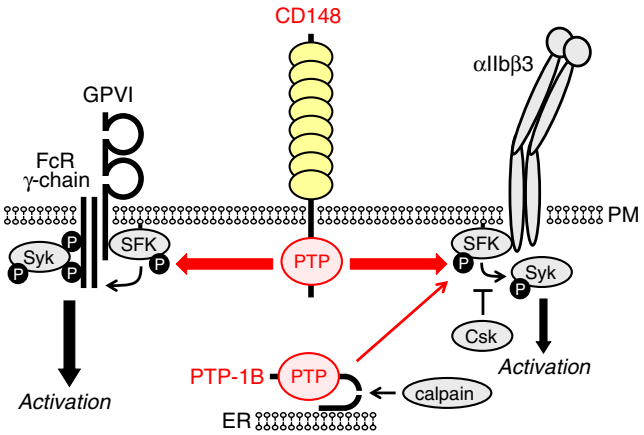

Fig. 4. PTP-1B is a selective positive regulator of αIIbβ3-associated Src family kinases (SFKs). PTP-1B translocates to the plasma membrane (PM) following calpain-mediated cleavage from the cytosolic surface of the endoplasmic reticulum (ER). CD148 positively regulates the GPVI-FcR γ-chain and outside-in integrin αIIbβ3 signaling by maintaining a pool of active SFKs in platelets.

ITAM-containing low affinity IgG receptor FcγRIIA in human platelets, suggesting that PTP-1B may regulate signaling from other tyrosine-kinase linked receptors in platelets. To resolve this issue and to investigate the relative contributions of CD148 and PTP-1B to platelet activation, we directly compared the activities of CD148- and PTP-1B-deficient platelets on the same genetic background to GPVI-, αIIbβ3- and CLEC-2-specific agonists [29]. Our findings revealed that CD148 and PTP-1B perform fundamentally different functions in platelets. CD148 is important for maintaining a pool of active SFKs in platelets, whereas PTP-1B plays a more specialized role, increasing platelet activity to fibrinogen and anti-CLEC-2 antibody (Table 3) [29]. Platelets lacking both PTP-1B and CD148 did not respond as well as single knockout platelets to fibrinogen and anti-CLEC-2 antibody, demonstrating minor additive effects. SFK and Syk activation were reduced in response to CRP, fibrinogen and anti-CLEC-2 antibody in CD148- and CD148/PTP-1B-deficient platelets, whereas SFK and Syk activity was only reduced in response to fibrinogen in PTP-1B-deficient platelets (Table 3) [29,51]. Intriguingly, PTP-1B-deficent platelets failed to form large aggregates when flowed over collagen at high shear conditions (3000 s^{-1}), found in small and stenotic arteries, but formed normal aggregates at intermediate shear conditions (1000 s^{-1}) [29]. This supported the notion of PTP-1B as a positive regulator of late-stage platelet activation and aggregation [45].

Shp1 and Shp2: more than just inhibitors of platelet activation

Shp1 and Shp2 are structurally related classical PTPs that consist of tandem SH2 domains in their N-termini and single PTP domains in their C-termini (Fig. 5). The tandem SH2 domains mediate intra- and inter-molecular interactions that regulate catalytic activity and compartmentalization, respectively. Structural and enzymological analysis of Shp2 has shown that the backside loop of the N-terminal SH2 domain, opposite the phospho-tyrosine binding pocket, blocks the catalytic site of the PTP domain in the inactive conformation (Fig. 5) [55–58]. This

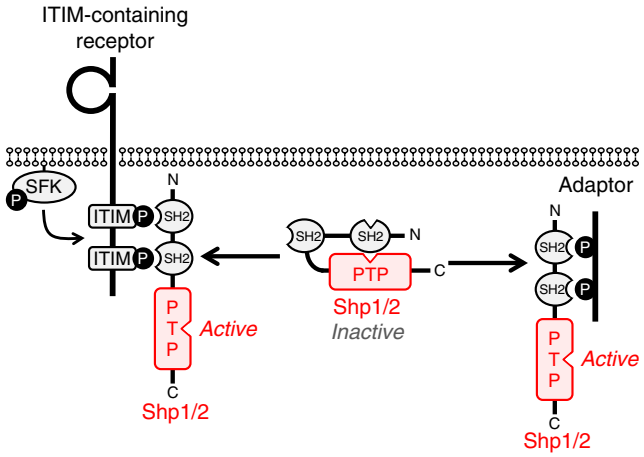

Fig. 5. Regulation of Shp1 and Shp2. Shp1 and Shp2 are regulated through intra-molecular interactions between the N-terminal Src homology 2 (SH2) domains and the protein-tyrosine phosphatase (PTP) domains, and inter-molecular interactions between the tandem SH2 domains and tyrosine phosphorylated immunoreceptor tyrosine-based inhibition motif (ITIM)-containing receptors and adaptor proteins. ITIM-containing receptors are phosphorylated by Src family kinases (SFKs).

also contorts the phospho-tyrosine binding pocket of the SH2 domain, preventing it from binding to its phospho-tyrosine ligand (Fig. 5). The C-terminal SH2 domain is, however, free to interact with its phospho-tyrosine ligand in this conformation. The same probably holds true for Shp1, which is structurally and enzymologically similar to Shp2 [55,58–60]. Binding of both SH2 domains to tandem tyrosine phosphorylated immunoreceptor tyrosine-based inhibition motifs (ITIMs, consensus sequence S/I/V/LxYxxV/L) separated by ~ 20 amino acids in the cytoplasmic tail of receptors strongly activates and localizes Shp1 and Shp2 to the plasma membrane [61,62]. Tandem binding sites are also found in adaptor proteins (Fig. 5).

Shp1 is highly expressed in hematopoietic and epithelial cells, and is generally regarded as a negative regulator of immune receptor signaling [62]. In contrast, Shp2 is ubiquitously expressed and is generally regarded as a positive regulator of growth factor and cytokine receptor signaling [62]. The different physiological functions of Shp1 and Shp2 are best illustrated by the different phenotypes of knockout mouse models. Mice lacking Shp1 exhibit a severe inflammatory and autoimmune phenotype, referred to as *motheaten*, due to patchy hair loss [63]. Mice expressing reduced levels of catalytically impaired Shp1 exhibit a less severe phenotype, referred to as *motheaten viable*, due to the longer life-span of the mice [63]. *Motheaten* and *motheaten viable* mice die due to severe tissue damage at 2–3 and 9–12 weeks of age, respectively. In contrast, Shp2-deficient mice die between E8.5 and E10.5 with a range of developmental defects [64–67]. Gain-of-function mutations in Shp2 result in a developmental condition referred to as Noonan syndrome, with an associated bleeding diathesis, and in some instances leukemia [68,69], depending on the mutation and the level of Shp2 activity. Loss-of-function mutations in Shp2 are associated with a Noonan syndrome-related condition referred to as LEOPARD syndrome [70].

Shp1 and Shp2 were initially hypothesized to inhibit platelet activation and thrombosis. This was mainly based on their association with the ITIM-containing receptor PECAM-1, which inhibits collagen- and thrombin-induced platelet activation and thrombus formation in mice [71,72]. Shp1 and Shp2 interact with the tandem tyrosine phosphorylated ITIMs in the cytoplasmic tail of PECAM-1 following collagen- and thrombin-induced platelet activation and antibody-mediated cross-linking of PECAM-1 [73–76]. Similarly, Shp1 and Shp2 also associate with the structurally related ITIM-containing receptor CEACAM1, which also inhibits platelet activation and thrombus formation in mice [77]. However, a much more complex picture is emerging of the functions of Shp1 and Shp2 in platelets and megakaryocytes. Initial studies by Pasquet *et al*. [78] suggested that Shp1 was in fact a positive regulator of platelet activation. This was based on findings from *motheaten viable* mice demonstrating that platelets expressing reduced levels of catalytically impaired Shp1 are less responsive to GPVI-mediated platelet activation [78]. However, the mechanism was not defined. More recently, Brass *et al*. [79] demonstrated that Shp1 negatively regulates G protein-coupled receptor signaling in platelets by mediating release of regulators of G protein signaling (RGS)10 and RGS18 from the scaffold protein spinophilin. Treatment of platelets with the non-selective Shp1/2 inhibitor NSC-87877 blocked the dephosphorylation and release of Shp1 and RGS18 from spinophilin, suggesting spinophilin is a substrate of Shp1. In a separate study, Tomiyama *et al*. [80] concluded that Shp1 positively regulates outside-in αIIbβ3 signaling by dephosphorylating and releasing the cytoskeletal protein α-actinin from αIIbβ3. Pretreatment of platelets with the Shp1/PTP-1B inhibitor PTPI-1 inhibited dephosphorylation of α-actinin [80]. Consistent with their findings, Haimovich *et al*. [81] previously showed that Shp1 directly dephosphorylates α-actinin, thus regulating α-actinin-mediated receptor tethering to the cytoskeleton and the extent of cross-linking of actin filaments. Finally, Gibbins *et al*. [82] proposed that Shp2 negatively regulates GPVI signaling through an adaptor-like function. According to their model, Shp2 sequesters PI3-kinase away from a LAT-Gab-1 complex found in lipid rafts, by binding to PECAM-1 present in non-lipid rafts [82]. A summary of these functions can be found in Table 2.

To determine the physiological functions of Shp1 and Shp2 in platelets, we generated megakaryocyte/platelet-specific Shp1 and Shp2 knockout mouse models [83]. Findings from these mice revealed novel and complex functions of both PTPs in megakaryocyte development and platelet production and function (Table 3) [83]. Intriguingly, despite their structural similarities, Shp1 and Shp2 perform mainly distinct functions in megakaryocytes and platelets. Surprisingly, Shp1-deficient platelets responded less well than control platelets to low-concentration CRP (Table 3). This was due to reduced GPVI expression and signaling via the SFK-Syk-PLCγ2 pathway. We anticipated the opposite as Shp1 is a well-established negative regulator of ITAM-containing receptor signaling in other hematopoietic cells [62]. The reduction in GPVI expression may reflect a negative feedback mechanism to down-regulate tonic GPVI signaling in the absence of Shp1. However, Shp1-deficient platelets did not spread to the same extent as control platelets on fibrinogen, despite expressing normal levels of αIIbβ3, and they responded normally to anti-CLEC-2 antibody [83]. By contrast, Shp2 conditional knockout mice were mildly macrothrombocytopenic and Shp2-deficient platelets responded normally to CRP, but hyper-responded to fibrinogen and anti-CLEC-2 antibody (Table 3). Shp1- and Shp2-deficient megakaryocytes were partially blocked at 2N/4N ploidy; however, only Shp2-deficient megakaryocytes exhibited concomitant reductions in proplatelet formation, thrombopoietin and integrin signaling. Deletion of both Shp1 and Shp2 resulted in severe

macrothrombocytopenia and reduced platelet surface glycoprotein expression, including GPVI, αIIbβ3 and GPIbα (Table 3) [83]. Megakaryocytes from these mice were blocked at 2N/4N ploidy and did not survive *in vitro*. Megakaryocyte/platelet-specific deletion of the ITIM-containing receptor G6b-B, which is constitutively tyrosine phosphorylated and associated with Shp1 and Shp2 in mature megakaryocytes and platelets, phenocopied multiple features of Shp1/2-deficient mice, including severe macrothrombocytopenia, suggesting G6b-B signals via and is a critical regulator of Shp1 and Shp2 [83,84]. Findings from this study establish Shp1 and Shp2 as major regulators of megakaryocyte development and platelet production and function, and suggest that the ITIM-containing receptor G6b-B is a critical regulator of Shp1 and Shp2 in mature megakaryocytes and platelets [83].

Another interesting finding to come out of our study is that Shp1 and Shp2 appear to have different functions in megakaryocytes and platelets [83]. For example, Shp1-deficient megakaryocytes spread normally on fibrinogen, whereas Shp1-deficient platelets spread less than control platelets. In addition, Shp2-deficient megakaryocytes did not spread to the same extent as control megakaryocytes on fibrinogen, whereas Shp2-deficient platelets spread to a greater extent than control platelets. The molecular basis of these differences remains undefined, but is likely to involve differential expression of regulatory proteins, such as G6b-B, that modulate Shp1 and Shp2 compartmentalization and activity at different stages of development [84].

One of the main functions of Shp2 in megakaryocytes is to positively regulate signaling via the thrombopoietin receptor Mpl. Shp2 was initially implicated in Mpl signaling by Kaushansky and Lacombe, who independently showed that it forms a complex with adaptor proteins Gab-1, Gab-2 and Shc, and is critical for activation of the PI3-kinase/Akt pathway in Mpl expressing cell lines [85,86]. We demonstrated that Shp2 also positively regulates the Ras/MAPK pathway downstream of Mpl in primary mouse megakaryocytes [84]; however, the proximal signaling mechanism remains ambiguous, but is likely to involve some of the adaptors described by Kaushansky and Lacombe [85,86]. Consistent with our findings, Cantor *et al.* [87] independently demonstrated that megakaryocyte/platelet-specific deletion of Shp2 results in mild macrothrombocytopenia in mice. They also describe a novel mechanism whereby the kinase-phosphatase pair Src-Shp2 regulates the phosphorylation-dephosphorylation and activity of the transcription factor RUNX-1 in hematopoietic cells [87]. Collectively, these findings demonstrate the central role played by Shp2 in regulating both proximal and distal Mpl signaling events in the megakaryocyte lineage.

A new frontier in platelet signal transduction

We are only just beginning to scratch the surface of this vast new area of platelet signal transduction. Not only is there still much to discover regarding CD148, PTP-1B, Shp1 and Shp2 in platelets and thrombosis, but there are many other classical PTPs waiting to be investigated (Table 1). An interesting candidate is the non-transmembrane PTP MEG2, which was recently reported to play a fundamental role in the biogenesis and fusion of vesicle membranes with the plasma membrane (Table 2) [88]. Mice lacking MEG2 exhibited large areas of hemorrhaging and cerebral infarction during embryogenesis (Table 3) [88]. Analysis of platelets from these mice revealed severely reduced responses to thrombin, whereas ADP-induced responses were slightly impaired, suggesting a secretion defect (Table 3) [88]. Ultrastructural analysis of T lymphocytes from these mice revealed almost the complete absence of mature secretory vesicles [88]. Comprehensive analyses of the megakaryocyte/platelet phenotype of these mice and the molecular basis of these defects remain to be done.

Another interesting PTP that warrants further investigation is the class II cysteine-based low-molecular-weight (LMW)-PTP, which is reported to be highly expressed in platelets and to dephosphorylate FcγRIIA and the adaptor protein LAT *in vitro* (Table 4) [89]. Knockdown and over-expression studies in the DAMI megakaryocytic cell line supported the role of LMW-PTP in regulating FcγRIIA dephosphorylation [89]. The physiological function of LMW-PTP remains ambiguous and the field would benefit from the generation of a LMW-PTP knockout mouse model.

Several other sub-families of protein phosphatases have also yet to be studied in detail in platelets, including the atypical, non-cysteine-based phosphatases, which contain aspartic acid or histidine residues in their active sites, the DSPs and the PPPs. The few studies published on members of these sub-families to date reveal unique and interesting functions in platelets and thrombosis. The histidine-based tyrosine phosphatase T cell ubiquitin ligand-2 (TULA-2) was recently reported to dephosphorylate and inactivate the tyrosine kinase Syk in platelets (Table 4) [90]. TULA-2-deficient mouse platelets hyper-respond to GPVI agonists, and TULA-2-deficient mice exhibit reduced bleeding times and enhanced thrombus formation and stability following ferric chloride-induced arterial injury [90]. Another interesting finding is that mice lacking the lipid phosphatase and tensin homologue (PTEN), which dephosphorylates the 3′-phosphate in the inositol ring of phosphatidylinositol 3,4,5-trisphosphate, generating phosphatidylinositol 4,5-bisphosphate, have increased platelet counts and decreased bleeding times (Table 4) [91]. PTEN-deficient platelets hyper-respond to collagen and exhibit enhanced collagen-induced Akt phosphorylation, which lies downstream of PI3-kinase [91]. This is not surprising as PTEN antagonizes the function of PI3-kinase, which phosphorylates the 3′-phosphate in the inositol ring of phosphatidylinositol 4,5-bisphosphate, and is essential for GPVI-FcR γ-chain signaling and downstream Akt phosphorylation [92].

Table 4 Functions of non-classical PTPs in platelets

Phosphatase	Localization	Function
Class I Cys-based lipid phosphatase		
PTEN	Associates with the cytosolic surface of the PM	Dephosphorylates PIP_3 to PIP_2 Negatively regulates GPVI-mediated platelet activation [91]
Class II Cys-based PTP		
LMW-PTP	Cytosolic	Dephosphorylates FcγRIIA and LAT [89] Implicated in down-regulating FcγRIIA-mediated platelet activation [89]
His-based PTP		
TULA-2	Cytosolic	Dephosphorylates and inhibits Syk downstream of GPVI [90]
Ser/Thr PPP		
PP1	Cytosolic	Implicated in regulating αIIbβ3 function and signaling [94,96]
	Catalytic subunit constitutively associated with the integrin αIIb subunit	Implicated in dephosphorylating and attenuating PKCζ activity downstream of αIIbβ3 [97]
PP2A	Cytosolic	Negatively regulates integrin αIIbβ3 function and signaling [95]
	Catalytic subunit constitutively associated with the integrin αIIb subunit	Implicated in dephosphorylating and attenuating PKCζ and PTP-1B activity downstream of αIIbβ3 [93,97]

PTP, protein-tyrosine phosphatase; PPP, phosphoprotein phosphatase; PM, plasma membrane; PTEN, phosphatase and tensin homolog; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; LMW-PTP, low molecular weight-protein-tyrosine phosphatase; LAT, linker of activated T cells; TULA-2, T-cell ubiquitin ligand-2; PP1, phosphoprotein phosphatase 1; PKC, protein kinase C; PP2A, phosphoprotein phosphatase 2A.

Finally, the serine/threonine-specific phosphoprotein phosphatases (PP)1 and PP2A have been implicated in regulating outside-in and PAR-4-induced inside-out integrin αIIbβ3 signaling (Table 4) [93–96]. The catalytic subunits of PP1 and PP2A were shown to be constitutively associated with the cytoplasmic tail of the integrin αIIb subunit (Table 4) [94–96]. Fibrinogen binding to αIIbβ3 causes the dissociation and activation of the PP1 catalytic domain and decreases αIIb-associated PP2A activity [95,96]. Both PP1 and PP2A have been implicated in dephosphorylation and attenuation of PKCζ activity downstream of the integrin αIIbβ3 (Table 4) [97]. Intriguingly, PP2A has also been proposed to negatively regulate outside-in αIIbβ3 signaling by dephosphorylating and attenuating PTP-1B activity, which in turn reduces Src activation [93]. This provides an interesting link between PPPs, PTPs and Src activation. Comprehensive analyses of the cellular and biochemical functions of these and other protein phosphatases in platelets are also waiting to be done.

A new class of antithrombotic drug targets?

Initial attempts to develop PTP-specific inhibitors were unsuccessful due mainly to the conservative approach taken to target the active site of PTPs, which are highly similar [12]. Hence, PTPs were dubbed 'undruggable' and the pharmaceutical industry steered clear of them. However, more recent attempts to target unique structural features surrounding their active sites have yielded more promising outcomes. Proof of concept can be found in the recent elegant study by Tonks *et al.* [98] demonstrating that conformation-sensing antibodies can be used to selectively stabilize PTP-1B in an inactive conformation. The ectodomains of receptor-like PTPs also provide unique and easily accessible targets. Domains and motifs essential for regulating compartmentalization and activity are also distinct possibilities. Non-selective PTP inhibitors may also prove to be efficacious in some instances, as is the case with some PTK inhibitors, including Dasatinib (Sprycel), which inhibits BCR/Abl, c-Kit and SFKs, and is used in patients with chronic myelogenous leukemia [99].

In light of the critical roles played by PTPs in regulating platelet activation and thrombosis, it would be prudent to test any PTP inhibitors currently under development for the treatment of diabetes, obesity, cancer and inflammation for unwanted bleeding or thrombotic side-effects. Whether specifically targeting platelet PTPs for the prevention and treatment of thrombosis is a good idea remains to be seen, but it is worth investigating. Although current antiplatelet therapies are highly effective, they have their shortcomings, including increased risk of bleeding and resistance in some patients. In addition, there is a need for more effective treatments in conditions such as stroke. Based on our findings to date, CD148 and PTP-1B are potential antithrombotic drug targets, but for different reasons. As a master regulator of platelet activity, inhibiting CD148 will markedly reduce platelet activation and thrombus formation under all conditions. In contrast, inhibiting PTP-1B should only reduce thrombus formation under high shear conditions found in stenotic blood vessels [29,51]. The ectodomain of CD148 is an obvious and easily accessible target, and because CD148 appears to be the main receptor-like PTP expressed in platelets, inhibiting it may have few side-effects on other hematopoietic cells that rely more on CD45. Recent findings by Takahashi *et al.* [100] have shown that antibody-induced cross-linking of CD148 inhibits endothelial cell growth and angiogenesis, providing proof of concept for using a similar approach to inhibit platelet activation.

Conclusions

A growing body of evidence has revealed that PTPs are critical and unique regulators of platelet activation and thrombosis. A combination of genetically modified mouse models, non-selective PTP inhibitors and cell line-based assays have been used to demonstrate the critical functions played by PTPs in regulating platelet activation and thrombosis. These findings also help to dispel the notion of PTPs as simply being inhibitors of platelet activation. Further work is needed to clarify the functions of all PTPs in platelets and determine ways in which they can be regulated. In addition, new avenues need to be opened into identifying and determining the functions of the numerous uncharacterized protein phosphatases in platelets. New discoveries will undoubtedly lead to a better understanding of the molecular basis of thrombosis, and may lead to the development of a new class of antithrombotic therapies targeting platelet PTPs.

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Disclosure of Conflict of Interests

The author states that he has no conflict of interests.

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