Fine-Tuning of Platelet Responses by Serine/Threonine Protein Kinases and Phosphatases—Just the Beginning

Yavar Shiravand[1] Ulrich Walter[2] Kerstin Jurk[2]

[1] Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, Naples, Italy
[2] Center for Thrombosis and Hemostasis (CTH), University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany

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Address for correspondence Kerstin Jurk, PhD, Center for Thrombosis and Hemostasis (CTH), University Medical Center of the Johannes Gutenberg University Mainz, Langenbeckstr. 1, 55131 Mainz, Germany (e-mail: kerstin.jurk@unimedizin-mainz.de).

Abstract

Comprehensive proteomic analyses of human and murine platelets established an extraordinary intracellular repertoire of signaling components, which control crucial functions. The spectrum of platelet serine/threonine protein kinases (more than 100) includes the AGC family (protein kinase A, G, C [PKA, PKG, PKC]), the mitogen-activated protein kinases (MAPKs), and others. PKA and PKG have multiple significantly overlapping substrates in human platelets, which possibly affect functions with clear "signaling nodes" of regulation by multiple protein kinases/phosphatases. Signaling nodes are intracellular Ca^{2+} stores, the contractile system (myosin light chains), and other signaling components such as G-proteins, protein kinases, and protein phosphatases. An example for this fine-tuning is the tyrosine kinase Syk, a crucial component of platelet activation, which is controlled by several serine/threonine and tyrosine protein kinases as well as phosphatases. Other protein kinases including PKA/PKG modulate protein phosphatase 2A, which may be a master regulator of MAPK signaling in human platelets. Protein kinases and in particular MAPKs are targeted by an increasing number of clinically used inhibitors. However, the precise regulation and fine-tuning of these protein kinases and their effects on other signaling components in platelets are only superficially understood—just the beginning. However, promising future approaches are in sight.

Keywords

- platelet signaling
- protein kinase networks
- protein phosphatases

Introduction

The discovery of adenosine diphosphate (ADP)-induced aggregation of human platelets in 1962[1] enabled subsequent discoveries that platelet-derived thromboxane A2 (TxA_2) stimulates[2] and endothelium-derived prostacyclin (PGI_2) inhibits[3,4] platelet activation. Multiple hormones, eicosanoids, and vasoactive factors are now known, which stimulate, inhibit, or modulate major platelet responses such as adhesion, motility, secretion, aggregation, and thrombin generation.[5–7] Most of these factors act via platelet membrane receptors, which stimulate intracellular signal transduction pathways.[6,8–10] Further advances in platelet receptor–mediated signaling developed with the "second messenger concept," which was originally reported for the effects of glucagon and adrenaline and their second messenger cyclic adenosine monophosphate (cAMP).[11,12] Two endothelial cell–derived factors, PGI_2 and nitric oxide (NO), increase the second messengers cAMP and cyclic guanosine monophosphate (cGMP), respectively, which activate their

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targets cAMP- and cGMP-dependent protein kinases (PKA, PKG), resulting in platelet inhibition.[3,4,10,13–17] Additional second messengers including 1,2-diacylglycerol (DAG), inositol 1,4,5-trisphosphate (IP3), phosphoinositides, and Ca^{2+}/calmodulins stimulate distinct serine/threonine protein kinases (ST-PKs), for example, protein kinase C (PKC), calmodulin-dependent protein kinases (CAMKs), myosin light chain kinase (MLCK), and protein kinase B (PKB/Akt), which support platelet activation.[6,18] Following the discovery of the protooncogene c-src in nondividing human platelets,[19] tyrosine protein kinases (TKs) c-src and other src family kinase (SFK) members (e.g., Lyn, Yes, Fyn, Fgr) were shown to mediate platelet activation in response of glycoprotein (GP) VI, GPIbα, integrins, and CLEC-2.[8,20] These membrane receptors also affect a sequential kinase cascade, the mitogen-activated protein kinases (MAPKs), in anucleate platelets.[21] A scheme of the major signaling pathways in platelets (►Fig. 1) illustrates the central role of ST-PKs in human platelets. However, this model, as many other models published, has important limitations. In most cases (as here), they show linear, "receptor-to-function signaling," which does not reflect the real signaling world within the platelet. In fact, each of the protein kinase shown in this model represents a family of closely related protein kinases, and altogether we have more than 100 platelet ST-PKs (►Supplementary Table S1 [online only]). Each of the kinase systems has multiple protein substrates, often with several phosphorylation sites, which are counterregulated by serine/threonine protein phosphatases (ST-PPs). Furthermore, these protein kinases and phosphatases and their substrates (often other regulatory molecules such as kinases and G-proteins) interact and integrate the signaling response to a functional response, not only at vertical (receptor-to function) level but also at horizontal level of signaling (interaction between various protein kinases). We are only at the beginning to understand this protein kinase network.

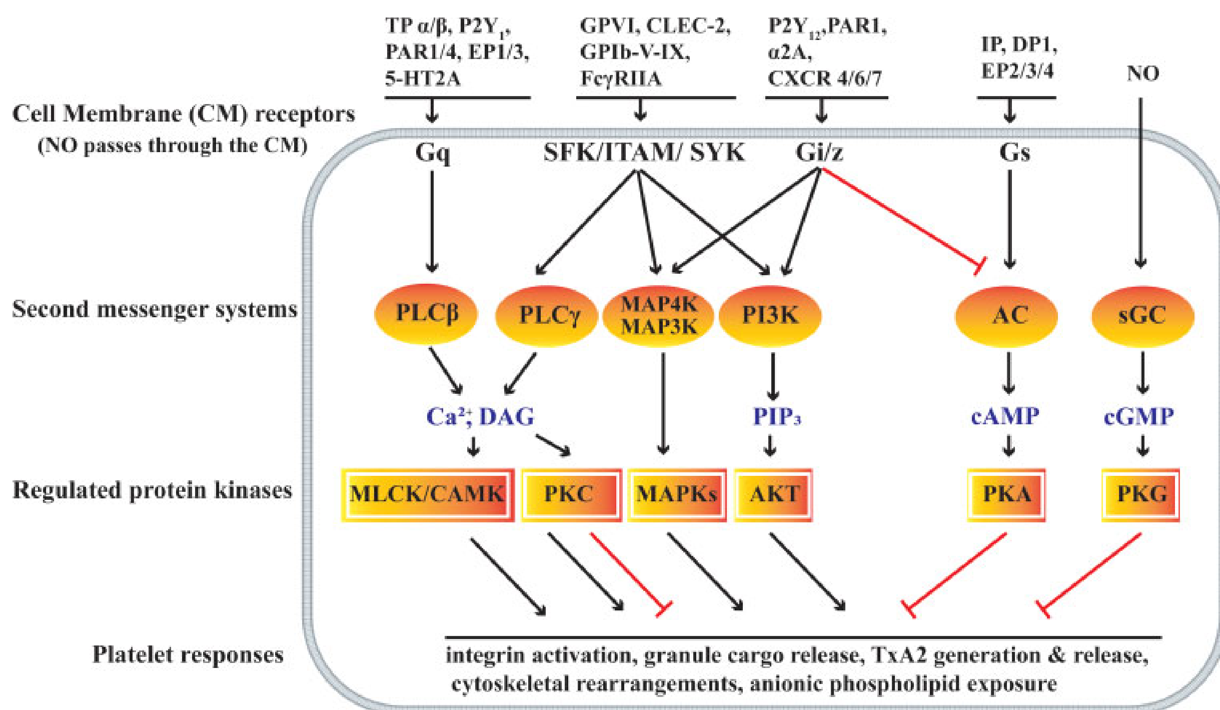

Fig. 1 Multiple intracellular serine/threonine protein kinases mediate the functional responses of platelet membrane receptor stimulation and subsequent second messenger signaling. Numerous physiological and pharmacological platelet activators or inhibitors achieve their effects via specific membrane receptors. G-protein–coupled receptors (GPCRs) activate distinct G-proteins (Gq, Gi/z, Gs), phospholipases C (PLC), and their specific second messenger systems (Ca^{2+}, DAG, PIP3, cAMP) and protein kinase targets. Platelet "pattern-recognition" receptors activate Src family tyrosine kinases (SFK)/immunoreceptor tyrosine-based activation motif (ITAM)/spleen tyrosine kinase (Syk) signaling complexes, which also lead to elevated cytoplasmic levels of second messengers and platelet activation. NO, an endothelium-derived gaseous platelet inhibitor, passes through the platelet membrane, stimulates the soluble guanylyl cyclase (sGC), and increases cytoplasmic cGMP levels. Both the cAMP/PKA and cGMP/PKG systems represent potent inhibitory pathways, which block agonist-induced platelet activation at several steps due to the phosphorylation of multiple substrates. The second messengers Ca^{2+}, DAG, and PIP3 also stimulate specific protein kinases, leading to partially overlapping platelet responses. Similar to PKA/PKG, most of these protein kinases also have multiple substrates, which have been only partially identified. This model shown here and many others published in the field have important limitations as discussed in the text. Platelets actually have hundreds of serine/threonine protein kinases (see ►Supplementary Table S1 [online only]) with thousands of substrates and interact with other components such as protein phosphatases to integrate the signaling response to a functional response. This not only occurs at a vertical (receptor-to-function) level, but also at horizontal level of signaling (interaction between various protein kinases). We are only at the beginning to understand this very dynamic and rapidly responding protein kinase network, which is addressed in this review. This model is based on previous models published.[6,100–104] 5-HT2A, serotonin receptor; CLEC-2, C-type lectin like-receptor 2; CXCR4/CXCR6/CXCR7, chemokine receptors; DP1, prostanoid receptor D1; EP 2,4, prostanoid receptors EP 2, 4; EP1, EP3, prostanoid receptors EP1 and EP3; GPIb–V–IX, glycoprotein Ib–V–IX complex; GPVI, glycoprotein VI; IP, prostanoid/prostacyclin receptor I; $P2Y_1$, ADP/P2 purinoceptor subtype Y1; $P2Y_{12}$, ADP/P2 purinoceptor subtype Y12; PAR 1/4, protease-activated receptor 1 and 4; TP, thromboxane receptor; α2A, α2A-adrenergic receptor.

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This and possible future approaches to elucidate this amazing network will be addressed in this review.

ST-PKs in Human and Murine Platelets—AGC (PKA, PKG, PKC)

The elucidation of the complete sequence of the human genome combined with bioinformatic approaches defined the entire PK and PP complement of the human genome, kinome, and phosphatome, respectively.[22,23] This fundamental step in signaling research established, based on their catalytic domain, the presence of 518 PK genes (+ 106 pseudogenes) and 189 PP genes (+ 79 pseudogenes) in humans. However, the genomic presence of PKs and PPs does not indicate that these proteins are expressed at the protein level. Advances in proteomics enabled comprehensive and quantitative analyses of the human and murine platelet proteome, including their major signaling components.[24,25] Both human and murine platelets express a large spectrum of PKs from essentially all classes.[24–26] Here, the quantitative expression of human and murine platelet ST PKs is compared for the first time (►**Supplementary Table S1** [online only]) together with a short update on the AGC protein kinase families (PKA/PKG/PKC). Then, specific examples of novel interactions between ST-PKs, TKs, and ST-PPs are discussed followed by a short update on platelet MAPKs. The quality and similarity of the original human and murine platelet proteomic data obtained by two different groups suggest that the protein kinase spectrum observed represent true positive results. On the other hand, it is known that a global proteomic approach may miss certain proteins, especially membrane proteins and proteins with very low expression. Therefore, platelets may have some additional protein kinases, which are not listed in our ►**Supplementary Table S1** (online only).

Human platelets express with more than 110 ST PKs approximately 25% of the human kinome (518 known PKs), in particular the PK families PKA, PKG, PKC, and CAMK.[22,27] PKA is the most extensively characterized kinase of this group. Under basal conditions, PKA is a heterotetrameric, inactive holoenzyme and consists of two regulatory and two catalytic subunits. With cAMP elevation, this cyclic nucleotide binds to the two regulatory subunits of the holoenzyme, causes a conformational change, and releases active catalytic subunits. PKA was the first example of a PK activated by second-messenger–induced loss of autoinhibition. Other mechanisms include the activation by phosphorylation/autophosphorylation (►**Supplementary Table S1** [online only]), which often also release autoinhibition. The human genome codes for seven different PKA subunits: three different catalytic subunits (PRKACA, PRKACB, PRKACG), two different regulatory subunits type I (PRKAR1A, PRKAR1B), and two different regulatory subunit type II (PRKAR2A, PRKAR2B). The main isoforms of regulatory and catalytic subunits expressed in human platelets are R1A, R1B, R2B, CA, and CB, which may form several distinct PKA holoenzymes.[16,24] However, very little is known regarding the contribution of the individual PKA subunits. PKA regulatory subunits influence the spatiotemporal PKA activation, due to RI/RII-specific A-kinase anchoring proteins (AKAPs).[28–31]

PKG is evolutionary, structurally and functionally closely related to PKA, but comprises a single polypeptide chain, containing both catalytic and two regulatory (cGMP-binding) domains, and there are two distinct forms, a soluble (type I) and a membrane-bound form (type II).[32] The human gene *PRKG1* (chromosome 10) encodes the soluble Iα/Iß isoforms produced by alternative transcript splicing, another gene on human chromosome 4, PRKG2, encodes the membrane-bound PRKG isoform II. Human and murine platelets do not express PKG type II, but PKG Iß at high (human) or low (murine) levels as shown in ►**Supplementary Table S1** (online only).[16,24]

Although there are other proteins, which specifically bind cAMP and/or cGMP, multiple lines of genetic, biochemical, physiological, and pharmacological evidence revealed that the inhibitory effects of cAMP- and cGMP-elevating agents on platelet activation and function are mediated by PKA and PKG, respectively, as reviewed elsewhere.[10,16,17] The platelet inhibitory effects of cGMP-elevating agents or membrane permeable cGMP analogs are strongly impaired in human or murine platelets lacking PKG I, whereas the cAMP/PKA system was not affected.[14,33] Previously, a small numbers of PKA and/or PKG substrates were identified in platelets such as G-proteins/G-protein-associated proteins, cytoskeletal proteins, proteins associated with intracellular Ca^{2+}-stores/-release, cell membranes, and cAMP/cGMP phosphodiesterases.[16,34] Selective stimulation of the human platelet cAMP/PKA system (by the PGI_2 analog iloprost) or of platelet PKG (by the soluble guanylyl cyclase stimulator riociguat) increased the phosphorylation of 299 (iloprost) and more than 150 (riociguat) proteins, and decreased the phosphorylation of more than 60 proteins (with riociguat).[10,35–37] Surprisingly, there was a large but not complete overlap between the PKA- and PKG-regulated phosphoproteins, in agreement with the earlier conventional studies.[10,16] This PKA/PKG overlap raises the hypothesis that each pathway may be, at least partially, a back-up for each other. Notably, there are cellular clusters (signaling nodes) with several PKA/PKG-regulated phosphoproteins, which often contain multiple phosphosites.[10,17,38] Remarkably, both PKA and PKG affect the phosphorylation of other PKs and PPs, as demonstrated with two specific examples, the TKs Src and Syk, and the protein phosphatase 2A (PP2A).[35,36]

Following cAMP/cGMP pathways, other second messengers such as diacylglycerol (DAG), inositol-3-phosphate (IP3), Ca^{2+}, phospholipids, and their target protein kinases PKC and MLCK were discovered. Ca^{2+}/calmodulin-dependent MLCK[39–41] and PKC[42–44] are closely associated with platelet activation, in particular shape change and secretion. MLCK phosphorylates myosin light chain (MLC) at S18/T19 and increases the contractile force of the myosin–F-actin complex, which is reversed by myosin light chain phosphatase (MLCP)-mediated MLC dephosphorylation. This MLCK–MLC–MLCP complex is tightly regulated by multiple PKs and PPs. MLCK is activated by Ca^{2+}/calmodulin binding, by

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TK- and PKC-mediated phosphorylation, but is inhibited by PKA and PKG, whereas MLCP is inhibited by Rho-associated protein kinase (ROCK)-mediated phosphorylation.[45,46] In platelets, many components of the MLCK–MLC–MLCP complex are major "signaling nodes" of motility regulation and phosphorylated at multiple sites by both PKA and PKG, resulting in decreased MLC phosphorylation and reduced contractile force.[10,47,48]

The classical PKC is one protein chain with a C-terminal kinase domain and N-terminal regulatory moieties, which bind the activating second messengers DAG and Ca^{2+}.[18,49] The tumor promotor phorbol ester is a potent activator of PKCs, binds to the DAG regulatory site, and is an excellent pharmacological tool to activate PKC. The entire PKC family consists of the classical/conventional forms (α, βI, βII, γ), novel forms (***δ***, ***θ***, ε, ŋ), and atypical forms (ζ, ***ι***/λ) with conserved catalytic domains, but different regulatory moieties.[44] Human and mouse platelets express PKC α, β, ***δ***, and ***θ*** at detectable levels, while the expression of PKCŋ is very low and PKCε is restricted to mouse platelets.[18,50] In vivo studies demonstrated that PKCα-deficient mice show a reduced phosphorylation of synaptosomal-associated protein 23 (SNAP-23, part of the soluble N-ethylmaleimide-sensitive factor attachment receptor [SNARE] complex) at S95, associated with decreased α-granule secretion and impaired dense granule biogenesis. Platelets from PKC ***δ***, ***θ***, and ε knockout mice exhibited impaired phosphorylation of vasodilator-stimulated phosphoprotein, pleckstrin, and syntaxin-4, respectively.[50] PKC isoforms (α and β) appear to have nonredundant roles in regulating α-granule secretion, integrin activation, and Ca^{2+} signaling, whereas PKC***δ*** and ***θ*** negatively regulate thrombus formation on collagen.[18,50,51] PKC***δ*** is suggested to be involved in collagen-induced platelet activation via the synthesis of TxA_2, mediated at least in part by mitogen-activated protein kinase kinase (MAP2K/MEK)/ extracellular signal-regulated kinase (ERK)/p38 MAPK pathways.[52] The observation that members of the PKC family have opposing functional roles in platelets suggests that these differences are due to distinct substrates, which need to be elucidated.[18,50]

Tyrosine (Y) PKs as Targets of ST-PKs and ST-PPs: Fine-Tuning of Syk

Various adhesion molecules activate platelets by binding to distinct transmembrane receptors (GPVI, GPIbα, CLEC-2, and αIIbβ3 integrin). Activation of intracellular, membrane-associated Src family tyrosine kinases (SFKs) increases Y-phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) and subsequent membrane recruitment and stimulation of the spleen Y kinase (Syk).[8,53] Recruitment of Syk to ITAM at the membrane via its two Src-homology 2 (SH2) domains and Y-phosphorylation of the sites Y352 (by SFKs) and Y525/526 (by autophosphorylation) abolishes Syk autoinhibition (►Fig. 2).[54–56] This sophisticated SFK-Syk cascade activates phospholipase (PLC) γ2, via Y-phosphorylation, leading to elevated DAG/IP3/Ca^{2+} and PKC activation.[53] Syk also stimulates ADP secretion and TxA_2 synthesis, which enhance initial platelet activation. Syk is the only ppITAM-activated TK in platelets and therefore represents an essential component of adhesion-dependent platelet activation. Many TKs are known to be phosphorylated by ST-PKs, but the functional role is often not known.

Interestingly, in our global phosphoproteomic studies with platelets, the cAMP/PKA pathway affected the phosphorylation of many protein kinases, including the TKs Src and Syk.[10,35,36] The observed cAMP/PK-stimulated Src Y530 phosphorylation is certainly not a direct PKA phosphosite and likely to be mediated by PKA phosphorylation of C-terminal Src kinase (CSK) at S364. This stimulates CSK to phosphorylate inhibitory tyrosine sites (e.g., Y530) at the C-terminus of Src and other SFKs.[57–59] This is one example that PKA affects a TK cascade (CSK, SFK, Syk) and their downstream targets. A recent study, using a different analytical approach (fluorescence two-dimensional gel electrophoresis) also reported that the cAMP/PKA system increases the phosphorylation status of several signaling components including protein phosphatase 1A (PPA1), Src kinase-associated phosphoprotein 2 (SKPA2), cGMP-dependent protein kinase 1 (PRKG1), integrin-linked protein kinase (ILK), and the dual specificity protein phosphatase 3 (DUSP3).[60] However, this method does not identify the identity of the phosphorylation sites, and the results are therefore not directly comparable to other mass spectrometry-based phosphoproteomic studies.

The PKA/PKG pathways downregulated basal and strongly inhibited ADP-stimulated Syk S297 phosphorylation in intact human platelets.[36,61] The S297 phosphosite is located within the Syk interdomain B, which maintains this TK in an autoinhibited state under basal conditions and participates in conformation-dependent Syk activation upon ITAM-binding and Y-phosphorylation as shown in ►Fig. 2.[54–56]

GPVI-/GPIbα-mediated platelet activation increased the well-established Syk Y-phosphorylation/activation and the stoichiometric, transient PKC-mediated Syk S297 phosphorylation as well. PKC inhibition abolished this Syk S297 phosphorylation, but enhanced GPVI-/GPIbα-increased Syk Y-phosphorylation/activity, indicating a possible feedback inhibition.[62] Furthermore, inhibition of the ST PP2A alone increased Syk S297 phosphorylation without affecting Syk Y-phosphorylation and activity, whereas PP2A inhibition preceding GPVI-mediated platelet activation downregulated Syk Y-phosphorylation and activity.[63] ►Fig. 2 summarizes that Syk, an established central component of adhesion-dependent platelet activation, is controlled and fine-tuned by multiple Y- and ST-PKs as well as by corresponding protein phosphatases. Upon activation, Syk interacts with Y-phosphorylates and activates further signaling components such as PLCγ2 (►Fig. 2). Furthermore, there are many more Syk substrates known in platelets and other Syk–containing cells such as B-cells, lymphoma, and breast cancer cells.[64,65] These developments lead to the recognition that Syk can function as an oncogene or tumor suppressor, depending on the cellular context.[66] Presently, there are major efforts ongoing to elucidate the Syk signaling network by functional and phosphoproteomic analyses combined

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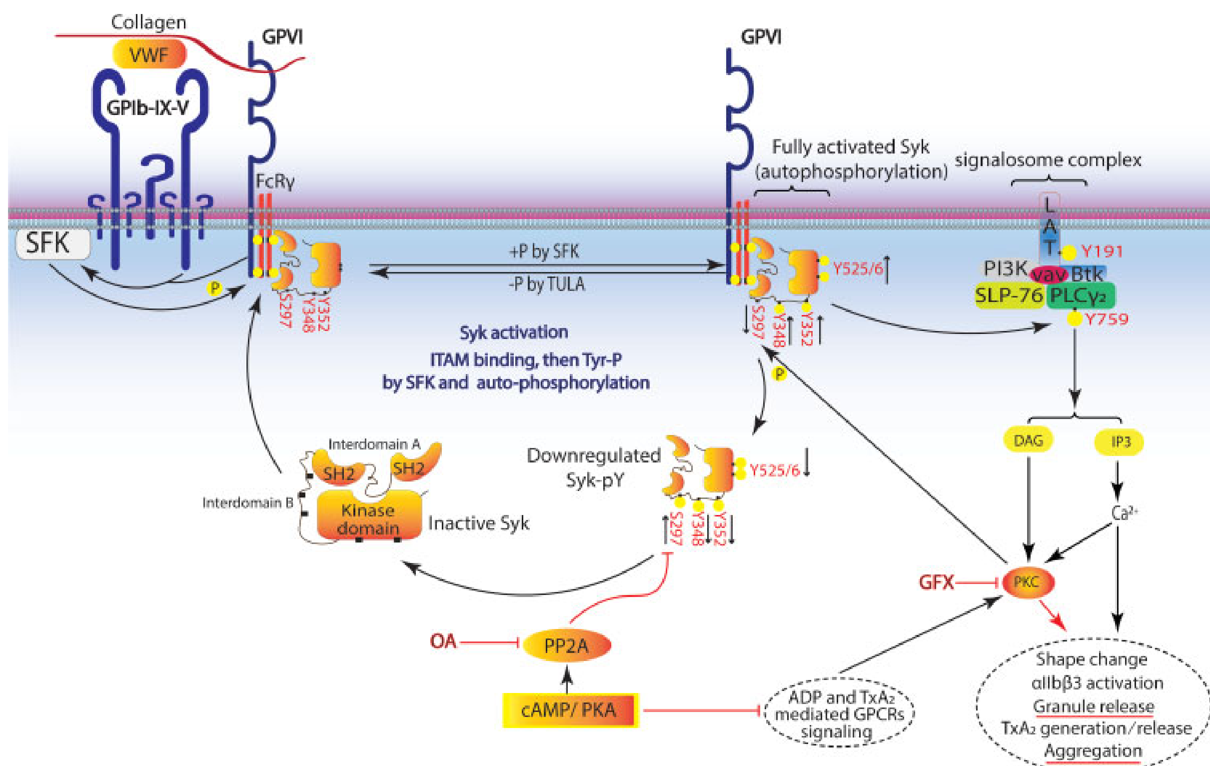

Fig. 2 Model showing activation, inhibition, and fine-tuning of adhesion-dependent Syk regulation in human platelets. Activated GPVI and GPIbα increase SFK-mediated dual phosphorylation of FcRγ ITAM motifs (ppITAM), which recruit inactive Syk (via its SH2 domains) from the cytosol to the cell membrane. This induces a conformational change leading to Syk activation and concomitant Y-phosphorylation of the interdomain B (Y352) and kinase domain (Y525/6), mainly by SFKs and autophosphorylation, respectively. Fully activated Syk and Syk interacting proteins such as LAT, Btk, PLCγ2, E3 ubiquitin-protein ligase CBL form a signalosome complex to trigger various cellular responses. Often, Syk tyrosine phosphorylates its interacting proteins in such complexes. GPVI ligands such as collagen, VWF, and convulxin also induce a Syk- and PKC-mediated S-phosphorylation (S297) within the interdomain B of Syk, which is negatively associated with Y-phosphorylation (Y352, Y525, Y526). Inhibition of the serine/threonine protein phosphatase PP2A by OA also results in Syk S297 phosphorylation due to inhibited dephosphorylation, introducing PP2A as novel factor for Syk regulation. PKA inhibits PKC-mediated Syk S297 phosphorylation at the level of generation and/or action of the secondary mediators ADP and TxA_2. The tyrosine phosphatase TULA-2 downregulates Syk tyrosine phosphorylation (mainly pY352) and activity. Syk and its interdomain B are "signaling nodes" for the regulation by multiple serine/threonine and tyrosine protein kinases and phosphatases. ADP, adenosine diphosphate; Btk, Bruton tyrosine kinase; cAMP, cyclic adenosine monophosphate; Cbl, casitas B-lineage lymphoma; cGMP, cyclic guanosine monophosphate; DAG, diacylglycerol; FcRγ, Fc receptor γ-chain; GPCRs, G-protein–coupled receptors; GPVI, glycoprotein VI; IP3, inositol trisphosphate; LAT, linker for activation of T-cells; OA, okadaic acid; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLCγ2, phospholipase Cγ2; PP2A, protein phosphatase 2A; SFK, src kinase family; SLP-76, Src homology (SH) 2 domain-containing leukocyte phosphoprotein of 76 kDa; Syk, spleen tyrosine kinase; TULA-2, T-cell ubiquitin ligand-2; TxA_2, thromboxane A_2; VWF, von Willebrand factor.

with bioinformatic network modeling.[65] It is very likely that these approaches can also be successfully applied to human platelets in the future.

ST-PPs as Targets of ST-PKs in Human Platelets

PPs reverse PK-mediated phosphorylations and are encoded by 189 PP genes in humans, based on their catalytic subunits.[23] However, the number of PP holoenzymes is much larger than the number of catalytic subunits due to additional regulatory subunits. PPs are classified into 10 different protein folds and 21 families, including Y-PPs (largest group) and ST-PPs (second largest group) as major families, and Y- and ST-PPs are widely expressed in human and murine platelets.[24–26] Four different dual-specificity (Y + ST) PPs (DUSPs) and 15 protein Y-phosphatases (PTPs) were detected, including some receptor Y-PP.[26,67] Platelet Y-PPs represent an important signaling system with a therapeutic potential as well reviewed by others.[67,68]

Human ST-PPs contain two major families, metallo-dependent PPs (PPM fold) and phosphoprotein phosphatases (PPP/PPPL fold, with subfamilies PPP 1–6), which are well expressed in human platelets.[69] The (P)PP1 subfamily regulates contraction in smooth muscle and platelets, as it reverses the action of MLC kinase (MLCK). This family is characterized by three distinct catalytic (α, β, γ) and sixteen different regulatory subunits.[46] Our platelet proteomic studies identified three catalytic subunits and seven different regulatory subunits of PP1, including the gene product PPP1R12A, the myosin phosphatase targeting subunit 1 (MYPT1). MLCP, a special PP1 holoenzyme, is a trimeric enzyme containing a catalytic subunit, a regulatory subunit of uncertain function, and MYPT1, which targets the MLCP holoenzyme to the MLC/myosin complex.[70] The regulatory switch of myosin contractility is the state of MLC

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phosphorylation, tightly controlled by the balance of MLCK and MLCP, which are themselves regulated by multiple mechanisms and multisite phosphorylation (signaling nodes).

The second major ST-PP, PP2A, has also been known for decades, but its structural and functional diversity was only more recently appreciated,[46,71] also in human platelets.[26,69] PP2A is expressed as heterotrimeric holoenzyme composed of a catalytic subunit (C), a scaffold subunit (A) and a targeting/regulatory subunit (B). In humans, A- and C-subunits of PP2A each have two variants (α, β), whereas B-subunits are encoded by 15 different genes, resulting in at least 23 isoforms due to alternative splicing. Based on this, human cells may contain up to 92 different trimeric PP2A holoenzymes. Previously, we presented an overview of PP2A heterogeneity in human platelets.[26]

Elegant studies with *Xenopus* oocytes, *Drosophila*, and mammalian cells established that cell mitosis is tightly controlled by multiple phosphorylation steps and a balance between PK and PP activities, in particular by the master mitotic cyclin-dependent kinase 1 (Cdk1) and its antagonizing PP, PP2A-B55. This balance is markedly altered, when PP2A-B55 is strongly inhibited by two regulatory phosphoproteins, α-endosulfine (ENSA) and cAMP-regulated phosphoprotein 19 (ARPP19). For this function, these proteins need to be phosphorylated at a centrally located, highly conserved FDSpGDY motif by Great wall kinase (Gwl; microtubule-associated serine/threonine kinase like [MASTL] in humans).[72–74] This Gwl–Arpp19–ENSA–PP2A-B55 pathway plays an essential role in the control of the cell cycle from yeast to human, but is thought to have also other functions.[74] Recently, we reported the presence and intracellular concentration of a larger spectrum of ST-PPs in human platelets and addressed in particular the diversity of the PP2A family.[69] Both human and murine platelets contain multiple PP2A subunits (two catalytic, one scaffolding, and seven regulatory) and two endogenous PP2A inhibitors, ENSA and ARPP19.[69] Furthermore, the entire MASTL(like)–ENSA/ARPP19–PP2A pathway was found to be present in human platelets and affected by both PKA and PKG.[69] Exciting questions concern the initiation of this novel pathway in human platelets and its phosphoprotein targets, which are expected to be different from the Cdk cell cycle system. Inhibition of PP2A in human platelets was found to increase the phosphorylation of several MAPKs, indicating their activation.[69] This is in line with the proposed PP2A function as tumor suppressor, as tumor cells often have a reduced and/or impaired PP2A activity resulting in increased MAPKs.[75] Considerable efforts were therefore undertaken in this field for some time to “repair” or elevate selective PP2A forms in diseases. These efforts were very recently rewarded when two major groups demonstrated that some novel phenothiazine derivatives (also called “small molecule activator of PP2A,” SMAP) reactivate specific PP2A isozymes with potential benefit in cancer and other diseases.[76–78]

It is still a major challenge to elucidate the PP2A isoform-specific signaling and their precise roles on platelet functions, including the observed effects on MAPK signaling. However, this will be aided now by isoform-specific endogenous inhibitors such as ENSA/ARPP19[69] and by novel, selective pharmacological PP2A activators.[78]

MAPKs and Their Interactions in Platelets

Recent proteome studies revealed that human and murine platelets abundantly express multiple members of the MAPK family, which belong to the CMGC (CDK, MAPK, GSK3 [glycogen synthase kinase 3], CDK-like kinase) class of serine/threonine kinases[24,25] (►**Supplementary Table S1** [online only]). The canonical MAPK cascades are initiated by a variety of platelet receptor signaling (►**Fig. 3**). PK-induced phosphorylation or small GTPase interaction lead to the activation of MAP kinase kinase kinase kinases (MAP4K, MEKKK) and MAP kinase kinase kinases (MAP3K, MEKK), which in turn activate MAP kinase kinases (MAP2K, MEK). Active MAP2Ks then activate MAPKs through dual phosphorylation of threonine/tyrosine residues within the activation loop of the kinase domain. The ERK1/2 (MAPK3/MAPK1) and ERK5 (MAPK7), the c-Jun N-terminal kinase JNK1/2 (MAPK8/MAPK9), and the p38 isoforms α and β (MAPK14) represent prominent MAPKs in human and murine platelets (►**Fig. 3**).

Despite advanced platelet MAPK research in the last years, the identification of proximal MAP3/4Ks, their underlying activation mechanisms, and signaling networks are still not well understood, although several members are present (►**Supplementary Table S1** [online only]). Murine platelets deficient in the MAP4K misshapen/Nck-interacting kinase–related kinase 1 (MINK1) are characterized by diminished MEK1/2, MKK3/6, ERK, and p38 phosphorylation, associated with decreased δ-granule release of ADP, especially in response to low concentrations of thrombin and collagen. This secretion defect might explain the prolonged bleeding time and impaired thrombus formation under arterial shear conditions in vitro and in vivo[79] (►**Fig. 3**). The impact of MINK1 on human platelet function has yet to be proven. Although the MAP3K3 (MEKK3) was not detected in human or murine platelets by proteomics studies,[24,25] there is evidence from MAP3K3-deficient mice that it regulates JNK1/2 and ERK1/2 activation.[80] The comprehensive identification and functional characterization of JNK1/2 and ERK1/2 substrates also bear challenges. Agonist-induced phosphorylation of cytosolic phospholipase A2 (cPLA2) at S505, representing cPLA2 activity and leading to TxA2 synthesis, was not affected in MAP3K3-deficient platelets,[80] whereas the MEK1/2 inhibitors PD184354 and U0126, used for the analysis of ERK1/2 regulation, clearly diminished cPLA2 S505 phosphorylation and TxA2 synthesis in stimulated human platelets.[81,82] As these inhibitors are also known to inhibit cyclooxygenase-1 and TxA_2-synthase, these off-target effects might be responsible for the conclusion that ERK1/2 is a regulator of TxA2 generation in platelets (►**Table 1**). Recently, another MAP3K, apoptosis signal-regulating kinase 1 (ASK1; MAP3K5), was identified to be crucially involved in hemostasis and arterial thrombosis in murine platelets.[83] In ASK1-deficient platelets, MKK3/6, MKK4, and p38 phosphorylation was

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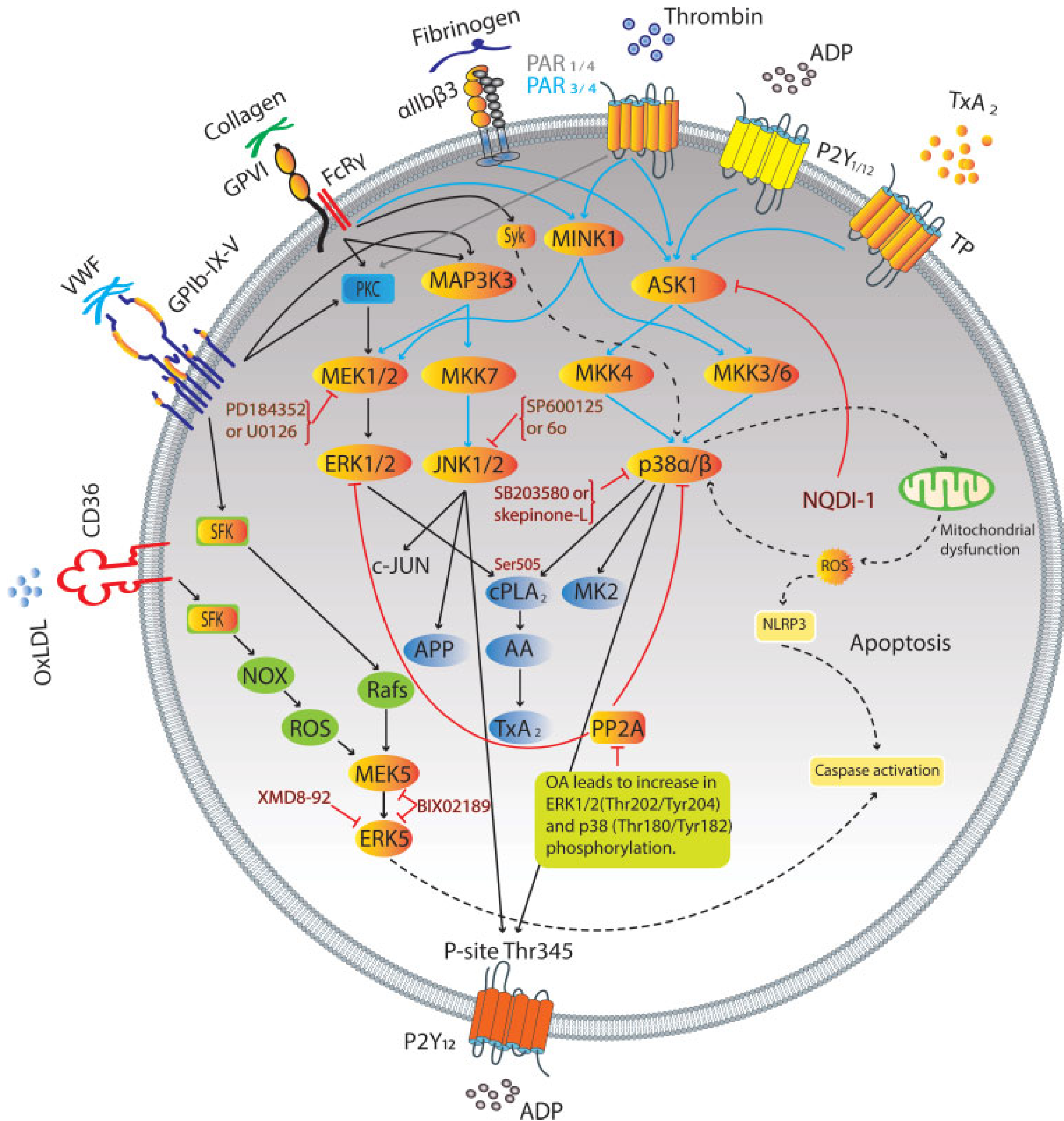

Fig. 3 Signaling network of MAPK in human and murine platelets. Black and red lines demonstrate common activatory and inhibitory signaling pathways in human and murine platelets, respectively. Mouse- and human-specific signaling pathways are shown in blue and gray, respectively. (Modified from various studies.[21,79,82,105–107]) AA, arachidonic acid; ADP, adenosine diphosphate; APP, amyloid precursor protein; ASK1 (MAP3K5), apoptosis signal-regulating kinase 1; JNK1/2, c-Jun N-terminal kinase 1/2; p38 (MAPK14), mitogen-activated protein kinase 14; cPLA2, cytosolic phospholipases A2; ERK 1/2 (MAPK3/MAPK1), mitogen-activated protein kinase 3 and 1, extracellular signal-regulated kinase 1 and 2; FcRγ, Fc receptor γ-chain; JNK1/2 (MAPK 8 and 9), mitogen-activated protein kinase 8 and 9; MAP3K3, mitogen-activated protein kinase kinase kinase 3; MEK (MAP2K) 1/2, 5, MAP kinase kinases 1/2 and 5; MINK1 (MAP4K6), Misshapen-like kinase 1; MK2, MAPK-activated protein kinase 2; MKK (MAP2K), MAP kinase kinases; NLRP3, NLR family pyrin domain containing 3; NOX, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; oxLDL, oxidized low-density lipoprotein; PARs, protease-activated receptors; PKC, protein kinase C; PP2A, protein phosphatase 2A; RAF, rapidly accelerated fibrosarcoma; ROS, reactive oxygen species; Syk, spleen tyrosine kinase; TP, thromboxane receptor; TxA_2 receptor; TxA_2, thromboxane A_2; VWF, von Willebrand factor.

decreased, cPLA2 was not phosphorylated at S505, and decreased TxA2 formation was observed in line with impaired platelet functions induced by submaximal agonist concentrations. ASK1 deficiency was recapitulated by using the pharmacological ASK1 inhibitor selonsertib in murine wild-type platelets. Conversely, selonsertib in human platelets only blocked early phosphorylation of p38 and its substrate MAPKAP-kinase 2 (MK2, MAPKAPK2), but not sustained phosphorylation, and neither cPLA2 phosphorylation, TxA2 formation, nor agonist-induced platelet activation was reduced.[82] These studies clearly demonstrate that MAPK signaling may differ between murine and human platelets, and effects of pharmacological MAPK inhibitors have to be validated in both species with respect to their specific

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Table 1 Off-target and functional in vitro effects of pharmacological MAP kinase inhibitors on human platelets

	Inhibitor (target)	*Inhibitor (off-target effects)*	*Functional effects in vitro*[a]
ERK1/2	U0126 (MEK1/2) PD98059 (MEK1/2) PD184161 (MEK1/2) PD184352 (MEK1/2) Cobimetinib (MEK1/2)	U0126 (MEK5) PD98059 (COX-1/2; TBXAS1; MEK5) PD184161 (NR) PD184352 (MEK5) Cobimetinib (NR)	Adhesion (venous, arterial shear) TxA_2 synthesis Agonist-specific granule secretion αIIbβ3 activation/aggregation Clot retraction
JNK1/2	SP600125 (JNK1/2/3) 6o (JNK1/2/3)	SP600125 (e.g., SGK; S6K1) 6o (NR)	Adhesion (arterial shear) α-Granule secretion (submaximal agonist concentrations) αIIbβ3 activation/aggregation
p38	SB203580 (p38α/β) SB202190 (p38α/β) Skepinone-L (p38α/β) VX-702 (p38α/β)	SB203580 (COX-1/2; TBXAS1) SB202190 (NR) Skepinone-L (NR) VX-702 (NR)	Adhesion (static, venous/arterial shear conditions) TxA_2 synthesis Granule secretion αIIbβ3 activation/aggregation Clot retraction
ERK5	XMD8–92 (ERK5) BIX02189 (MEK5; ERK5)	XMD8–92 (e.g., TNK1; Pik4) BIX02189 (NR)	Substrate-specific adhesion (arterial shear) Agonist-specific αIIbβ3 Activation/aggregation Agonist-specific PS exposure

Abbreviations: COX 1/2, cyclooxygenase 1/2; ERK1/2, extracellular signal-regulated kinase 1/2; JNK1/2, c-Jun N-terminal kinase 1/2; MEK 1/2, mitogen-activated protein kinase kinase; NR, not reported; Pik4, polo-like kinase 4; PS, phosphatidylserine; S6K1, ribosomal protein S6 kinase β-1; SGK, serum and glucocorticoid inducible protein kinases; TBXAS1, thromboxane A synthase 1; TNK1, nonreceptor tyrosine-protein kinase 1; TxA_2, thromboxane A_2.
Source: Modified from Patel and Naik.[21]
[a]Off-target effects cannot be excluded.

signaling and potential off-target effects. A recent comprehensive overview summarizes our current knowledge of MAPK signaling and function in murine and human platelets[21] (►Fig. 3, ►Table 1).

Interestingly, the crosstalk between MAPKs and S/T-PPs is insufficiently known, too. Thrombin-induced ERK1/2 phosphorylation is increased by ASK1 or p38 inhibitors in human platelets,[82] which was also observed for murine platelets.[83,84] Potentially, a crosstalk between p38 and S/T-PPs such as PP2A might be responsible for the negative regulation of ERK1/2 by p38.[85] In accordance with these data, we showed that specific inhibition of PP2A by okadaic acid results in increased phosphorylation of ERK1/2 but also of p38 in human platelets, suggesting that PP2A represents an important regulator of distal platelet MAPKs[69] (►Fig. 3).

Clinically established cancer therapeutics targeting Y-PKs such as Syk or Btk have been intensively analyzed regarding their antiplatelet properties due to their bleeding risk.[86–88] Interestingly, current clinical trials for the approvement of MAPK inhibitors as anticancer drugs revealed that MEK1/2 (ERK1/2) inhibitors are associated with higher bleeding risk than p38 inhibitors.[21] Much less is known about platelet effects of clinically used anticancer drugs targeting the S/T-PP PP2A.[89,90] In this regard, it is important to note that novel therapeutics targeting more specifically MAPKs and PP2A may have unwarranted, off-target but also beneficial effects on platelets, megakaryopoiesis, and platelet production. There is evidence that the MEK1/2-ERK1/2 pathway is important for the physiological regulation of megakaryocyte maturation and proplatelet production induced by thrombopoietin via c-MPL (myeloproliferative leukemia virus oncogene)-receptor/JAK2 (janus kinase-2) signaling.[91,92] On the other hand, inherited ankyrin repeat domain-containing protein 26 (ANKRD26)-related thrombocytopenia is associated with defective proplatelet formation, but with persistent megakaryocyte MEK1/2-ERK1/2 activation and ANKRD26 expression. Interestingly, the MAPK inhibitor PD98059 could correct impaired proplatelet formation of the patients' megakaryocytes.[93] Accordingly, persistent p38-MAPK activation induced by Flt3 (fms-related receptor tyrosine kinase 3)-mediated pathways independent of JAK2 seems to be involved in the control of dysmegakaryopoiesis in primary myelofibrosis.[94] A recent study analyzing MASTL-mutant mice suggested that a distinct pathogenic gain-of-function variant in *MASTL* related to autosomal dominant thrombocytopenia, E167D, mediates decreased activity of PP2A-B55, leading to hyperphosphorylation of multiple actin cytoskeleton components, hyperstabilized pseudopods, and actin polymerization defects.[95]

Conclusion and perspective

Our understanding of Y-PKs and MAPK regulation by ST-PKs and ST-PPs in platelets is just beginning. Their crosstalk is not characterized by linear pathways from "receptor-to-function signaling," but has to be considered as complex network. Multiple PKs, PPs of different families, and their regulatory substrates interact and integrate signaling processes to a functional outcome. Only multi-pathway interactions between the Y-PK Syk and ST-PKs as well as ST-PP promote a fine-tuned regulation of platelet Syk activation/activity, emphasizing the crucial role of Syk in "pattern-recognition

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receptor"-mediated hemostatic, inflammatory, and immune functions of platelets. In the future, there is a need for advanced phosphoarrays and/or mass spectrometry-based quantitative phosphoproteomics combined with sophisticated bioinformatics and function tools for high-throughput analyses to establish a deep profile of platelet signaling–function relations. Is this far away and/or unrealistic? Recently, rapid and site-specific deep phosphoproteome profiling by data-independent acquisition without the need for spectral libraries was reported and successfully applied to characterize the epidermal growth factor signaling pathway of two human cell lines.[96] Clinical application is not too far away since the profile of hundreds of phosphoproteomes could be obtained in a few days from a starting protein material as low as 200 µg or less. Interestingly, the signaling pathways in this study were validated by a panel of 30, highly selective protein kinase inhibitors. Since the first successful use of a protein kinase inhibitor (imatinib/ Gleevec) to treat leukemia patients in 2001, 52 FDA-approved small-molecule protein kinase inhibitors are listed as of 2020 for a diverse spectrum of therapeutic indications, among them also the Syk inhibitor fostamatinib (Tavalisse) to treat chronic immune thrombocytopenia.[97] PK inhibitors and more recently drugs targeting PPs have come a long way, are now target-designed, and powerful reagents to control and repair diseased signal transduction pathways. However, their targeted pathways need to be understood and assessed with valid assays. Such emerging strategies[65,98,99] are promising for the individualized screening of platelet-related diseases and drugs. Human platelets can serve here as prominent cellular model, but also as therapeutic and diagnostic target.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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