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## **A comprehensive tyrosine phosphoproteomic analysis reveals novel components of the platelet CLEC-2 signaling cascade**

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### 17 **Abstract**

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20 C-type lectin-like receptor 2 (CLEC-2) plays a crucial role in different platelet-related  
21 physiological and pathological processes. It signals through a tyrosine kinase-mediated  
22 pathway that is highly dependent on the positive feedback exerted by the platelet-derived  
23 secondary mediators ADP and thromboxane A2 (TXA2). Here, we aimed to analyse the  
24 tyrosine phosphoproteome of platelets activated with the CLEC-2 agonist rhodocytin in order  
25 to identify relevant phosphorylated tyrosine residues (p-Tyr) and proteins involved in platelet  
26 activation downstream of this receptor. We identified 363 differentially p-Tyr residues,  
27 corresponding to the majority of proteins previously known to participate in CLEC-2  
28 signalling and also novel ones, including adaptors (e.g. DAPP1, Dok1/3, CASS4, Nck1/2);  
29 kinases/phosphatases (e.g. FAK1, FES, FGR, JAK2, SHIP2); and membrane proteins (e.g.  
30 G6F, JAM-A, PECAM-1, TLT-1). In order to elucidate the contribution of ADP and TXA2 at  
31 different points of the CLEC-2 signalling cascade, we evaluated p-Tyr levels of residues  
32 identified in the analysis and known to be essential for the catalytic activity of kinases Syk(p-  
33 Tyr<sup>525+526</sup>) and Src(p-Tyr<sup>419</sup>), and for PLC $\gamma$ 2 activity (p-Tyr<sup>759</sup>). We demonstrated that Syk  
34 phosphorylation at Tyr<sup>525+526</sup> also happens in the presence of ADP and TXA2 inhibitors,  
35 which is not the case for Src-pTyr<sup>419</sup> and PLC $\gamma$ 2-pTyr<sup>759</sup>. Kinetics studies for the three  
36 phosphoproteins **show some differences in the phosphorylation profile**. Ca<sup>2+</sup> mobilization  
37 assays confirmed the relevance of ADP and TXA2 for full CLEC-2-mediated platelet  
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3 activation. The present study provides significant insights into the intracellular events that  
4 take place following CLEC-2 activation in platelets, contributing to elucidate in detail the  
5 CLEC-2 signalosome.  
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10 **Key words:** platelets, CLEC-2 signalling, tyrosine phosphoproteome  
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## 16 **Summary Table**

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### 19 What is known on this topic:

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- 22 • CLEC-2 plays a crucial role in maintaining vascular integrity, embryonic lymphatic  
23 vessel development, hematogenous metastasis and thrombus stabilization.
- 24 • CLEC-2 signals through a protein tyrosine-kinase-mediated pathway; the only  
25 endogenous ligand known so far is podoplanin.  
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### 32 What this paper adds:

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- 35 • First tyrosine phosphoproteomic analysis of the CLEC-2 signalling cascade, analyzing  
36 in detail the impact of secondary mediators.
- 37 • Novel CLEC-2 signalling proteins and p-Tyr sites identified allowing a more complete  
38 and updated overview of the CLEC-2 signalosome.
- 39 • Kinetic studies show differences in the phosphorylation profiles of Syk-p-Tyr<sup>525+526</sup>,  
40 Src-pTyr<sup>419</sup>, and PLCγ2-pTyr<sup>759</sup> following CLEC-2 activation.  
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## 1. Introduction

C-type lectin-like receptor 2 (CLEC-2) has been demonstrated to play a crucial role in maintaining vascular integrity<sup>1</sup> and embryonic lymphatic vessel development<sup>2</sup>, and also participates in platelet-mediated hematogenous metastasis<sup>3</sup>. CLEC-2 plays a minor role in haemostasis<sup>4</sup>, but contributes to thrombus stabilization under flow<sup>2</sup>. This latter process is a key step in the development of ischemic events associated with unwanted platelet activation.

CLEC-2 signals through a protein tyrosine-kinase-mediated pathway. The only endogenous agonist for CLEC-2 identified to date is podoplanin, a glycoprotein expressed on the surface of lymphatic endothelial cells, podocytes, type-I alveolar cells, and some tumour cells. In addition, rhodocytin, a protein isolated from the venom of the Malayan pit viper *Calloselasma rhodostoma*, is another CLEC-2 agonist, capable of inducing a potent platelet aggregation response following receptor engagement<sup>5,6</sup>.

The CLEC-2 signalling cascade starts with the tyrosine- phosphorylation of its immunoreceptor tyrosine-based activation motif (hemITAM), defined as a singular YxxL sequence within the cytoplasmic tail of the receptor. Cell signalling through ITAM motifs requires the presence of two YxxL sequences, which allows receptor interaction with a Src homology 2 domain (SH2)-containing tyrosine-kinase. Therefore, bringing two hemITAM motifs in close vicinity through CLEC-2 dimerization is necessary for signal transduction. Once the hemITAM motifs of dimeric CLEC-2 have been phosphorylated, the tyrosine-kinase Syk interacts with the cytoplasmic tails of the dimer through its SH2 domains, reinforcing hemITAM phosphorylation and contributing to signal transduction. Subsequent biochemical events that take place in the intracellular space include the phosphorylation of the adapters LAT and SLP-76, and the activation of PLC $\gamma$ 2, leading to platelet aggregation<sup>7-10</sup>. The events taking place for primary phosphorylation of the hemITAM motifs are not yet completely

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3 understood, but may involve the participation of Src-family tyrosine kinases (SFKs)<sup>11</sup>.  
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5 Interestingly, CLEC-2 signalling in platelets has been found to be highly dependent in  
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7 pathway reinforcement induced by the secondary mediators Adenosine diphosphate (ADP)  
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9 and thromboxane A<sub>2</sub> (TXA<sub>2</sub>), released from platelets upon activation and acting in an  
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11 autocrine and paracrine manner<sup>12</sup>. Positive feedback by these secondary mediators is highly  
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13 relevant in the amplification of the most proximal events that occur upon receptor activation,  
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15 presumable through activation of SFKs and Syk that mediate CLEC-2 phosphorylation<sup>13</sup>.  
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20 Recently, we performed a gel-based proteomic analysis of the CLEC-2 signalling  
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22 pathway<sup>14</sup>. This previous study was limited by the gel-based approach, which was far from  
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24 being quantitative. Recent advances in mass spectrometry leading to confident identification  
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26 of phosphorylation sites in complex protein samples, and the development of antibodies  
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28 capable of recognizing specific phosphosites, have opened the path for more sensitive and  
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30 quantitative proteomic analyses of signalling cascades in platelets. In this context, we have  
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32 performed an extensive phosphotyrosine proteomic analysis of platelets stimulated through  
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34 the CLEC-2 receptor in presence and absence of inhibitors of secondary mediators, in  
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36 comparison with resting state, in order to identify new effectors in the signalling pathway  
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38 coupled to this receptor and to elucidate the impact of secondary mediators in the tyrosine-  
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40 phosphorylation response. In this way, it was possible to provide a much complete picture of  
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42 the CLEC-2 signalosome, including identifying new proteins and mapping the precise  
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44 phosphosites responsible for platelet activation through this receptor.  
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## 53 **2. Materials and methods**

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56 A schematic representation of the methodology applied is shown in **Figure 1**.  
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## 2.1. Platelet isolation and activation

Fresh blood was obtained from healthy volunteers not under chronic medication, and not under anti-platelet drugs for the previous 10 days. The study was conducted according to the declaration of Helsinki, and with approval by the Galician Investigation Ethics Committee (code 2009-270). The age range of the donors was 22-51 years old; 50% of them were female. Blood was collected in coagulation 3.2% sodium citrate tubes (Vacuette), and samples were processed within the first 30 minutes after extraction.

Platelets isolation was following a well-established procedure that limits contamination with other blood cells and plasma proteins<sup>15</sup>. After washing steps, platelets were resuspended in HEPES-Tyrodes (134mM NaCl; 0.34mM Na<sub>2</sub>HPO<sub>4</sub>; 2.9 mM KCl; 12mM NaHCO<sub>3</sub>; 20mM HEPES; 5mM glucose; 1mM MgCl<sub>2</sub>; pH 7.3) at a concentration of 8 x10<sup>8</sup> platelets/ml and allowed to rest for 30 minutes at room temperature.

From each healthy donor included in the proteomic study (two men and two women), we obtained platelet samples under three different stimulation conditions: basal (non-activated in absence of secondary mediators' inhibitors), "Rhod" (rhodocytin- activated in absence of secondary mediators' inhibitors), and "Rhod inh" (rhodocytin- activated in presence of secondary mediators' inhibitors). We did not include a "Basal inh" condition in our proteomic analysis due to sample limitation issues to keep biological replicates and considering both types of basal samples elicit a similar tyr phosphoproteome profiles, with marginally lower phosphorylation levels in the presence of the inhibitors. Activations were performed for 5 minutes with 200nM rhodocytin at 37°C and 1200rpm stirring in a Chrono-log aggregometer. Optimal incubation times were based on previous data<sup>14</sup> and the dose was adjusted in dose response experiments based on measuring the Tyr- phosphorylation protein profile by Western blotting (**Supplementary Figure 1**). All activations were performed after the addition of 9µM Integrilin® (GlaxoSmithKline), in order to avoid platelet aggregation and

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3 the activation of the  $\alpha$ IIB $\beta$ 3 integrin signalling cascade. For inhibiting platelet stimulation by  
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5 ADP and TXA<sub>2</sub>, 2U/ml of apyrase (ADPase that catalyses the hydrolysis of ADP), and 10 $\mu$ M  
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7 of indomethacin (that inhibits COX-1 and thus the synthesis of TXA<sub>2</sub>), both from Sigma-  
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9 Aldrich, were added to the platelet suspension prior to CLEC-2 stimulation.  
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12 For proteomic analysis, lysates were obtained by directly adding 2% SDS (final  
13  
14 concentration) to 500  $\mu$ l of washed platelet suspensions. For validation analyses by Western  
15  
16 blotting, lysates were obtained by supplementing platelet suspensions with an equal volume  
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18 (500 $\mu$ l) of lysis buffer 2x (0.3M sodium chloride; 20mM Tris; 2mM EGTA; 2mM EDTA; 2%  
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20 v/v NP-40; 10 $\mu$ g/ml aprotinin; 1 $\mu$ g/ml pepstatin; 10 $\mu$ g/ml leupeptin; 400 $\mu$ g/ml AEBFS; 5mM  
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22 sodium orthovanadate; pH 7.5).  
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## 29 **2.2. Protein quantification and digestion for proteomic analysis**

30 For proteomic analysis, four biological replicates were analysed per condition. Protein  
31  
32 concentration in the sample was quantified with the micro BCA<sup>TM</sup> protein assay kit (Pierce)  
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34 following the manufacturer's protocol. After that, 800 $\mu$ g of protein per sample were digested  
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36 with trypsin following a large-scale filter aided sample preparation (LFASP) method on 10  
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38 kDa filters (Amicon<sup>TM</sup> Ultra-15, Millipore)<sup>16</sup>. Tryptic digests were dried and resuspended in  
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40 700 $\mu$ l of IAP buffer (50mM MOPS pH 7.2; 10mM disodium phosphate; 50mM sodium  
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42 chloride).  
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## 49 **2.3. Phosphotyrosine peptide enrichment and LC-MS/MS analysis**

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52 Immunoaffinity enrichment of phosphotyrosine peptides was accomplished with PTMScan<sup>®</sup>  
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54 Phospho-Tyrosine Rabbit mAb (P-Tyr-1000) Kit (Cell Signaling), as indicated in the  
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56 Supplemental Methods.  
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3 Samples containing phospho-Tyr peptides were loaded in a nano Easy-nLC 1000  
4 chromatographic system (Proxeon), and mass spectra acquired on an LTQ-Orbitrap Velos  
5 (ThermoScientific). The spectra were searched against the human SwissProt database and  
6 validation of phosphorylation sites was performed by ptmRS and Percolator algorithms.  
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8 Protein p-sites included in the analysis were those identified by at least two peptide spectrum  
9 matches (PSMs). Fold changes were calculated as the ratio of the number of PSMs that led to  
10 the identification of the corresponding protein p-site for the two compared conditions. Further  
11 details on the mass spec analysis and phosphosite identification can be found in the  
12 Supplemental Methods section.  
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#### 26 **2.4. Database search and data integration**

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28 Phosphosite Plus<sup>®17</sup> database was scrutinized to find detailed information about Tyrosine-  
29 phosphorylation sites detected in the proteins identified in the study.  
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34 String<sup>©18</sup> and PhosphoPath<sup>19</sup> tools, powered by Cytoscape v3.7<sup>20</sup>, were used to  
35 investigate potential interactions between differentially phosphorylated proteins, and to  
36 identify potential kinases responsible for the Tyr-phosphorylations detected in those proteins.  
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42 UniProtKB (UniProt Protein Knowledgebase)<sup>21</sup> was consulted in order to obtain  
43 detailed information about relevant proteins identified in the study.  
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#### 49 **2.6. Ca<sup>2+</sup> mobilization *in vitro* assays**

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51 Washed platelets (2x10<sup>7</sup> platelets/ml) from healthy donors were incubated with FLIPR  
52 Calcium 4 reagent (Molecular devices<sup>®</sup>), supplemented with Probenecid 0.07% and  
53 incubated for 1 hour at 37°C in a 384-well transparent bottom plate. Inhibitors and agonists  
54 were dispensed by FDSS7000EX (Hamamatsu), automatically. Platelets were incubated for  
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3 10 minutes with 9 $\mu$ M integrilin (eptifibatide) and, when necessary, with apyrase 2 U/ml, and  
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5 indomethacin 10 $\mu$ M. After that, agonists (3 $\mu$ g/ml CRP, 0.75 U/ml thrombin, 100 nM or 200  
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7 nM rhodocytin) were added to platelets. Changes in fluorescence were measured in real time  
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9 for 15 minutes after the addition of the agonists and using emission and excitation wave  
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11 lengths of 480nm and 540nm, respectively.  
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## 18 **2.7. Western blotting**

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21 For validation by Western blotting, four samples corresponding to four biological replicates  
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23 from different cohorts of healthy donors were analysed per condition and experimental  
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25 setting.  
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29 Proteins were precipitated from lysates in 20% trichloroacetic acid in acetone as  
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31 previously described<sup>15</sup>, and protein pellets were resuspended in 50 $\mu$ l of sample buffer (65mM  
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33 CHAPS, 5M urea, 2M thiourea, 0.15M NDSB-256, 30mM Tris, 1mM sodium vanadate,  
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35 0.1mM sodium fluoride, and 1mM benzamidine). Protein quantitation was done with  
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37 Coomassie Plus protein reagent (Thermo Scientific) following manufacturer's protocol.  
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41 Protein separation by SDS-PAGE and subsequent immunoblotting were carried out as  
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43 indicated in the Supplemental Methods. The following primary antibodies were used: rabbit  
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45 anti-human p-PLC $\gamma$ 2 (Y759) (MAB7377, R&D systems) dilution 1/300; rabbit anti-p-Syk  
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47 (Y525+Y526) (ab58575, Abcam) dilution 1/1000; rabbit anti-p-Src(Y418) (44660G, Fisher  
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49 Scientific), dilution 1/1000; mouse anti-PLC $\gamma$ 2 (sc-5283, Santa Cruz Biotechnology) dilution  
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51 1/500; mouse anti-Syk (sc-1240, Santa Cruz Biotechnology) dilution 1/1000; rabbit anti-Src  
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53 pan (44-656G, Invitrogen), dilution 1/1000.  
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57 Further information on the statistical analysis can be found in the Supplemental  
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59 Methods.  
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### 3. Results

#### 3.1. Tyrosine- phosphoproteome analysis of resting and CLEC-2 activated platelets.

From the 12 samples analysed (corresponding to 4 biological replicates per condition), we identified a total number of 757 p-Tyr peptides, 466 p-Tyr residues and 264 proteins (**Table 1**). More proteins, p-Tyr peptides, and pTyr sites were identified in “Rhod” samples (rhodocytin-activated platelets in absence of inhibitors of secondary mediators) (**Table 1; Figure. 2A,B**). Interestingly, from the 757 p-Tyr peptides identified, 258 were exclusively present in “Rhod” samples (**Figure 2A**). Considering the p-Tyr residues identified per condition, 458 were identified in “Rhod” samples, a number that decreased to 313 when platelets were activated in the presence of secondary mediators’ inhibitors (“Rhod inh”) (**Figure 2B; Table 1**). The overall results highlight the relevance of secondary mediators on achieving a robust intracellular tyrosine-phosphorylation response when platelets are activated through CLEC-2.

Interestingly, our LC-MS/MS analysis led to the identification of p-Tyr residues in 20 proteins that were not reported to undergo phosphorylation in those residues to date, some of them playing a relevant role in several intracellular signalling cascades, such as Btk, FES, Nck2, and ROCK2, and Talin, among others (**Supplemental table 1**).

#### 3.2. Phosphotyrosine fingerprint on CLEC-2 activated platelets: new signalling proteins identified.

As our data confirmed that the contribution of secondary mediators is essential for a powerful activation of the CLEC-2 coupled signalling pathway through tyrosine- phosphorylation, we performed our comparative analysis with the data obtained from “Rhod” and basal samples.

From the 235 proteins that were identified with at least one p-Tyr residue differentially regulated between the aforementioned conditions (Fold change  $\geq 1.5$ ), 21% are known to

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3 participate in cytoskeleton remodelling process, and 58% are involved in signal transduction  
4 upon receptor activation (**Figure 2C**). From these signalling proteins, more than a half of  
5 them belong to protein kinases (23%) plus adaptors/scaffolds (30%) (**Figure 2C**). **Table 2**  
6 contains a selection of relevant signalling proteins and their p-Tyr residues identified in the  
7 study (fold change  $\geq 1.5$ ). In total, 363 differentially regulated p-Tyr residues were identified,  
8 encompassing almost all the proteins previously known to be key effectors in the CLEC-2  
9 signalling pathway, including: CLEC-2 itself, the adapters LAT, SLP-76, Vav, Grb2, ADAP,  
10 CrkL, Gads, Dok2, and SKAP2; the kinases Syk, the SFKs Lyn and Fyn, Btk, Csk, FER, and  
11 PIK3R1; the phosphatases SHP-1, SHP-2, SHIP1, and PTP1B; the phospholipase PLC $\gamma$ 2; and  
12 the receptors CD148, and G6b-B.  
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26 Furthermore, we identified differentially-regulated p-Tyr residues in a number of  
27 proteins that are relevant for intracellular signal transduction in platelets and other blood cell  
28 types. Here, we show for the first time that several of them are involved in the CLEC-2  
29 signalling cascade, including: the adapters Gab1, DAPP1, Dok1, Dok3, CASS4, EFS, Pag1,  
30 Nck1 and Nck2; the kinases FAK1, FES, FGR, and JAK2, the phosphoinositide phosphatase  
31 SHIP2, and the membrane proteins G6F, JAM-A, PECAM-1, and TLT-1 (**Table 2**).  
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### **3.3. Secondary mediators (ADP and TXA<sub>2</sub>) are required for powerful tyrosine-phosphorylation response upon CLEC-2 activation.**

Our phosphoproteomic data indicate secondary mediators are essential for a potent tyrosine-phosphorylation intracellular response mediated by CLEC-2 activation (**Figure 2; Table 1**). To further investigate this issue, we compared the fold changes (FC) obtained when comparing “Rhod” with basal samples, and “Rhod” with “Rhod inh” samples. We used this fold change comparison as an indirect measurement of the Tyr-phosphorylation levels between “Rhod inh” and resting platelets. Thus, when FC-values between both stimulation

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3 conditions (“Rhod” vs. “Rhod inh”) are smaller than those obtained between “Rhod” and  
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5 basal samples, we can conclude that rhodocytin stimulation itself is capable of altering Tyr-  
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7 phosphorylation levels of the corresponding protein phosphorylation site. The smaller the  
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9 “Rhod” vs. “Rhod inh” FC is, the higher the stimulation effect of Rhod inh. Therefore, from  
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11 the 150 p-Tyr residues from relevant signalling proteins that we found differentially regulated  
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13 (FC  $\geq 1.5$ ) in “Rhod” samples (**Table 2**), only 28 were directly regulated by rhodocytin-  
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15 CLEC-2 engagement (FC Rhod/Rhod inh < FC Rhod/basal; FC Rhod/basal / FC Rhod/Rhod  
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17 inh  $\geq 1.5$ ). Among these conditions, we identified p-Tyr residues for Syk, Gads, Dok2, SFKs,  
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19 PLC $\gamma$ 2, SHP-1, and CASS4 (**Supplemental table 2**).  
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### 26 **3.4. CLEC-2 activation-mediated Ca<sup>2+</sup> release is highly dependent on secondary** 27 28 **mediators**

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30 In order to investigate more in detail the impact of secondary mediators in achieving full  
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32 CLEC-2-mediated platelet activation, we developed a miniaturized calcium mobilization  
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34 assay for platelets. All experiments were under non-aggregating conditions (with integrillin).  
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36 Firstly, we checked a couple of Rhodocytin doses for platelet activation in presence and  
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38 absence of secondary mediators inhibitors (apyrase and indomethacin). We demonstrated  
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40 good levels of Ca<sup>2+</sup> release with a 100 nM dose of Rhodocytin, similar to 200 nM.  
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42 Importantly, Ca<sup>2+</sup> release was drastically diminished in the presence of inhibitors (**Figure**  
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44 **3A**). We next compared the latter effect with the impact of secondary mediators on platelet  
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46 activation by the collagen receptor GPVI (activated with collagen-related peptide (CRP)), and  
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48 PAR receptors (activated with thrombin). Such impact on Ca<sup>2+</sup> release was minor compared  
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50 to what happens in the case of CLEC-2 activation (**Figure 3B**). Finally, we explored the  
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52 specific contribution of ADP and TXA<sub>2</sub>, individually, to CLEC-2 activation-mediated  
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54 calcium release. Ca<sup>2+</sup> release diminishes in the presence of apyrase or indomethacin, **with a**  
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3 higher impact of the former. Maximum inhibition of  $\text{Ca}^{2+}$  release following CLEC-2  
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5 activation takes place when both inhibitors of secondary mediators are present at the same  
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7 time (Figure 3C).  
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### 10 11 12 **3.5. Contribution of secondary mediators to different steps essential for CLEC-2** 13 14 **signalling**

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16 In order to further elucidate the contribution of ADP and TXA2 at different points of the  
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18 signalling pathway downstream of CLEC-2, we evaluated p-Tyr levels of residues that were  
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20 identified in the analysis and are known to be essential for the catalytic activity of kinases Syk  
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22 (p-Tyr<sup>525+526</sup>) and Src (p-Tyr<sup>419</sup>), and for PLC $\gamma$ 2 activity (p-Tyr<sup>759</sup>). To do so, we performed a  
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24 western blot with protein samples obtained after activating the platelets in the presence or  
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26 absence of apyrase and indomethacin under non aggregating conditions. In parallel, we just  
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28 checked the aggregation profiles of platelets activated with Rhodcytin in the presence and  
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30 absence of the secondary mediators inhibitors; as expected, the latter prevented aggregation  
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32 (Figure 4A).  
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38 As it is shown in Figure 4B,C, phosphorylation of Syk on Tyr<sup>525/526</sup>, which is one of  
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40 the primary events occurring upon CLEC-2 activation following hemITAM phosphorylation,  
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42 is robustly increased in response to positive feedback by released ADP and TXA2.  
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44 Nonetheless, CLEC-2 stimulation itself, in the presence of inhibitors of secondary mediators,  
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46 led to a significant increase in phosphorylation of Syk Tyr<sup>525/526</sup>.  
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50 Robust phosphorylation of Src Tyr<sup>419</sup>, was also critically dependent on released ADP  
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52 and TXA2. In this case, we also observed high levels of that phosphoprotein in resting (basal)  
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54 platelets compared to the other two proteins analysed (Figure 4B,C).  
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57 Finally, we also investigated the activation of PLC $\gamma$ 2, which enables  $\text{Ca}^{2+}$  release from  
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59 intracellular stores and cytoskeleton remodelling process, so we evaluated its phosphorylation  
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3 on Tyr<sup>759</sup>. Optimal phosphorylation of PLCγ2 Tyr<sup>759</sup> is only achieved when ADP and TXA2  
4 are not inhibited from exerting their positive feedback effects on CLEC-2 signalling (**Figure**  
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7 **4B,C**).

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10 One question that arises from the above data is whether the increase in tyrosine  
11 phosphorylation observed for the proteins validated is directly due to Rhodocytin-CLEC-2  
12 engagement with signalling reinforced by the secondary mediators' positive feedback, or if  
13 such mediators could cause part of the increase by themselves. In order to clarify this issue,  
14 we activated platelets with ADP and arachidonic acid (AA), in comparison with Rhodocytin  
15 activation, in the absence of apyrase and indomethacin. Interestingly, ADP and AA are not  
16 able to induce an increase in the levels of Syk (p-Tyr<sup>525+526</sup>), Src (p-Tyr<sup>419</sup>), and PLCγ2 (p-  
17 Tyr<sup>759</sup>), which confirms the differences observed in our analysis are mediated by CLEC-2  
18 activation (**Fig. 5**).

### 3.6. Tyrosine phosphorylation kinetic profiles show variations depending on the CLEC-2 signalling protein analysed

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33 To complete the signalling studies, we carried out a small scale kinetic analysis focusing on  
34 the three phosphoproteins mentioned above. As shown in **Figure 6**, tyrosine phosphorylation  
35 levels start to increase after 30 sec of platelet stimulation with 200 nM Rhodocytin and  
36 significant levels are still maintained after 5 minutes; however the kinetic profile is different  
37 depending on the protein. Thus, Syk, which plays a relevant role in early signalling events, is  
38 phosphorylated in Y<sup>525</sup> and Y<sup>526</sup> rapidly, reaching maximum levels after one minute **and**  
39 **decreasing steadily after 90 sec**. Regarding Src (p-Tyr<sup>419</sup>), basal levels are high compared with  
40 the other two proteins; nevertheless, there is an increase in phosphorylation levels following  
41 activation reaching the maximum after 1 min and maintaining such level for 3 min when it  
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3 starts to decrease gradually. Regarding PLC $\gamma$ 2 (p-Tyr<sup>759</sup>), maximum phosphorylation levels  
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5 are also achieved between 1 and 3 min.  
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#### 10 **4. Discussion**

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12 The CLEC-2 receptor is known to mediate platelet activation through the stimulation of a  
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14 phosphotyrosine signalling cascade that is triggered by phosphorylation of the tyrosine  
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16 residue within its hemITAM<sup>5</sup>. In the present study, we aimed to analyse the phosphotyrosine  
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18 proteome of CLEC-2-activated platelets. To do so, we performed an immunoaffinity  
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20 enrichment of p-Tyr peptides in our samples, and we analysed them in an LTQ Orbitrap Velos  
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22 mass spectrometer, an experimental tandem that allows high sensitivity on the identification  
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24 of specific protein p-Tyr residues.  
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29 Results from our study emphasize the crucial synergism between the secondary  
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31 mediators, ADP and TXA2, and CLEC-2 to induce a powerful activation of the receptor. To  
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33 further explore this data, we evaluated the relevance of ADP and TXA2 in intracellular  
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35 calcium release mediated by CLEC-2-Rhodocytin activation, and also by platelet activation  
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37 with other agonists, such as CRP (GPVI agonist) and thrombin (PAR1/4 agonist). Our results  
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39 clearly demonstrate the relevance of both ADP and TXA2 (together) to achieve full CLEC-2  
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41 platelet activation, which does not happen in the case of GPVI or PAR1/4 activation. In  
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43 addition, we also studied the phosphorylation levels of tyrosine sites that are crucial for the  
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45 enzymatic activity of three proteins that are relevant for signal transduction upon CLEC-2  
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47 activation: Syk p-Tyr<sup>525+526</sup>, Src p-Tyr<sup>419</sup> and PLC $\gamma$ 2 p-Tyr<sup>759</sup>. Interestingly, we found that,  
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49 although powerful platelet activation through the stimulatory effect of secondary mediators is  
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51 essential for optimal Syk- kinase domain phosphorylation, rhodocytin stimulation of CLEC-2  
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53 itself is sufficient to significantly increase Syk Tyr<sup>525+526</sup> phosphorylation. In contrast, optimal  
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55 phosphorylation of PLC $\gamma$ 2 Tyr<sup>759</sup> is highly dependent on ADP and TXA2, indicating that the  
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3 initial steps of CLEC-2 signalling, including Syk phosphorylation, are not as dependent on  
4 ADP and TXA2 as downstream events. In resume, secondary mediators inhibitors are able to  
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7 fully inhibit Src and PLC $\gamma$ 2 tyrosine phosphorylation in the indicated residues, and can only  
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9 partially inhibit Syk Tyr<sup>525+526</sup>.

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12 To complement the above data, we also analysed the direct effect of ADP and AA to  
13 induce phosphorylation of Syk p-Tyr<sup>525+526</sup>, Src p-Tyr<sup>419</sup> and PLC $\gamma$ 2 p-Tyr<sup>759</sup>, observing no  
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16 effect, which indicates phosphorylation changes observed when activating platelets with  
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19 rhodocytin in absence of apyrase and indomethacin are due to CLEC-2-activation and  
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22 secondary mediators contribute only with a positive feedback to achieve full CLEC-2 tyrosine  
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25 phosphorylation and signalling activation. Although it was previously suggested that the latter  
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28 is mediated by Rac activation<sup>12</sup>, further research is needed to confirm the precise mechanism  
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30 of this positive feedback in line with the present study.

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33 Complementary data on the three phosphoproteins mentioned above demonstrate  
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36 differences in the kinetic profile of p-Tyr depending on the signalling protein analysed. **For**  
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39 **example, maximum phosphorylation levels of Syk on Tyr<sup>525+526</sup> decrease faster than in the**  
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42 **case of PLC $\gamma$ 2 Tyr<sup>759</sup>.** The observed differences in the kinetic profile indicate that further  
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45 kinetic studies by quantitative western blotting or proteomics are needed to have a more  
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48 precise picture of the assembling of the CLEC-2 signalosome depicted in the present study.

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51 Intriguingly, in the case of phosphorylation of Src Tyr<sup>419</sup>, which is located within the  
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54 kinase domain responsible of Src-catalytic activity, the levels of basal samples were high  
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57 compared to the other validated proteins, especially in the presence of secondary mediators  
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60 **(Figs. 4-6)**. This suggests the existence of a pool of active Src in platelets, reinforced by a  
basal release of secondary mediators. Further research is needed to explore this possibility;  
moreover, other SFKs besides Src, including Fyn, Lyn or Fgr, may contribute to signalling  
downstream of CLEC-2.



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3 Considering the above mentioned relevance of ADP and TXA2 in reinforcing CLEC-2  
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5 signalling, as our aim was to obtain a broader picture of the biochemical events that are taking  
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7 place in the signalling cascade downstream of CLEC-2 activation, we focused on the  
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9 differentially regulated p-Tyr sites ( $FC \geq 1.5$ ) identified in “Rhod” samples (rhodocytin-  
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11 activated in absence of inhibitors of secondary mediators) compared to unstimulated samples.  
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13 We demonstrate differentially regulated p-Tyr residues for almost all the proteins known to  
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15 participate in the CLEC-2 signalling pathway to date (**Table 2**). Interestingly, a number of  
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17 those proteins were known to be phosphorylated upon CLEC-2 activation, but the specific  
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19 tyrosine residues that undergo this modification were not described to date. Here, we have  
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21 identified p-Tyr sites that are known to have great impact in signal transduction. CLEC-2 was  
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23 found to be tyrosine phosphorylated on hemITAM motif -Tyr<sup>7-5</sup>; phosphorylation of LAT  
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25 Tyr<sup>220</sup> was found to be up- regulated, which promotes its interaction with Grb2, Gads, and  
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27 PIK3R1<sup>22</sup>; Syk was phosphorylated at Tyr<sup>348</sup> and Tyr<sup>352</sup>, both of which are essential for  
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29 regulating Syk kinase activity, and at Tyr<sup>525</sup> and Tyr<sup>526</sup>, required for catalytic activity<sup>23</sup>; the  
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31 protein- tyrosine phosphatase SHP-1 was phosphorylated at Tyr<sup>536</sup> and Tyr<sup>564</sup>, which have  
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33 been implicated in regulating its activity <sup>24</sup>; and CD148 was shown to be phosphorylated on  
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35 Tyr<sup>1311</sup> and Tyr<sup>1320</sup>, previously reported to be mediated by Src and Fyn<sup>25</sup> and essential for  
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37 SHP-1 interaction with CD148 SH2 domains. Moreover, the identification of several proteins  
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39 tyrosine- phosphorylated on SH3, SH2, tyrosine- kinase, and phosphatase domains, as well as  
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41 in ITIM motifs, highlights the potential relevance of the identified p-Tyr sites in mediating  
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43 signal transduction upon CLEC-2 receptor activation (**Table 2**).

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45 Furthermore, we have identified differentially regulated p-Tyr residues from proteins  
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47 that have never been reported to play a role downstream of CLEC-2, but that are known to  
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49 participate in regulating signal transduction in platelets and other blood cells. Some of these  
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51 proteins have been described to interact and regulate the function of other known regulators of  
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3 CLEC-2 signalling. As a compendium of our proteomic data and the previous knowledge  
4 about protein interactions and functions, we have uncovered the more detailed picture of the  
5 CLEC-2 signalosome to date, represented in **Figure 7**, which includes key signalling proteins  
6 and their differentially regulated p-Tyr residues identified in our study. Interestingly, the set  
7 of proteins we show to be differentially- phosphorylated in our study suggests a delicate  
8 balance between stimulatory and inhibitory intracellular signals, which may be crucial for  
9 maintaining platelet activation without triggering an exacerbated response. Some of these  
10 novel phosphorylation sites and signalling proteins are likely to be clinically relevant, due to  
11 the potential interest of this receptor as a therapeutic target, since it seems to be crucial for  
12 thrombus stabilization, but not for haemostasis maintenance.  
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26 Novel players on the CLEC-2 signalling cascade identified to be regulated by tyrosine-  
27 phosphorylation include the adapters Dok1 and Dok3<sup>26</sup>, Nck1 and Nck2<sup>27,28</sup>, and Crk-  
28 associated substrate (CAS) family members CASS4 and EFS<sup>29</sup>, among others (**Table 2**;  
29 **Figure 7**). Moreover, we report a number of up- regulated phosphorylated platelet kinases  
30 that were not previously described to participate in the CLEC-2 signalling cascade, but that  
31 are related to further signalling pathways, such as FAK, differentially phosphorylated at  
32 Tyr<sup>925</sup>, phosphorylation of which is mediated by FER allowing FAK interaction with Grb2  
33 and SHP-2<sup>30</sup>; FES; and FGR<sup>31</sup>, among others (**Table 2**; **Figure 7**).  
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45 We also identified numerous p-Tyr residues differentially- regulated in proteins that  
46 are known to maintain platelet activation within physiological limits by exerting an inhibitory  
47 action during intracellular signalling processes. The most important effectors on regulating  
48 signal transduction upon ITAM-receptor activation are the immunoreceptor tyrosine-based  
49 inhibition motif (ITIM)-containing receptors. Tyrosine-phosphorylation of these domains  
50 provides docking sites for SH2 domain- containing phosphatases, such as SHIP1, SHP-1 and  
51 SHP-2, all of which were found to contain differentially regulated p-Tyr residues in our study  
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3 (Table 2; Figure 7), localizing the phosphatases in close proximity with ITAM-containing  
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5 receptors that can then dephosphorylate and inactivate<sup>32</sup>. G6b-B is an ITIM-containing  
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7 membrane protein that has been already reported to participate in CLEC-2 mediated  
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9 signalling<sup>33,34</sup>, and that we found to be further phosphorylated on ITIM Tyr<sup>211</sup> and Tyr<sup>237</sup> upon  
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11 rhodocytin stimulation. Moreover, we identified up-regulated tyrosine- phosphorylated  
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13 residues for the other two known platelet ITIM-containing receptors, PECAM-1 and TLT-1.  
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15 PECAM-1 was found to be phosphorylated on Tyr<sup>663</sup>, Tyr<sup>690</sup>, and Tyr<sup>713</sup>, the last two located  
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17 on the ITIM domain of the protein, and both known to be phosphorylated by Lyn<sup>35</sup>. PECAM-  
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19 1 was reported to negatively regulate platelet GPVI-activation, as a feedback mechanism to  
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21 limit thrombus growth, through the binding of its ITIM domains to SHP-2<sup>36,37</sup>. Interestingly,  
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23 PECAM-1 is thought to exert its inhibitory activity by an additional mechanism, consisting in  
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25 the recruitment of PIK3R1 regulatory subunit of PI3K that we also found to be differentially  
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27 tyrosine phosphorylated, through its interaction with SHP-2, negatively regulating the LAT  
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29 signalosome by decreasing the interaction of LAT, Gab1 and PIK3R1<sup>38</sup>. Gab1 is another  
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31 protein that we are reporting to participate in the CLEC-2 signalling pathway for the first  
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33 time, since we found differentially regulated p-Tyr residues of this protein following  
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35 stimulation with rhodocytin. Gab1 is an adaptor protein that regulates signal transduction  
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37 downstream of PI3K by directly interacting with this kinase. Finally, phosphorylation of  
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39 Tyr<sup>245</sup> in the atypical ITIM-containing receptor TLT-1 was found to be up-regulated in  
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41 rhodocytin-stimulated platelets. This residue was not previously reported to undergo  
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43 phosphorylation, which may regulate downstream signalling<sup>39</sup>. Another kinase identified,  
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45 Csk, phosphorylates the C-terminal tyrosine of SFKs inactivating them, a mechanism that is  
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47 in part compensated by the phosphatase CD148, previously reported to participate in CLEC-2  
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49 downstream signalling events<sup>40,41</sup>, and phosphorylation of which we found to be up-regulated  
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51 in our proteomic analysis (Table 2; Figure 7).  
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3 Of the phosphatases found to be phosphorylated in rhodocytin-stimulated platelets,  
4 CD148, SHP-1, SHP-2, SHIP1, and PTP1B were previously reported to participate in the  
5 CLEC-2 signalling<sup>14,42-44</sup>. In addition, we also found p-Tyr residues within SHIP2, PTPRA,  
6 PTN18, and PTN12 (**Table 2; Figure 7**) to be up-regulated, suggesting these phosphatases  
7 may also contribute to the regulation of CLEC-2 signalling. Further, another protein that was  
8 reported to negatively regulate platelet signalling, and more precisely GPVI- mediated  
9 signalling downstream PI3K, is the adapter DAPP1<sup>45</sup>. Interestingly, we found  
10 phosphorylation of Tyr<sup>139</sup> within DAPP1 to be up-regulated, which is required for its  
11 interaction with PLC $\gamma$ 2 and protein function<sup>46,47</sup>.

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24 In conclusion, we have performed the most extensive tyrosine phosphoproteomic  
25 analysis of the CLEC-2 signalling pathway in platelets to date. We identified differentially  
26 regulated p-Tyr sites relevant for the activity of known as well as novel proteins in the CLEC-  
27 2 signalling cascade. We also demonstrated a crucial role of AA and ADP (together) to  
28 achieve full CLEC-2-mediated platelet activation, and phosphorylation of relevant tyr sites in  
29 CLEC-2 signalling proteins. We also show that the kinetic profiles of phosphorylation of key  
30 tyrosine residues in some relevant CLEC-2 signalling proteins - such as Syk (Tyr<sup>525+526</sup>), Src  
31 (Tyr<sup>419</sup>) and PLC $\gamma$ 2 (Tyr<sup>759</sup>) - vary slightly depending on the precise phosphoprotein analysed,  
32 which provides clues on the role of each protein in the pathway. Overall, we believe that our  
33 study provides significant insights into the intracellular events that take place following  
34 CLEC-2 activation in platelets, contributing to elucidate in detail the CLEC-2 signalosome.

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### Authorship contributions

- Irene Izquierdo: performed research, analyzed data and wrote the paper.
- María N Barrachina, Lidia Hermida-Nogueira, Vanessa Casas, Luis A. Morán, Serena Lacerenza, Roberto Pinto-Llorente, and Vivian de los Rios: performed research.
- J. Ignacio Casal, Montserrat Carrascal, and Joaquín Abián: contributed with key analytical tools and analyzed and integrated mass spec data.
- Johannes A. Eble: provided vital reagents.
- **Eduardo Domínguez, and María I. Loza provided vital reagents and analytical tools for the calcium release experiments.**
- Ángel García: designed research, analyzed data, provided vital reagents and analytical tools, and wrote the paper.

### Disclosure of Conflicts of interest

The authors declare no conflicts of interest.

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## 22 Tables

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25 **Table 1. Results from LC-MS/MS analysis.**  
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| 27 CONTITION       | 28 P-TYR PEPTIDES | 29 P-TYR SITES | 30 P-TYR PROTEINS |
|--------------------|-------------------|----------------|-------------------|
| 31 <b>Total</b>    | 32 757            | 33 466         | 34 264            |
| 35 <b>Basal</b>    | 36 374            | 37 288         | 38 187            |
| 39 <b>Rhod inh</b> | 40 421            | 41 313         | 42 198            |
| 43 <b>Rhod</b>     | 44 688            | 45 458         | 46 258            |

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48 **Rhod inh:** rhodocytin- activated in presence of secondary mediators' inhibitors; **Rhod:**  
49 rhodocytin- activated in absence of secondary mediators' inhibitors.  
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**Table 2. Tyrosine- phosphorylation sites of relevant signalling proteins identified in “Rhod” vs. “Basal” samples with a fold change (FC)  $\geq$  1.5.**

| Uniprot code            | Protein names   | P-Site    | FC       | Relevant Domain |
|-------------------------|---|-----------|----------|-----------------|
| <b>Adaptor/Scaffold</b> |   |           |          |                 |
| ABI1_HUMAN*             | Abl interactor 1 (ABI1)   | Y213      | $\infty$ |                 |
|                         |   | Y455      | $\infty$ | SH3             |
| BANK1_HUMAN*            | B-cell scaffold protein with ankyrin repeats (BANK1)                                  | Y443      | $\infty$ |                 |
|                         |   | Y737      | 3.00     |                 |
| CASS4_HUMAN*            | Cas scaffolding protein family member 4 (CASS4)                                       | Y350      | $\infty$ |                 |
|                         |   | Y174      | $\infty$ |                 |
|                         |   | Y195      | $\infty$ |                 |
| CRKL_HUMAN              | Crk-like protein (CRKL)   | Y207      | $\infty$ |                 |
|                         |   | Y132      | $\infty$ | SH3             |
|                         |   | Y127      | $\infty$ | SH3             |
|                         |   | Y198+Y207 | $\infty$ |                 |
|                         |   | Y251      | 4.00     | SH3             |
| DAPP1_HUMAN*            | Dual adapter for phosphotyrosine and 3-phosphotyrosine and 3-phosphoinositide (DAPP1) | Y139      | 3.00     |                 |
| DOK1_HUMAN*             | Docking protein 1 (DOK1)  | Y377      | $\infty$ | Pro rich        |
|                         |   | Y296      | $\infty$ | Pro rich        |
|                         |   | Y362+Y377 | $\infty$ | Pro rich        |
|                         |   | Y409      | 3.50     | Pro rich        |
|                         |   | Y449      | 1.50     |                 |
| DOK2_HUMAN              | Docking protein 2 (DOK2)  | Y139      | $\infty$ |                 |
|                         |   | Y402      | $\infty$ |                 |
| DOK3_HUMAN*             | Docking protein 3 (DOK3)  | Y398      | 2.44     | Pro rich        |
| EFS_HUMAN*              | Embryonal Fyn-associated substrate (EFS)  | Y163      | $\infty$ | Pro rich        |
| FYB1_HUMAN              | FYN-binding protein 1 (FYB1; ADAP; FYB; SLAP130)                                      | Y387      | 5.00     |                 |
|                         |   | Y757      | 3.00     | SH3             |
| GAB1_HUMAN*             | GRB2-associated-binding protein 1 (GAB1)  | Y659      | $\infty$ |                 |
| GRAP2_HUMAN             | GRB2-related adapter protein 2 (GRAP2; GADS)  | Y207      | $\infty$ |                 |
|                         |   | Y45       | 3.00     | SH3             |
| GRB2_HUMAN              | Growth factor receptor-bound protein 2 (GRB2)   | Y209      | $\infty$ | SH3             |
| LAT_HUMAN               | Linker for activation of T-cells family member 1 (LAT)                                | Y45       | 4.67     |                 |
|                         |   | Y220      | 3.40     |                 |
| LCP2_HUMAN              | Lymphocyte cytosolic protein 2 (LCP2; SLP-76; SLP76)                                  | Y532      | $\infty$ |                 |
|                         |   | Y39       | 2.00     |                 |
| NCK1_HUMAN*             | Cytoplasmic protein NCK1 (NCK1; NCK)  | Y105      | 1.56     |                 |

| Uniprot code            | Protein names  | P-Site    | FC        | Relevant Domain      |
|-------------------------|--|-----------|-----------|----------------------|
| <b>Adaptor/Scaffold</b> |  |           |           |                      |
| NCK2_HUMAN*             | Cytoplasmic protein NCK2 (NCK2)  | Y50       | $\infty$  | SH3                  |
|                         |  | Y251      | $\infty$  | SH3                  |
|                         |  | Y110      | 2.91      |                      |
| NTAL_HUMAN*             | Linker for activation of T-cells family member 2 (LAT2; NTAL)                        | Y136      | $\infty$  |                      |
|                         |  | Y58       | $\infty$  |                      |
| PARD3_HUMAN*            | Partitioning defective 3 homolog (PARD3; PAR3)                                       | Y719      | $\infty$  |                      |
|                         |  | Y1080     | 4.00      |                      |
| PHAG1_HUMAN*            | Phosphoprotein associated with glycosphingolipid-enriched microdomains 1 (PAG1; PAG) | Y317      | 2.50      | Interaction with CSK |
|                         |  | Y163      | 2.00      |                      |
|                         |  | Y341      | 1.50      |                      |
| REPS2_HUMAN*            | RalBP1-associated Eps domain-containing protein 2 (REPS2)                            | Y558      | $\infty$  |                      |
| SKAP2_HUMAN             | Src kinase-associated phosphoprotein 2 (SKAP2)                                       | Y169      | $\infty$  |                      |
|                         |  | Y334      | 2.00      | SH3                  |
|                         |  | Y151/Y152 | -2.00     |                      |
| SPN90_HUMAN*            | NCK-interacting protein with SH3 domain (NCKIPSD; SPIN90)                            | Y161      | $-\infty$ |                      |
| STAM2_HUMAN*            | Signal transducing adapter molecule 2 (STAM2)  | Y374      | 2.00      | ITAM                 |
| VAV_HUMAN               | Proto-oncogene vav (VAV1; VAV)   | Y844      | $\infty$  |                      |
|                         |  | Y791      | $\infty$  | SH3                  |
|                         |  | Y110      | 11.00     |                      |
| WASP_HUMAN*             | Wiskott-Aldrich syndrome protein (WAS)   | Y212      | $\infty$  |                      |
|                         |  | Y291      | 3.33      |                      |
| WIPF1_HUMAN*            | WAS/WASL-interacting protein family member 1 (WIPF1)                                 | Y455      | $\infty$  |                      |
| WIPF2_HUMAN*            | WAS/WASL-interacting protein family member 2 (WIPF2)                                 | Y400      | $\infty$  |                      |
| <b>Kinase</b>           |  |           |           |                      |
| ABL2_HUMAN*             | Tyrosine-protein kinase ABL2 (ABL2)  | Y439      | $\infty$  | Tyr- kinase          |
| ACK1_HUMAN*             | Activated CDC42 kinase 1 (TNK2; ACK1)  | Y518      | 2.00      |                      |
|                         |  | Y827      | -1.67     |                      |
| BTK_HUMAN               | Tyrosine-protein kinase BTK (BTK)  | Y334      | $\infty$  | SH2                  |
|                         |  | Y223      | 5.27      | SH3                  |
|                         |  | Y361      | 3.00      | SH2                  |
|                         |  | Y315      | 2.00      | SH2                  |
| CSK_HUMAN*              | Tyrosine-protein kinase CSK (CSK)  | Y18       | $\infty$  | SH3                  |

| Uniprot code  | Protein names   | P-Site      | FC    | Relevant Domain |
|---------------|---|-------------|-------|-----------------|
| <b>Kinase</b> |   |             |       |                 |
| FAK1_HUMAN*   | Focal adhesion kinase 1 (PTK2; FAK; FAK1)                             | Y925        | 2.50  |                 |
| FER_HUMAN     | Tyrosine-protein kinase Fer (FER)                                     | Y402        | ∞     |                 |
|               |   | Y714        | 3.33  | Tyr- kinase     |
| FES_HUMAN*    | Tyrosine-protein kinase Fes/Fps (FES; FPS)                            | Y513        | ∞     |                 |
|               |   | Y713        | 5.00  | Tyr- kinase     |
| FGR_HUMAN*    | Tyrosine-protein kinase Fgr (FGR; SRC2)                               | Y208/Y209   | ∞     | SH2             |
| FYN_HUMAN     | Tyrosine-protein kinase Fyn (FYN)                                     | Y213        | ∞     | SH2             |
| JAK2_HUMAN*   | Tyrosine-protein kinase JAK2 (JAK2)                                   | Y570        | 1.83  | Tyr- kinase     |
| KALRN_HUMAN*  | Kalirin (KALRN)   | Y2265       | 4.00  |                 |
| KGP1_HUMAN*   | cGMP-dependent protein kinase 1 (PRKG1)                               | Y336        | ∞     |                 |
| KPCD_HUMAN    | Protein kinase C delta type (PRKCD)                                   | Y64         | ∞     |                 |
|               |   | Y313        | 3.00  |                 |
|               |   | Y334        | 2.40  |                 |
| KSYK_HUMAN    | Tyrosine-protein kinase SYK (SYK)                                     | Y296        | ∞     |                 |
|               |   | Y323        | 19.00 |                 |
|               |   | Y352        | 15.00 |                 |
|               |   | Y348+Y352   | 10.00 |                 |
|               |   | Y525+Y526   | 3.50  | Tyr- kinase     |
| Y526          | 1.50  | Tyr- kinase |       |                 |
| LYN_HUMAN     | Tyrosine-protein kinase Lyn (LYN)                                     | Y306        | 4.00  | Kinase          |
| MK01_HUMAN    | Mitogen-activated protein kinase 1 (MAPK1; ERK2)                      | Y187        | 6.00  | Tyr- kinase     |
| MK03_HUMAN    | Mitogen-activated protein kinase 3 (MAPK3; ERK1)                      | Y204        | ∞     | Kinase          |
| MK08_HUMAN    | Mitogen-activated protein kinase 8 (MAPK8; JNK1)                      | Y185        | ∞     | Kinase          |
| MK09_HUMAN    | Mitogen-activated protein kinase 9 (MAPK9; JNK2)                      | Y185        | 1.82  | Kinase          |
| MK12_HUMAN    | Mitogen-activated protein kinase 12 (MAPK12; ERK6)                    | Y185        | 6.00  | Kinase          |
| MK14_HUMAN    | Mitogen-activated protein kinase 14 (MAPK14; CSBP)                    | Y182        | 2.15  | Kinase          |
| MRCKB_HUMAN*  | Serine/threonine-protein kinase MRCK beta (CDC42BPB)                  | Y1638       | 3.00  |                 |
| P85A_HUMAN    | Phosphatidylinositol 3-kinase regulatory subunit alpha (PIK3R1; GRB1) | Y467        | 2.00  |                 |
| PI4KA_HUMAN*  | Phosphatidylinositol 4-kinase alpha (PI4KA; PIK4)                     | Y1154       | -4.00 |                 |
| ROCK2_HUMAN*  | Rho-associated protein kinase 2 (ROCK2)                               | Y722        | 5.00  |                 |
|               |   | Y450        | 5.00  |                 |
| SRC_HUMAN     | Proto-oncogene tyrosine-protein kinase Src (SRC; SRC1)                | Y187        | -2.00 | SH2             |

| Uniprot code         | Protein names  | P-Site      | FC    | Domain      |
|----------------------|--|-------------|-------|-------------|
| <b>Phosphatase</b>   |  |             |       |             |
| PTN1_HUMAN           | Tyrosine-protein phosphatase non-receptor type 1 (PTPN1; PTP1B)                | Y46         | 4.00  | Phosphatase |
| PTN11_HUMAN          | Tyrosine-protein phosphatase non-receptor type 11 (PTPN11; SHP-2)              | Y584        | 1.57  |             |
| PTN12_HUMAN*         | Tyrosine-protein phosphatase non-receptor type 12 (PTPN12; PTPG1)              | Y64         | ∞     | Phosphatase |
| PTN18_HUMAN*         | Tyrosine-protein phosphatase non-receptor type 18 (PTPN18)                     | Y426        | ∞     |             |
|                      |  | Y389        | 2.50  |             |
| PTN6_HUMAN           | Tyrosine-protein phosphatase non-receptor type 6 (PTPN6; SHP-1)                | Y64         | 3.00  | SH2         |
|                      |  | Y564        | 9.00  |             |
|                      |  | Y98         | 2.00  | SH2         |
|                      |  | Y536        | 1.78  |             |
| SHIP1_HUMAN          | Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1 (INPP5D; SHIP; SHIP1) | Y1022       | ∞     | Pro rich    |
| SHIP2_HUMAN*         | Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2 (INPPL1; SHIP2)       | Y986        | ∞     | Pro rich    |
|                      |  | Y1135       | ∞     |             |
|                      |  | Y886        | ∞     |             |
| <b>Phospholipase</b> |  |             |       |             |
| PLCG2_HUMAN          | 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-2 (PLCG2)      | Y1217       | ∞     |             |
|                      |  | Y1245       | ∞     |             |
|                      |  | Y753        | ∞     |             |
|                      |  | Y495        | ∞     |             |
|                      |  | Y759        | 23.50 |             |
| <b>Receptor</b>      |  |             |       |             |
| CLC1B_HUMAN          | C-type lectin domain family 1 member B (CLEC1B; CLEC2)                         | Y7          | ∞     | ITAM        |
| FCERG_HUMAN*         | High affinity immunoglobulin epsilon receptor subunit gamma (FCER1G)           | Y58         | 2.00  | ITAM        |
| FCG2A_HUMAN*         | Low affinity immunoglobulin gamma Fc region receptor II-a (FCGR2A)             | Y304        | 3.00  |             |
| G6B_HUMAN            | Megakaryocyte and platelet inhibitory receptor G6b (MPIG6B; G6B; G6B-B)        | Y211        | 4.33  | ITIM        |
|                      |  | Y237        | 2.00  | ITIM        |
| PTPRA_HUMAN*         | Receptor-type tyrosine-protein phosphatase alpha (PTPRA; PTPA)                 | Y798        | 2.00  |             |
| PTPRJ_HUMAN          | Receptor-type tyrosine-protein phosphatase eta (PTPRJ; CD148)                  | Y1311+Y1320 | ∞     |             |
|                      |  | Y1311       | 9.00  |             |
| JAM1_HUMAN*          | Junctional adhesion molecule A (JAM-A)   | Y280        | -3.00 |             |
| TRML1_HUMAN*         | Trem-like transcript 1 protein (TLT-1)   | Y245        | 3.06  | Pro rich    |

| Uniprot code        | Protein names  | P-Site | FC        | Domain |
|---------------------|--|--------|-----------|--------|
| <b>Small GTPase</b> |  |        |           |        |
| RAB13_HUMAN*        | Ras-related protein Rab-13 (RAB13)   | Y5     | $\infty$  |        |
| RAB1B_HUMAN*        | Ras-related protein Rab-1B (RAB1B)   | Y5     | $\infty$  |        |
| RAN_HUMAN*          | GTP-binding nuclear protein Ran (RAN)  | Y39    | 1.50      |        |
| <b>GAP</b>          |  |        |           |        |
| ARAP1_HUMAN*        | Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 1 (ARAP1) | Y737   | 3.50      |        |
| IQGA2_HUMAN*        | Ras GTPase-activating-like protein IQGAP2 (IQGAP2)                                 | Y93    | $\infty$  |        |
| RASA1_HUMAN*        | Ras GTPase-activating protein 1 (RASA1)  | Y615   | 2.00      |        |
| RASA3_HUMAN*        | Ras GTPase-activating protein 3 (RASA3)  | Y66    | $\infty$  |        |
| RHG18_HUMAN*        | Rho GTPase-activating protein 18 (ARHGAP18)  | Y41    | 4.00      |        |
| RHG35_HUMAN*        | Rho GTPase-activating protein 35 (ARHGAP35)  | Y1087  | $\infty$  |        |
|                     |  | Y1105  | 1.75      |        |
| <b>GDI</b>          |  |        |           |        |
| GDIR2_HUMAN*        | Rho GDP-dissociation inhibitor 2 (ARHGDI2)   | Y130   | $-\infty$ |        |
|                     |  | Y24    | $\infty$  |        |
| <b>GEF</b>          |  |        |           |        |
| DEN2C_HUMAN         | DENN domain-containing protein 2C (DENND2C)  | Y322   | $\infty$  |        |
|                     |  | Y237   | $\infty$  |        |
|                     |  | Y475   | $\infty$  |        |
|                     |  | Y195   | $\infty$  |        |
|                     |  | Y102   | $\infty$  |        |
|                     |  | Y310   | $\infty$  |        |
|                     |  | Y50    | $\infty$  |        |
|                     |  | Y219   | $\infty$  |        |
|                     |  | Y228   | $\infty$  |        |
|                     | Y185   | 2.75   |           |        |
| GRP2_HUMAN*         | RAS guanyl-releasing protein 2 (RASGRP2)   | Y523   | -2.00     |        |
| <b>Other</b>        |  |        |           |        |
| LY66F_HUMAN*        | Lymphocyte antigen 6 complex locus protein G6f (LY6G6F; G6F)                       | Y281   | 4.50      |        |
| PECA1_HUMAN*        | Platelet endothelial cell adhesion molecule (PECAM-1)                              | Y663   | 3.67      |        |
|                     |  | Y690   | $\infty$  | ITIM   |

A positive fold change indicates that the variation is favourable to “Rhod” samples (rhodocytin- activated in absence of secondary mediators’ inhibitors), and a negative fold change indicates that the variation is favourable to basal samples. Fold changes were calculated as the quotient of the total number of PSMs that led to the identification of the protein p-site. GAP: GTPase-activating protein; GDI: GDP dissociation inhibitor; GEF: Guanine nucleotide exchange factor.  $\infty$  means infinity.

\* Means that the protein was not previously reported to participate in CLEC-2 signalling pathway.

## Figure legends

**Figure 1. Experimental workflow.** Schematic representation of the methodology applied and the equipment employed in this study.

**Figure 2. P-Tyr peptides, and p-Tyr sites identified by LC-MS/MS. Function of proteins with differentially regulated p-Tyr sites in CLEC-2-activated platelets.**

Venn diagrams represent the total number of p-Tyr peptides (**A**), and the corresponding p-Tyr sites (**B**), that were identified in each analysed condition. **C**) Functions of proteins that were identified with differentially regulated p-Tyr sites in CLEC-2-activated vs. basal platelets. Charts represent protein functions distribution ( $FC \geq 1.5$ ).

**Figure 3. Ca<sup>2+</sup> mobilization following CLEC-2 activation is dependent on secondary mediators.** A) Secondary mediators play a fundamental role in CLEC-2-

mediated Ca<sup>2+</sup> release induced by platelet activation with Rhodocytin. **B**) Secondary mediators are needed for a full platelet activation induced by Rhodocytin, in contrast with what occurs in the case of platelet activation with CRP or thrombin. **C**) Both ADP and TXA2 together are needed for full CLEC-2 activation. Rhod, Rhodocytin; CRP, collagen-related peptide; THR, thrombin. **All measurements were done in triplicate (biological replicates). For further experimental details please see section 2.6.**

**Figure 4. Contribution of ADP and TXA2 to phosphorylation of relevant tyrosine residues in Src, Syk, and PLC $\gamma$ 2 downstream of CLEC-2.** A) Aggregation profile of

platelets stimulated with 200nM rhodocytin in absence and presence of secondary mediators inhibitors (2U/mL apyrase and indomethacin 10 $\mu$ M). Representative western blot images (**B**), and densitometry graphs (**C**), representing tyrosine phosphorylation levels of Src p-Tyr<sup>419</sup>, Syk p-Tyr<sup>525+526</sup>, and PLC $\gamma$ 2 p-Tyr<sup>759</sup>. \*\*  $p < 0.01$  (RM one way



1  
2  
3 **Anova with Tukey's post-hoc multiple comparisons**). Experiments were done with  
4  
5 samples obtained from 4 different healthy donors (biological replicates).  
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7

8 **Figure 5. ADP and arachidonic acid do not induce tyrosine phosphorylation of Src,**  
9 **Syk, and PLC $\gamma$ 2 in the same residues CLEC-2 activation does.** A) Aggregation  
10  
11 profile of platelets stimulated with 200nM rhodocytin, 10  $\mu$ M ADP, and 100  $\mu$ M AA.  
12  
13 Representative western blot images (B) and densitometry graphs (C) showing tyrosine  
14  
15 phosphorylation levels of Src p-Tyr<sup>419</sup>, Syk p-Tyr<sup>525+526</sup>, and PLC $\gamma$ 2 p-Tyr<sup>759</sup> in  
16  
17 response to Rhodocytin and AA+ADP. **Comparisons were between each stimulus and**  
18  
19 **its respective control.** \*\* p < 0.01 (paired Student's t-test). Experiments were done with  
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21 samples obtained from 4 different healthy donors (biological replicates).  
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28 **Figure 6. Kinetic studies on essential tyrosine residues in Src, Syk, and PLC $\gamma$ 2**  
29 **downstream of CLEC-2 point out differences in the phosphorylation pattern.**  
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31 Representative western blot images (A), and densitometry graphs (B) showing tyrosine  
32  
33 phosphorylation profiles of Src p-Tyr<sup>419</sup>, Syk p-Tyr<sup>525+526</sup>, and PLC $\gamma$ 2 p-Tyr<sup>759</sup>  
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35 following platelet activation with rhodocytin for 30s, 60s, 120s, 180s and 300s. **For**  
36  
37 **statistics, comparisons were always between each activation time point and basal**  
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39 **(control platelets).** \*p < 0.05, \*\* p < 0.01, and \*\*\* p < 0.001 (paired Student's t-test).  
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43 Experiments were done with samples obtained from 4 different healthy donors  
44  
45 (biological replicates).  
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49 **Figure 7. The CLEC-2 signalling pathway in platelets: solving the puzzle.** New  
50  
51 proposal for the platelet CLEC-2 signalling cascade in which relevant signalling  
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53 proteins and their corresponding differentially regulated p-Tyr sites identified in the  
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55 present study are highlighted. This figure only includes those Tyr residues that were  
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57 identified as differentially regulated in the present proteomic study (Fold change  $\geq$  1.5).  
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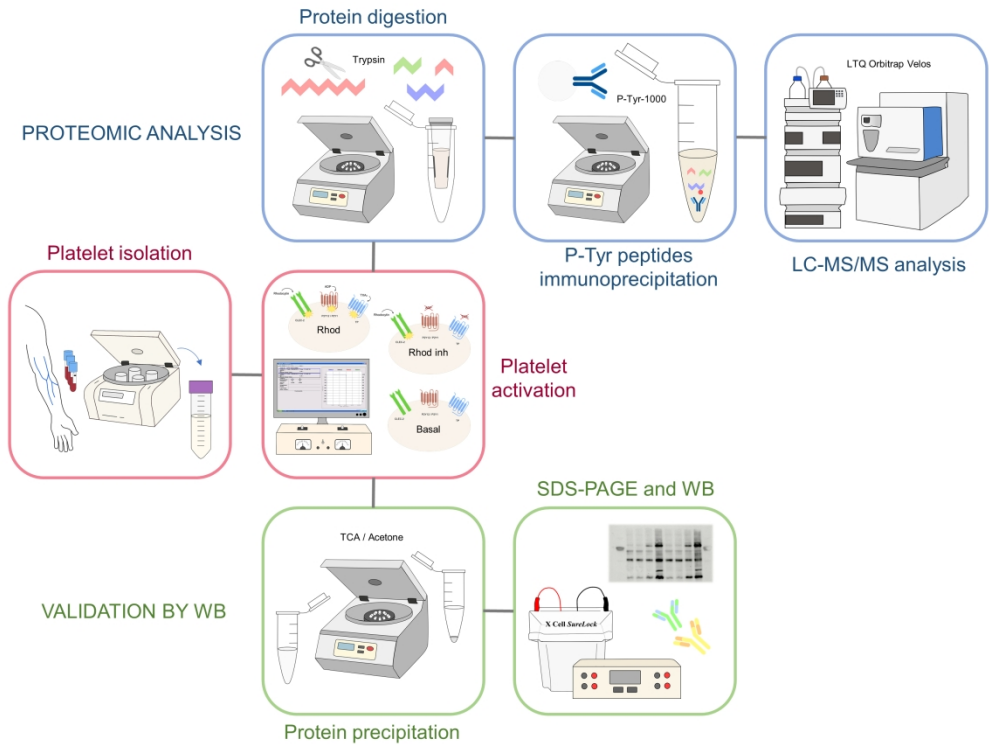


Figure 1

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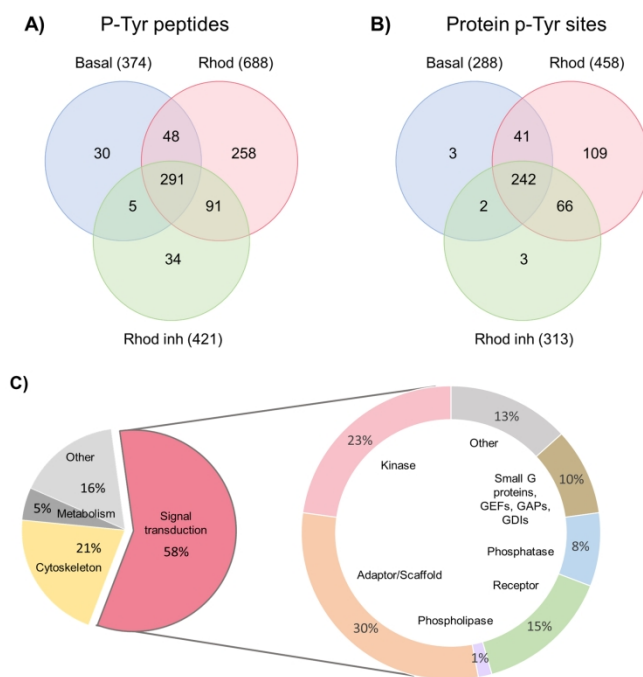


Figure 2

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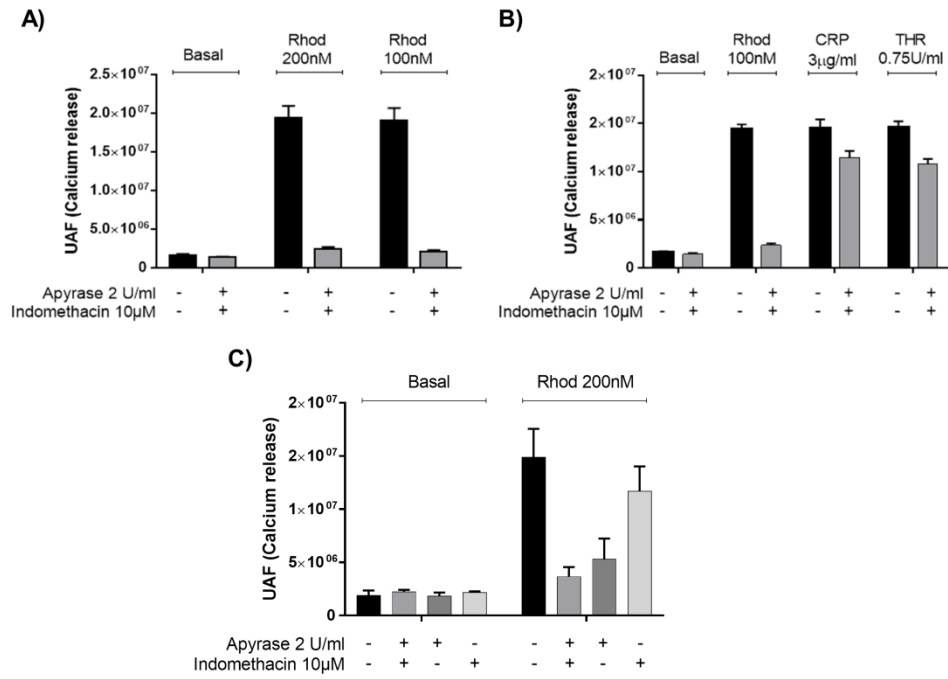


Figure 3

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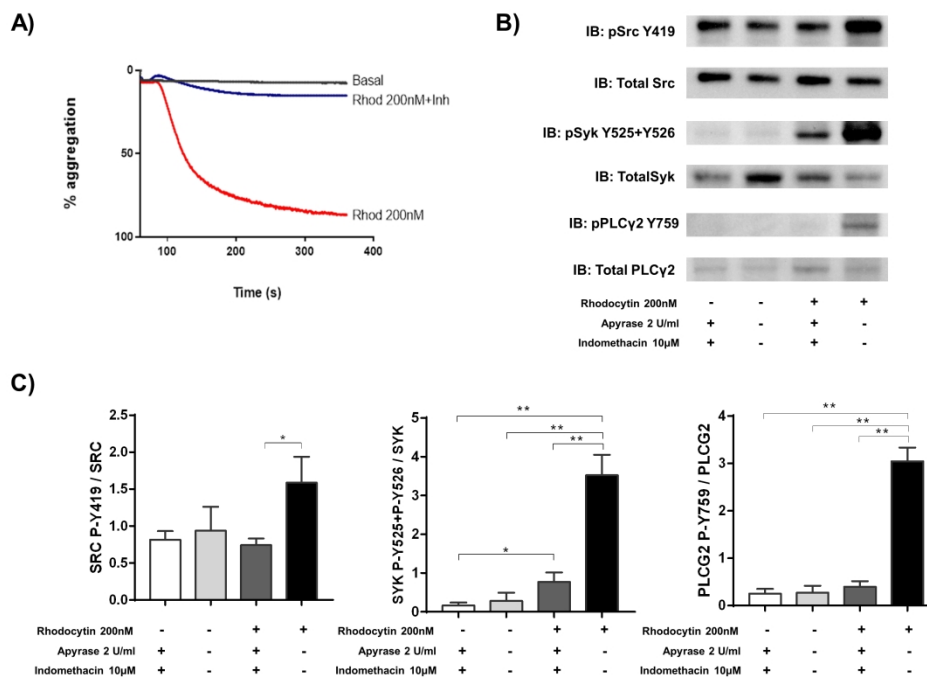


Figure 4

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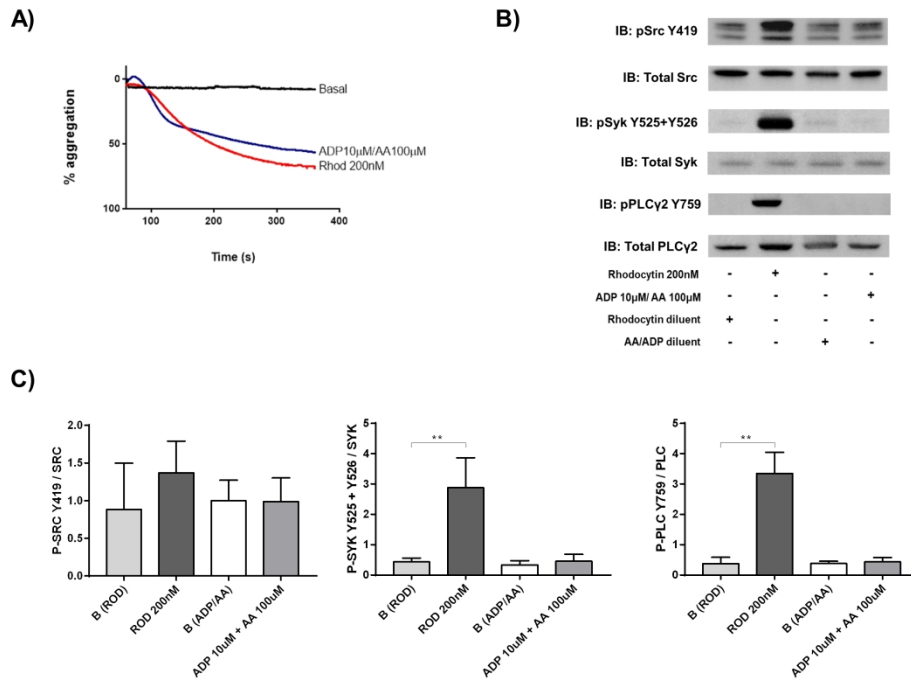


Figure 5

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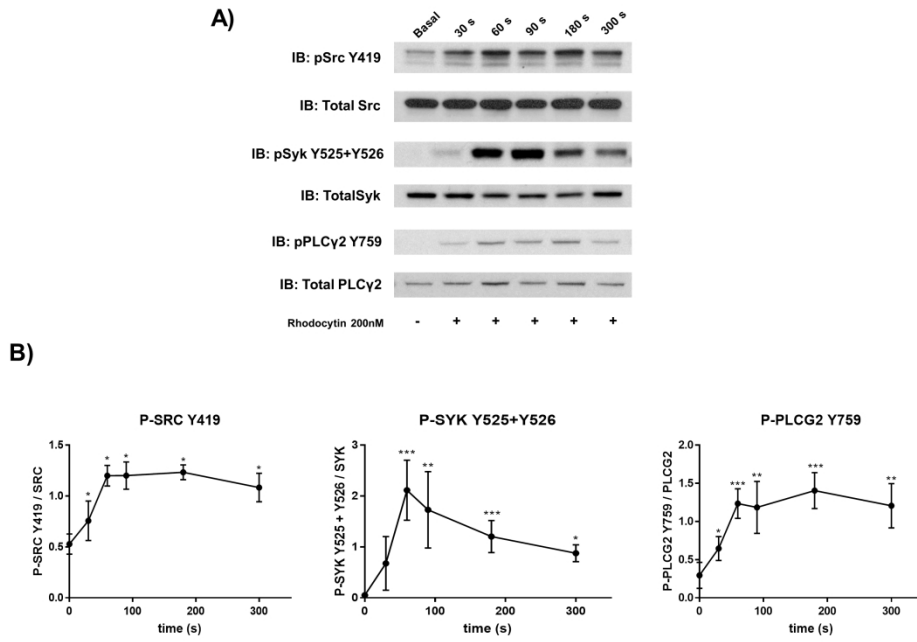


Figure 6

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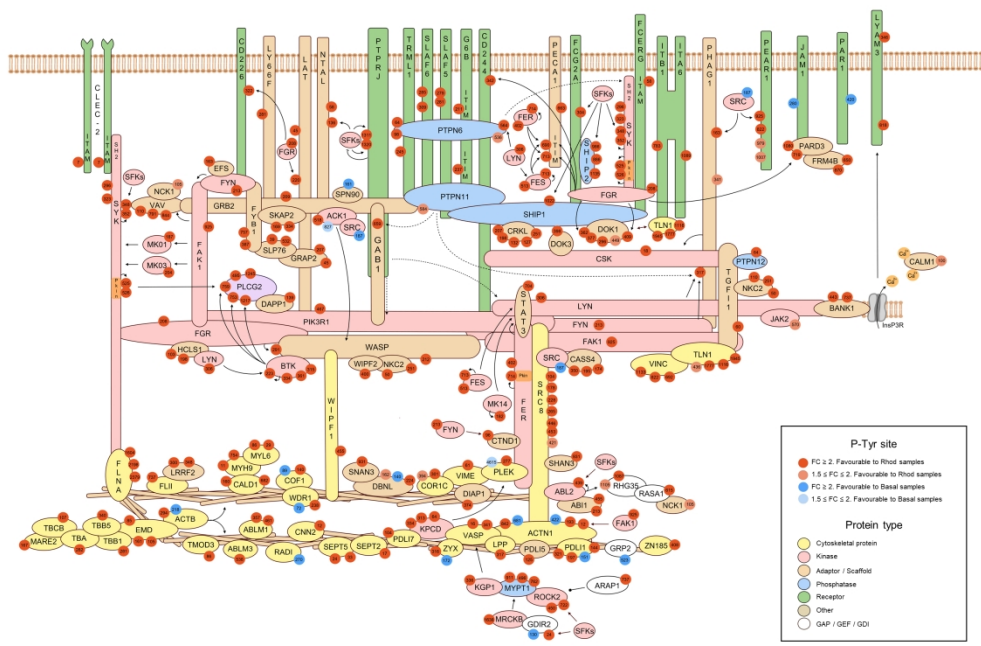
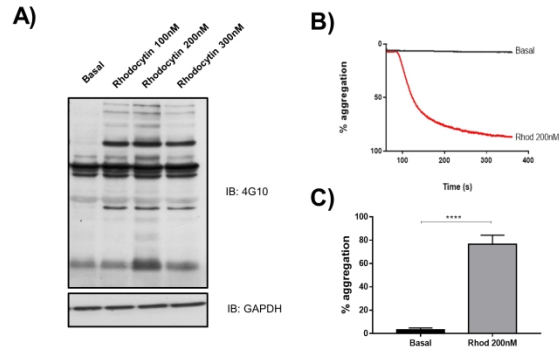


Figure 7

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Supplementary Figure 1

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3 **A comprehensive tyrosine phosphoproteomic analysis reveals novel components of the**  
4 **platelet CLEC-2 signaling cascade**  
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10 Irene Izquierdo<sup>1</sup>, María N. Barrachina<sup>1</sup>, Lidia Hermida-Nogueira<sup>1</sup>, Vanessa Casas<sup>2</sup>, Luis A.  
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5 **Word count of body:** 4923  
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8 **Word count of abstract:** 249  
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## 16 **Abstract**

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20 C-type lectin-like receptor 2 (CLEC-2) plays a crucial role in different platelet-related  
21 physiological and pathological processes. It signals through a tyrosine kinase-mediated  
22 pathway that is highly dependent on the positive feedback exerted by the platelet-derived  
23 secondary mediators ADP and thromboxane A2 (TXA2). Here, we aimed to analyse the  
24 tyrosine phosphoproteome of platelets activated with the CLEC-2 agonist rhodocytin in order  
25 to identify relevant phosphorylated tyrosine residues (p-Tyr) and proteins involved in platelet  
26 activation downstream of this receptor. We identified 363 differentially p-Tyr residues,  
27 corresponding to the majority of proteins previously known to participate in CLEC-2  
28 signalling and also novel ones, including adaptors (e.g. DAPP1, Dok1/3, CASS4, Nck1/2);  
29 kinases/phosphatases (e.g. FAK1, FES, FGR, JAK2, SHIP2); and membrane proteins (e.g.  
30 G6F, JAM-A, PECAM-1, TLT-1). In order to elucidate the contribution of ADP and TXA2 at  
31 different points of the CLEC-2 signalling cascade, we evaluated p-Tyr levels of residues  
32 identified in the analysis and known to be essential for the catalytic activity of kinases Syk(p-  
33 Tyr<sup>525+526</sup>) and Src(p-Tyr<sup>419</sup>), and for PLC $\gamma$ 2 activity (p-Tyr<sup>759</sup>). We demonstrated that Syk  
34 phosphorylation at Tyr<sup>525+526</sup> also happens in the presence of ADP and TXA2 inhibitors,  
35 which is not the case for Src-pTyr<sup>419</sup> and PLC $\gamma$ 2-pTyr<sup>759</sup>. Kinetics studies for the three  
36 phosphoproteins show some differences in the phosphorylation profile. Ca<sup>2+</sup> mobilization  
37 assays confirmed the relevance of ADP and TXA2 for full CLEC-2-mediated platelet  
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3 activation. The present study provides significant insights into the intracellular events that  
4 take place following CLEC-2 activation in platelets, contributing to elucidate in detail the  
5 CLEC-2 signalosome.  
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10 **Key words:** platelets, CLEC-2 signalling, tyrosine phosphoproteome  
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## 16 **Summary Table**

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### 18 What is known on this topic:

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- 20 • CLEC-2 plays a crucial role in maintaining vascular integrity, embryonic lymphatic  
21 vessel development, hematogenous metastasis and thrombus stabilization.  
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- 23 • CLEC-2 signals through a protein tyrosine-kinase-mediated pathway; the only  
24 endogenous ligand known so far is podoplanin.  
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### 32 What this paper adds:

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- 34 • First tyrosine phosphoproteomic analysis of the CLEC-2 signalling cascade, analyzing  
35 in detail the impact of secondary mediators.  
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- 38 • Novel CLEC-2 signalling proteins and p-Tyr sites identified allowing a more complete  
39 and updated overview of the CLEC-2 signalosome.  
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- 42 • Kinetic studies show differences in the phosphorylation profiles of Syk-p-Tyr<sup>525+526</sup>,  
43 Src-pTyr<sup>419</sup>, and PLCγ2-pTyr<sup>759</sup> following CLEC-2 activation.  
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## 1. Introduction

C-type lectin-like receptor 2 (CLEC-2) has been demonstrated to play a crucial role in maintaining vascular integrity<sup>1</sup> and embryonic lymphatic vessel development<sup>2</sup>, and also participates in platelet-mediated hematogenous metastasis<sup>3</sup>. CLEC-2 plays a minor role in haemostasis<sup>4</sup>, but contributes to thrombus stabilization under flow<sup>2</sup>. This latter process is a key step in the development of ischemic events associated with unwanted platelet activation.

CLEC-2 signals through a protein tyrosine-kinase-mediated pathway. The only endogenous agonist for CLEC-2 identified to date is podoplanin, a glycoprotein expressed on the surface of lymphatic endothelial cells, podocytes, type-I alveolar cells, and some tumour cells. In addition, rhodocytin, a protein isolated from the venom of the Malayan pit viper *Calloselasma rhodostoma*, is another CLEC-2 agonist, capable of inducing a potent platelet aggregation response following receptor engagement<sup>5,6</sup>.

The CLEC-2 signalling cascade starts with the tyrosine- phosphorylation of its immunoreceptor tyrosine-based activation motif (hemITAM), defined as a singular YxxL sequence within the cytoplasmic tail of the receptor. Cell signalling through ITAM motifs requires the presence of two YxxL sequences, which allows receptor interaction with a Src homology 2 domain (SH2)-containing tyrosine-kinase. Therefore, bringing two hemITAM motifs in close vicinity through CLEC-2 dimerization is necessary for signal transduction. Once the hemITAM motifs of dimeric CLEC-2 have been phosphorylated, the tyrosine-kinase Syk interacts with the cytoplasmic tails of the dimer through its SH2 domains, reinforcing hemITAM phosphorylation and contributing to signal transduction. Subsequent biochemical events that take place in the intracellular space include the phosphorylation of the adapters LAT and SLP-76, and the activation of PLC $\gamma$ 2, leading to platelet aggregation<sup>7-10</sup>. The events taking place for primary phosphorylation of the hemITAM motifs are not yet completely

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3 understood, but may involve the participation of Src-family tyrosine kinases (SFKs)<sup>11</sup>.  
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5 Interestingly, CLEC-2 signalling in platelets has been found to be highly dependent in  
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7 pathway reinforcement induced by the secondary mediators Adenosine diphosphate (ADP)  
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9 and thromboxane A<sub>2</sub> (TXA<sub>2</sub>), released from platelets upon activation and acting in an  
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11 autocrine and paracrine manner<sup>12</sup>. Positive feedback by these secondary mediators is highly  
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13 relevant in the amplification of the most proximal events that occur upon receptor activation,  
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15 presumable through activation of SFKs and Syk that mediate CLEC-2 phosphorylation<sup>13</sup>.  
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20 Recently, we performed a gel-based proteomic analysis of the CLEC-2 signalling  
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22 pathway<sup>14</sup>. This previous study was limited by the gel-based approach, which was far from  
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24 being quantitative. Recent advances in mass spectrometry leading to confident identification  
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26 of phosphorylation sites in complex protein samples, and the development of antibodies  
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28 capable of recognizing specific phosphosites, have opened the path for more sensitive and  
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30 quantitative proteomic analyses of signalling cascades in platelets. In this context, we have  
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32 performed an extensive phosphotyrosine proteomic analysis of platelets stimulated through  
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34 the CLEC-2 receptor in presence and absence of inhibitors of secondary mediators, in  
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36 comparison with resting state, in order to identify new effectors in the signalling pathway  
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38 coupled to this receptor and to elucidate the impact of secondary mediators in the tyrosine-  
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40 phosphorylation response. In this way, it was possible to provide a much complete picture of  
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42 the CLEC-2 signalosome, including identifying new proteins and mapping the precise  
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44 phosphosites responsible for platelet activation through this receptor.  
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## 53 **2. Materials and methods**

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56 A schematic representation of the methodology applied is shown in **Figure 1**.  
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## 2.1. Platelet isolation and activation

Fresh blood was obtained from healthy volunteers not under chronic medication, and not under anti-platelet drugs for the previous 10 days. The study was conducted according to the declaration of Helsinki, and with approval by the Galician Investigation Ethics Committee (code 2009-270). The age range of the donors was 22-51 years old; 50% of them were female. Blood was collected in coagulation 3.2% sodium citrate tubes (Vacuette), and samples were processed within the first 30 minutes after extraction.

Platelets isolation was following a well-established procedure that limits contamination with other blood cells and plasma proteins<sup>15</sup>. After washing steps, platelets were resuspended in HEPES-Tyrodes (134mM NaCl; 0.34mM Na<sub>2</sub>HPO<sub>4</sub>; 2.9 mM KCl; 12mM NaHCO<sub>3</sub>; 20mM HEPES; 5mM glucose; 1mM MgCl<sub>2</sub>; pH 7.3) at a concentration of 8 x10<sup>8</sup> platelets/ml and allowed to rest for 30 minutes at room temperature.

From each healthy donor included in the proteomic study (two men and two women), we obtained platelet samples under three different stimulation conditions: basal (non-activated in absence of secondary mediators' inhibitors), "Rhod" (rhodocytin- activated in absence of secondary mediators' inhibitors), and "Rhod inh" (rhodocytin- activated in presence of secondary mediators' inhibitors). We did not include a "Basal inh" condition in our proteomic analysis due to sample limitation issues to keep biological replicates and considering both types of basal samples elicit a similar tyr phosphoproteome profiles, with marginally lower phosphorylation levels in the presence of the inhibitors. Activations were performed for 5 minutes with 200nM rhodocytin at 37°C and 1200rpm stirring in a Chrono-log aggregometer. Optimal incubation times were based on previous data<sup>14</sup> and the dose was adjusted in dose response experiments based on measuring the Tyr- phosphorylation protein profile by Western blotting (**Supplementary Figure 1**). All activations were performed after the addition of 9µM Integrilin® (GlaxoSmithKline), in order to avoid platelet aggregation and



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3 the activation of the  $\alpha$ IIb $\beta$ 3 integrin signalling cascade. For inhibiting platelet stimulation by  
4 ADP and TXA<sub>2</sub>, 2U/ml of apyrase (ADPase that catalyses the hydrolysis of ADP), and 10 $\mu$ M  
5 of indomethacin (that inhibits COX-1 and thus the synthesis of TXA<sub>2</sub>), both from Sigma-  
6 Aldrich, were added to the platelet suspension prior to CLEC-2 stimulation.  
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12 For proteomic analysis, lysates were obtained by directly adding 2% SDS (final  
13 concentration) to 500  $\mu$ l of washed platelet suspensions. For validation analyses by Western  
14 blotting, lysates were obtained by supplementing platelet suspensions with an equal volume  
15 (500 $\mu$ l) of lysis buffer 2x (0.3M sodium chloride; 20mM Tris; 2mM EGTA; 2mM EDTA; 2%  
16 v/v NP-40; 10 $\mu$ g/ml aprotinin; 1 $\mu$ g/ml pepstatin; 10 $\mu$ g/ml leupeptin; 400 $\mu$ g/ml AEBFS; 5mM  
17 sodium orthovanadate; pH 7.5).  
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## 29 **2.2. Protein quantification and digestion for proteomic analysis**

30 For proteomic analysis, four biological replicates were analysed per condition. Protein  
31 concentration in the sample was quantified with the micro BCA<sup>TM</sup> protein assay kit (Pierce)  
32 following the manufacturer's protocol. After that, 800 $\mu$ g of protein per sample were digested  
33 with trypsin following a large-scale filter aided sample preparation (LFASP) method on 10  
34 kDa filters (Amicon<sup>TM</sup> Ultra-15, Millipore)<sup>16</sup>. Tryptic digests were dried and resuspended in  
35 700 $\mu$ l of IAP buffer (50mM MOPS pH 7.2; 10mM disodium phosphate; 50mM sodium  
36 chloride).  
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## 49 **2.3. Phosphotyrosine peptide enrichment and LC-MS/MS analysis**

50 Immunoaffinity enrichment of phosphotyrosine peptides was accomplished with PTMScan<sup>®</sup>  
51 Phospho-Tyrosine Rabbit mAb (P-Tyr-1000) Kit (Cell Signaling), as indicated in the  
52 Supplemental Methods.  
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3 Samples containing phospho-Tyr peptides were loaded in a nano Easy-nLC 1000  
4 chromatographic system (Proxeon), and mass spectra acquired on an LTQ-Orbitrap Velos  
5 (ThermoScientific). The spectra were searched against the human SwissProt database and  
6 validation of phosphorylation sites was performed by ptmRS and Percolator algorithms.  
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8 Protein p-sites included in the analysis were those identified by at least two peptide spectrum  
9 matches (PSMs). Fold changes were calculated as the ratio of the number of PSMs that led to  
10 the identification of the corresponding protein p-site for the two compared conditions. Further  
11 details on the mass spec analysis and phosphosite identification can be found in the  
12 Supplemental Methods section.  
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#### 26 **2.4. Database search and data integration**

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28 Phosphosite Plus<sup>®17</sup> database was scrutinized to find detailed information about Tyrosine-  
29 phosphorylation sites detected in the proteins identified in the study.  
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34 String<sup>©18</sup> and PhosphoPath<sup>19</sup> tools, powered by Cytoscape v3.7<sup>20</sup>, were used to  
35 investigate potential interactions between differentially phosphorylated proteins, and to  
36 identify potential kinases responsible for the Tyr-phosphorylations detected in those proteins.  
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42 UniProtKB (UniProt Protein Knowledgebase)<sup>21</sup> was consulted in order to obtain  
43 detailed information about relevant proteins identified in the study.  
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#### 49 **2.6. Ca<sup>2+</sup> mobilization *in vitro* assays**

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51 Washed platelets (2x10<sup>7</sup> platelets/ml) from healthy donors were incubated with FLIPR  
52 Calcium 4 reagent (Molecular devices<sup>®</sup>), supplemented with Probenecid 0.07% and  
53 incubated for 1 hour at 37°C in a 384-well transparent bottom plate. Inhibitors and agonists  
54 were dispensed by FDSS7000EX (Hamamatsu), automatically. Platelets were incubated for  
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3 10 minutes with 9 $\mu$ M integrilin (eptifibatide) and, when necessary, with apyrase 2 U/ml, and  
4 indomethacin 10 $\mu$ M. After that, agonists (3 $\mu$ g/ml CRP, 0.75 U/ml thrombin, 100 nM or 200  
5 nM rhodocytin) were added to platelets. Changes in fluorescence were measured in real time  
6 for 15 minutes after the addition of the agonists and using emission and excitation wave  
7 lengths of 480nm and 540nm, respectively.  
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## 18 **2.7. Western blotting**

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21 For validation by Western blotting, four samples corresponding to four biological replicates  
22 from different cohorts of healthy donors were analysed per condition and experimental  
23 setting.  
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29 Proteins were precipitated from lysates in 20% trichloroacetic acid in acetone as  
30 previously described<sup>15</sup>, and protein pellets were resuspended in 50 $\mu$ l of sample buffer (65mM  
31 CHAPS, 5M urea, 2M thiourea, 0.15M NDSB-256, 30mM Tris, 1mM sodium vanadate,  
32 0.1mM sodium fluoride, and 1mM benzamidine). Protein quantitation was done with  
33 Coomassie Plus protein reagent (Thermo Scientific) following manufacturer's protocol.  
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41 Protein separation by SDS-PAGE and subsequent immunoblotting were carried out as  
42 indicated in the Supplemental Methods. The following primary antibodies were used: rabbit  
43 anti-human p-PLC $\gamma$ 2 (Y759) (MAB7377, R&D systems) dilution 1/300; rabbit anti-p-Syk  
44 (Y525+Y526) (ab58575, Abcam) dilution 1/1000; rabbit anti-p-Src(Y418) (44660G, Fisher  
45 Scientific), dilution 1/1000; mouse anti-PLC $\gamma$ 2 (sc-5283, Santa Cruz Biotechnology) dilution  
46 1/500; mouse anti-Syk (sc-1240, Santa Cruz Biotechnology) dilution 1/1000; rabbit anti-Src  
47 pan (44-656G, Invitrogen), dilution 1/1000.  
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57 Further information on the statistical analysis can be found in the Supplemental  
58 Methods.  
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### 3. Results

#### 3.1. Tyrosine- phosphoproteome analysis of resting and CLEC-2 activated platelets.

From the 12 samples analysed (corresponding to 4 biological replicates per condition), we identified a total number of 757 p-Tyr peptides, 466 p-Tyr residues and 264 proteins (**Table 1**). More proteins, p-Tyr peptides, and pTyr sites were identified in “Rhod” samples (rhodocytin-activated platelets in absence of inhibitors of secondary mediators) (**Table 1; Figure. 2A,B**). Interestingly, from the 757 p-Tyr peptides identified, 258 were exclusively present in “Rhod” samples (**Figure 2A**). Considering the p-Tyr residues identified per condition, 458 were identified in “Rhod” samples, a number that decreased to 313 when platelets were activated in the presence of secondary mediators’ inhibitors (“Rhod inh”) (**Figure 2B; Table 1**). The overall results highlight the relevance of secondary mediators on achieving a robust intracellular tyrosine-phosphorylation response when platelets are activated through CLEC-2.

Interestingly, our LC-MS/MS analysis led to the identification of p-Tyr residues in 20 proteins that were not reported to undergo phosphorylation in those residues to date, some of them playing a relevant role in several intracellular signalling cascades, such as Btk, FES, Nck2, and ROCK2, and Talin, among others (**Supplemental table 1**).

#### 3.2. Phosphotyrosine fingerprint on CLEC-2 activated platelets: new signalling proteins identified.

As our data confirmed that the contribution of secondary mediators is essential for a powerful activation of the CLEC-2 coupled signalling pathway through tyrosine- phosphorylation, we performed our comparative analysis with the data obtained from “Rhod” and basal samples.

From the 235 proteins that were identified with at least one p-Tyr residue differentially regulated between the aforementioned conditions (Fold change  $\geq 1.5$ ), 21% are known to

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3 participate in cytoskeleton remodelling process, and 58% are involved in signal transduction  
4 upon receptor activation (**Figure 2C**). From these signalling proteins, more than a half of  
5 them belong to protein kinases (23%) plus adaptors/scaffolds (30%) (**Figure 2C**). **Table 2**  
6 contains a selection of relevant signalling proteins and their p-Tyr residues identified in the  
7 study (fold change  $\geq 1.5$ ). In total, 363 differentially regulated p-Tyr residues were identified,  
8 encompassing almost all the proteins previously known to be key effectors in the CLEC-2  
9 signalling pathway, including: CLEC-2 itself, the adapters LAT, SLP-76, Vav, Grb2, ADAP,  
10 CrkL, Gads, Dok2, and SKAP2; the kinases Syk, the SFKs Lyn and Fyn, Btk, Csk, FER, and  
11 PIK3R1; the phosphatases SHP-1, SHP-2, SHIP1, and PTP1B; the phospholipase PLC $\gamma$ 2; and  
12 the receptors CD148, and G6b-B.  
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26 Furthermore, we identified differentially-regulated p-Tyr residues in a number of  
27 proteins that are relevant for intracellular signal transduction in platelets and other blood cell  
28 types. Here, we show for the first time that several of them are involved in the CLEC-2  
29 signalling cascade, including: the adaptors Gab1, DAPP1, Dok1, Dok3, CASS4, EFS, Pag1,  
30 Nck1 and Nck2; the kinases FAK1, FES, FGR, and JAK2, the phosphoinositide phosphatase  
31 SHIP2, and the membrane proteins G6F, JAM-A, PECAM-1, and TLT-1 (**Table 2**).  
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### **3.3. Secondary mediators (ADP and TXA<sub>2</sub>) are required for powerful tyrosine-phosphorylation response upon CLEC-2 activation.**

Our phosphoproteomic data indicate secondary mediators are essential for a potent tyrosine-phosphorylation intracellular response mediated by CLEC-2 activation (**Figure 2; Table 1**). To further investigate this issue, we compared the fold changes (FC) obtained when comparing “Rhod” with basal samples, and “Rhod” with “Rhod inh” samples. We used this fold change comparison as an indirect measurement of the Tyr-phosphorylation levels between “Rhod inh” and resting platelets. Thus, when FC-values between both stimulation

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3 conditions (“Rhod” vs. “Rhod inh”) are smaller than those obtained between “Rhod” and  
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5 basal samples, we can conclude that rhodocytin stimulation itself is capable of altering Tyr-  
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7 phosphorylation levels of the corresponding protein phosphorylation site. The smaller the  
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9 “Rhod” vs. “Rhod inh” FC is, the higher the stimulation effect of Rhod inh. Therefore, from  
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11 the 150 p-Tyr residues from relevant signalling proteins that we found differentially regulated  
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13 (FC  $\geq 1.5$ ) in “Rhod” samples (**Table 2**), only 28 were directly regulated by rhodocytin-  
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15 CLEC-2 engagement (FC Rhod/Rhod inh < FC Rhod/basal; FC Rhod/basal / FC Rhod/Rhod  
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17 inh  $\geq 1.5$ ). Among these conditions, we identified p-Tyr residues for Syk, Gads, Dok2, SFKs,  
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19 PLC $\gamma$ 2, SHP-1, and CASS4 (**Supplemental table 2**).  
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### 26 **3.4. CLEC-2 activation-mediated Ca<sup>2+</sup> release is highly dependent on secondary** 27 **mediators**

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30 In order to investigate more in detail the impact of secondary mediators in achieving full  
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32 CLEC-2-mediated platelet activation, we developed a miniaturized calcium mobilization  
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34 assay for platelets. All experiments were under non-aggregating conditions (with integrillin).  
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36 Firstly, we checked a couple of Rhodocytin doses for platelet activation in presence and  
37  
38 absence of secondary mediators inhibitors (apyrase and indomethacin). We demonstrated  
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40 good levels of Ca<sup>2+</sup> release with a 100 nM dose of Rhodocytin, similar to 200 nM.  
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42 Importantly, Ca<sup>2+</sup> release was drastically diminished in the presence of inhibitors (**Figure**  
43  
44 **3A**). We next compared the latter effect with the impact of secondary mediators on platelet  
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46 activation by the collagen receptor GPVI (activated with collagen-related peptide (CRP)), and  
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48 PAR receptors (activated with thrombin). Such impact on Ca<sup>2+</sup> release was minor compared  
49  
50 to what happens in the case of CLEC-2 activation (**Figure 3B**). Finally, we explored the  
51  
52 specific contribution of ADP and TXA<sub>2</sub>, individually, to CLEC-2 activation-mediated  
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54 calcium release. Ca<sup>2+</sup> release diminishes in the presence of apyrase or indomethacin, with a  
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3 higher impact of the former. Maximum inhibition of  $\text{Ca}^{2+}$  release following CLEC-2  
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5 activation takes place when both inhibitors of secondary mediators are present at the same  
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7 time (**Figure 3C**).  
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### 10 11 12 **3.5. Contribution of secondary mediators to different steps essential for CLEC-2** 13 14 **signalling**

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16 In order to further elucidate the contribution of ADP and TXA2 at different points of the  
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18 signalling pathway downstream of CLEC-2, we evaluated p-Tyr levels of residues that were  
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20 identified in the analysis and are known to be essential for the catalytic activity of kinases Syk  
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22 (p-Tyr<sup>525/526</sup>) and Src (p-Tyr<sup>419</sup>), and for PLC $\gamma$ 2 activity (p-Tyr<sup>759</sup>). To do so, we performed a  
23  
24 western blot with protein samples obtained after activating the platelets in the presence or  
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26 absence of apyrase and indomethacin under non aggregating conditions. In parallel, we just  
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28 checked the aggregation profiles of platelets activated with Rhodcytin in the presence and  
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30 absence of the secondary mediators inhibitors; as expected, the latter prevented aggregation  
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32 (**Figure 4A**).  
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38 As it is shown in **Figure 4B,C**, phosphorylation of Syk on Tyr<sup>525/526</sup>, which is one of  
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40 the primary events occurring upon CLEC-2 activation following hemITAM phosphorylation,  
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42 is robustly increased in response to positive feedback by released ADP and TXA2.  
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44 Nonetheless, CLEC-2 stimulation itself, in the presence of inhibitors of secondary mediators,  
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46 led to a significant increase in phosphorylation of Syk Tyr<sup>525/526</sup>.  
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50 Robust phosphorylation of Src Tyr<sup>419</sup>, was also critically dependent on released ADP  
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52 and TXA2. In this case, we also observed high levels of that phosphoprotein in resting (basal)  
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54 platelets compared to the other two proteins analysed (**Figure 4B,C**).  
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57 Finally, we also investigated the activation of PLC $\gamma$ 2, which enables  $\text{Ca}^{2+}$  release from  
58  
59 intracellular stores and cytoskeleton remodelling process, so we evaluated its phosphorylation  
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3 on Tyr<sup>759</sup>. Optimal phosphorylation of PLCγ2 Tyr<sup>759</sup> is only achieved when ADP and TXA2  
4 are not inhibited from exerting their positive feedback effects on CLEC-2 signalling (**Figure**  
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7 **4B,C**).

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10 One question that arises from the above data is whether the increase in tyrosine  
11 phosphorylation observed for the proteins validated is directly due to Rhodocytin-CLEC-2  
12 engagement with signalling reinforced by the secondary mediators' positive feedback, or if  
13 such mediators could cause part of the increase by themselves. In order to clarify this issue,  
14 we activated platelets with ADP and arachidonic acid (AA), in comparison with Rhodocytin  
15 activation, in the absence of apyrase and indomethacin. Interestingly, ADP and AA are not  
16 able to induce an increase in the levels of Syk (p-Tyr<sup>525+526</sup>), Src (p-Tyr<sup>419</sup>), and PLCγ2 (p-  
17 Tyr<sup>759</sup>), which confirms the differences observed in our analysis are mediated by CLEC-2  
18 activation (**Fig. 5**).

### 3.6. Tyrosine phosphorylation kinetic profiles show variations depending on the CLEC-2 signalling protein analysed

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21 To complete the signalling studies, we carried out a small scale kinetic analysis focusing on  
22 the three phosphoproteins mentioned above. As shown in **Figure 6**, tyrosine phosphorylation  
23 levels start to increase after 30 sec of platelet stimulation with 200 nM Rhodocytin and  
24 significant levels are still maintained after 5 minutes; however the kinetic profile is different  
25 depending on the protein. Thus, Syk, which plays a relevant role in early signalling events, is  
26 phosphorylated in Y<sup>525</sup> and Y<sup>526</sup> rapidly, reaching maximum levels after one minute and  
27 decreasing steadily after 90 sec. Regarding Src (p-Tyr<sup>419</sup>), basal levels are high compared with  
28 the other two proteins; nevertheless, there is an increase in phosphorylation levels following  
29 activation reaching the maximum after 1 min and maintaining such level for 3 min when it  
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3 starts to decrease gradually. Regarding PLC $\gamma$ 2 (p-Tyr<sup>759</sup>), maximum phosphorylation levels  
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5 are also achieved between 1 and 3 min.  
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#### 10 **4. Discussion**

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12 The CLEC-2 receptor is known to mediate platelet activation through the stimulation of a  
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14 phosphotyrosine signalling cascade that is triggered by phosphorylation of the tyrosine  
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16 residue within its hemITAM<sup>5</sup>. In the present study, we aimed to analyse the phosphotyrosine  
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18 proteome of CLEC-2-activated platelets. To do so, we performed an immunoaffinity  
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20 enrichment of p-Tyr peptides in our samples, and we analysed them in an LTQ Orbitrap Velos  
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22 mass spectrometer, an experimental tandem that allows high sensitivity on the identification  
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24 of specific protein p-Tyr residues.  
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29 Results from our study emphasize the crucial synergism between the secondary  
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31 mediators, ADP and TXA2, and CLEC-2 to induce a powerful activation of the receptor. To  
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33 further explore this data, we evaluated the relevance of ADP and TXA2 in intracellular  
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35 calcium release mediated by CLEC-2-Rhodocytin activation, and also by platelet activation  
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37 with other agonists, such as CRP (GPVI agonist) and thrombin (PAR1/4 agonist). Our results  
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39 clearly demonstrate the relevance of both ADP and TXA2 (together) to achieve full CLEC-2  
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41 platelet activation, which does not happen in the case of GPVI or PAR1/4 activation. In  
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43 addition, we also studied the phosphorylation levels of tyrosine sites that are crucial for the  
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45 enzymatic activity of three proteins that are relevant for signal transduction upon CLEC-2  
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47 activation: Syk p-Tyr<sup>525+526</sup>, Src p-Tyr<sup>419</sup> and PLC $\gamma$ 2 p-Tyr<sup>759</sup>. Interestingly, we found that,  
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49 although powerful platelet activation through the stimulatory effect of secondary mediators is  
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51 essential for optimal Syk- kinase domain phosphorylation, rhodocytin stimulation of CLEC-2  
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53 itself is sufficient to significantly increase Syk Tyr<sup>525+526</sup> phosphorylation. In contrast, optimal  
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55 phosphorylation of PLC $\gamma$ 2 Tyr<sup>759</sup> is highly dependent on ADP and TXA2, indicating that the  
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3 initial steps of CLEC-2 signalling, including Syk phosphorylation, are not as dependent on  
4 ADP and TXA2 as downstream events. In resume, secondary mediators inhibitors are able to  
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7 fully inhibit Src and PLC $\gamma$ 2 tyrosine phosphorylation in the indicated residues, and can only  
8  
9 partially inhibit Syk Tyr<sup>525+526</sup>.

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12 To complement the above data, we also analysed the direct effect of ADP and AA to  
13 induce phosphorylation of Syk p-Tyr<sup>525+526</sup>, Src p-Tyr<sup>419</sup> and PLC $\gamma$ 2 p-Tyr<sup>759</sup>, observing no  
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16 effect, which indicates phosphorylation changes observed when activating platelets with  
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19 rhodocytin in absence of apyrase and indomethacin are due to CLEC-2-activation and  
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22 secondary mediators contribute only with a positive feedback to achieve full CLEC-2 tyrosine  
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25 phosphorylation and signalling activation. Although it was previously suggested that the latter  
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28 is mediated by Rac activation<sup>12</sup>, further research is needed to confirm the precise mechanism  
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30 of this positive feedback in line with the present study.

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33 Complementary data on the three phosphoproteins mentioned above demonstrate  
34 differences in the kinetic profile of p-Tyr depending on the signalling protein analysed. For  
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37 example, maximum phosphorylation levels of Syk on Tyr<sup>525+526</sup> decrease faster than in the  
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40 case of PLC $\gamma$ 2 Tyr<sup>759</sup>. The observed differences in the kinetic profile indicate that further  
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43 kinetic studies by quantitative western blotting or proteomics are needed to have a more  
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46 precise picture of the assembling of the CLEC-2 signalosome depicted in the present study.

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49 Intriguingly, in the case of phosphorylation of Src Tyr<sup>419</sup>, which is located within the  
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52 kinase domain responsible of Src-catalytic activity, the levels of basal samples were high  
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55 compared to the other validated proteins, especially in the presence of secondary mediators  
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58 (**Figs. 4-6**). This suggests the existence of a pool of active Src in platelets, reinforced by a  
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61 basal release of secondary mediators. Further research is needed to explore this possibility;  
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64 moreover, other SFKs besides Src, including Fyn, Lyn or Fgr, may contribute to signalling  
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67 downstream of CLEC-2.

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3 Considering the above mentioned relevance of ADP and TXA2 in reinforcing CLEC-2  
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5 signalling, as our aim was to obtain a broader picture of the biochemical events that are taking  
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7 place in the signalling cascade downstream of CLEC-2 activation, we focused on the  
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9 differentially regulated p-Tyr sites ( $FC \geq 1.5$ ) identified in “Rhod” samples (rhodocytin-  
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11 activated in absence of inhibitors of secondary mediators) compared to unstimulated samples.  
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13 We demonstrate differentially regulated p-Tyr residues for almost all the proteins known to  
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15 participate in the CLEC-2 signalling pathway to date (**Table 2**). Interestingly, a number of  
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17 those proteins were known to be phosphorylated upon CLEC-2 activation, but the specific  
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19 tyrosine residues that undergo this modification were not described to date. Here, we have  
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21 identified p-Tyr sites that are known to have great impact in signal transduction. CLEC-2 was  
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23 found to be tyrosine phosphorylated on hemITAM motif -Tyr<sup>7-5</sup>; phosphorylation of LAT  
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25 Tyr<sup>220</sup> was found to be up- regulated, which promotes its interaction with Grb2, Gads, and  
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27 PIK3R1<sup>22</sup>; Syk was phosphorylated at Tyr<sup>348</sup> and Tyr<sup>352</sup>, both of which are essential for  
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29 regulating Syk kinase activity, and at Tyr<sup>525</sup> and Tyr<sup>526</sup>, required for catalytic activity<sup>23</sup>; the  
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31 protein- tyrosine phosphatase SHP-1 was phosphorylated at Tyr<sup>536</sup> and Tyr<sup>564</sup>, which have  
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33 been implicated in regulating its activity <sup>24</sup>; and CD148 was shown to be phosphorylated on  
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35 Tyr<sup>1311</sup> and Tyr<sup>1320</sup>, previously reported to be mediated by Src and Fyn<sup>25</sup> and essential for  
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37 SHP-1 interaction with CD148 SH2 domains. Moreover, the identification of several proteins  
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39 tyrosine- phosphorylated on SH3, SH2, tyrosine- kinase, and phosphatase domains, as well as  
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41 in ITIM motifs, highlights the potential relevance of the identified p-Tyr sites in mediating  
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43 signal transduction upon CLEC-2 receptor activation (**Table 2**).

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45 Furthermore, we have identified differentially regulated p-Tyr residues from proteins  
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47 that have never been reported to play a role downstream of CLEC-2, but that are known to  
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49 participate in regulating signal transduction in platelets and other blood cells. Some of these  
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51 proteins have been described to interact and regulate the function of other known regulators of  
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3 CLEC-2 signalling. As a compendium of our proteomic data and the previous knowledge  
4 about protein interactions and functions, we have uncovered the more detailed picture of the  
5 CLEC-2 signalosome to date, represented in **Figure 7**, which includes key signalling proteins  
6 and their differentially regulated p-Tyr residues identified in our study. Interestingly, the set  
7 of proteins we show to be differentially- phosphorylated in our study suggests a delicate  
8 balance between stimulatory and inhibitory intracellular signals, which may be crucial for  
9 maintaining platelet activation without triggering an exacerbated response. Some of these  
10 novel phosphorylation sites and signalling proteins are likely to be clinically relevant, due to  
11 the potential interest of this receptor as a therapeutic target, since it seems to be crucial for  
12 thrombus stabilization, but not for haemostasis maintenance.

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Novel players on the CLEC-2 signalling cascade identified to be regulated by tyrosine-  
phosphorylation include the adapters Dok1 and Dok3<sup>26</sup>, Nck1 and Nck2<sup>27,28</sup>, and Crk-  
associated substrate (CAS) family members CASS4 and EFS<sup>29</sup>, among others (**Table 2**;  
**Figure 7**). Moreover, we report a number of up- regulated phosphorylated platelet kinases  
that were not previously described to participate in the CLEC-2 signalling cascade, but that  
are related to further signalling pathways, such as FAK, differentially phosphorylated at  
Tyr<sup>925</sup>, phosphorylation of which is mediated by FER allowing FAK interaction with Grb2  
and SHP-2<sup>30</sup>; FES; and FGR<sup>31</sup>, among others (**Table 2**; **Figure 7**).

We also identified numerous p-Tyr residues differentially- regulated in proteins that  
are known to maintain platelet activation within physiological limits by exerting an inhibitory  
action during intracellular signalling processes. The most important effectors on regulating  
signal transduction upon ITAM-receptor activation are the immunoreceptor tyrosine-based  
inhibition motif (ITIM)-containing receptors. Tyrosine-phosphorylation of these domains  
provides docking sites for SH2 domain- containing phosphatases, such as SHIP1, SHP-1 and  
SHP-2, all of which were found to contain differentially regulated p-Tyr residues in our study

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3 (Table 2; Figure 7), localizing the phosphatases in close proximity with ITAM-containing  
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5 receptors that can then dephosphorylate and inactivate<sup>32</sup>. G6b-B is an ITIM-containing  
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7 membrane protein that has been already reported to participate in CLEC-2 mediated  
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9 signalling<sup>33,34</sup>, and that we found to be further phosphorylated on ITIM Tyr<sup>211</sup> and Tyr<sup>237</sup> upon  
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11 rhodocytin stimulation. Moreover, we identified up-regulated tyrosine- phosphorylated  
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13 residues for the other two known platelet ITIM-containing receptors, PECAM-1 and TLT-1.  
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15 PECAM-1 was found to be phosphorylated on Tyr<sup>663</sup>, Tyr<sup>690</sup>, and Tyr<sup>713</sup>, the last two located  
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17 on the ITIM domain of the protein, and both known to be phosphorylated by Lyn<sup>35</sup>. PECAM-  
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19 1 was reported to negatively regulate platelet GPVI-activation, as a feedback mechanism to  
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21 limit thrombus growth, through the binding of its ITIM domains to SHP-2<sup>36,37</sup>. Interestingly,  
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23 PECAM-1 is thought to exert its inhibitory activity by an additional mechanism, consisting in  
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25 the recruitment of PIK3R1 regulatory subunit of PI3K that we also found to be differentially  
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27 tyrosine phosphorylated, through its interaction with SHP-2, negatively regulating the LAT  
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29 signalosome by decreasing the interaction of LAT, Gab1 and PIK3R1<sup>38</sup>. Gab1 is another  
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31 protein that we are reporting to participate in the CLEC-2 signalling pathway for the first  
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33 time, since we found differentially regulated p-Tyr residues of this protein following  
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35 stimulation with rhodocytin. Gab1 is an adaptor protein that regulates signal transduction  
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37 downstream of PI3K by directly interacting with this kinase. Finally, phosphorylation of  
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39 Tyr<sup>245</sup> in the atypical ITIM-containing receptor TLT-1 was found to be up-regulated in  
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41 rhodocytin-stimulated platelets. This residue was not previously reported to undergo  
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43 phosphorylation, which may regulate downstream signalling<sup>39</sup>. Another kinase identified,  
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45 Csk, phosphorylates the C-terminal tyrosine of SFKs inactivating them, a mechanism that is  
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47 in part compensated by the phosphatase CD148, previously reported to participate in CLEC-2  
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49 downstream signalling events<sup>40,41</sup>, and phosphorylation of which we found to be up-regulated  
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51 in our proteomic analysis (Table 2; Figure 7).  
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3 Of the phosphatases found to be phosphorylated in rhodocytin-stimulated platelets,  
4 CD148, SHP-1, SHP-2, SHIP1, and PTP1B were previously reported to participate in the  
5 CLEC-2 signalling<sup>14,42-44</sup>. In addition, we also found p-Tyr residues within SHIP2, PTPRA,  
6 PTN18, and PTN12 (**Table 2; Figure 7**) to be up-regulated, suggesting these phosphatases  
7 may also contribute to the regulation of CLEC-2 signalling. Further, another protein that was  
8 reported to negatively regulate platelet signalling, and more precisely GPVI- mediated  
9 signalling downstream PI3K, is the adapter DAPP1<sup>45</sup>. Interestingly, we found  
10 phosphorylation of Tyr<sup>139</sup> within DAPP1 to be up-regulated, which is required for its  
11 interaction with PLC $\gamma$ 2 and protein function<sup>46,47</sup>.  
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24 In conclusion, we have performed the most extensive tyrosine phosphoproteomic  
25 analysis of the CLEC-2 signalling pathway in platelets to date. We identified differentially  
26 regulated p-Tyr sites relevant for the activity of known as well as novel proteins in the CLEC-  
27 2 signalling cascade. We also demonstrated a crucial role of AA and ADP (together) to  
28 achieve full CLEC-2-mediated platelet activation, and phosphorylation of relevant tyr sites in  
29 CLEC-2 signalling proteins. We also show that the kinetic profiles of phosphorylation of key  
30 tyrosine residues in some relevant CLEC-2 signalling proteins - such as Syk (Tyr<sup>525+526</sup>), Src  
31 (Tyr<sup>419</sup>) and PLC $\gamma$ 2 (Tyr<sup>759</sup>) - vary slightly depending on the precise phosphoprotein analysed,  
32 which provides clues on the role of each protein in the pathway. Overall, we believe that our  
33 study provides significant insights into the intracellular events that take place following  
34 CLEC-2 activation in platelets, contributing to elucidate in detail the CLEC-2 signalosome.  
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#### **Authorship contributions**

- Irene Izquierdo: performed research, analyzed data and wrote the paper.
- María N Barrachina, Lidia Hermida-Nogueira, Vanessa Casas, Luis A. Morán, Serena Lacerenza, Roberto Pinto-Llorente, and Vivian de los Rios: performed research.
- J. Ignacio Casal, Montserrat Carrascal, and Joaquín Abián: contributed with key analytical tools and analyzed and integrated mass spec data.
- Johannes A. Eble: provided vital reagents.
- Eduardo Domínguez, and María I. Loza provided vital reagents and analytical tools for the calcium release experiments.
- Ángel García: designed research, analyzed data, provided vital reagents and analytical tools, and wrote the paper.



## Disclosure of Conflicts of interest

The authors declare no conflicts of interest.

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## 22 Tables

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25 **Table 1. Results from LC-MS/MS analysis.**  
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| 27 CONTITION       | 28 P-TYR PEPTIDES | 29 P-TYR SITES | 30 P-TYR PROTEINS |
|--------------------|-------------------|----------------|-------------------|
| 31 <b>Total</b>    | 32 757            | 33 466         | 34 264            |
| 35 <b>Basal</b>    | 36 374            | 37 288         | 38 187            |
| 39 <b>Rhod inh</b> | 40 421            | 41 313         | 42 198            |
| 43 <b>Rhod</b>     | 44 688            | 45 458         | 46 258            |

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48 **Rhod inh:** rhodocytin- activated in presence of secondary mediators' inhibitors; **Rhod:**  
49 rhodocytin- activated in absence of secondary mediators' inhibitors.  
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**Table 2. Tyrosine- phosphorylation sites of relevant signalling proteins identified in “Rhod” vs. “Basal” samples with a fold change (FC)  $\geq 1.5$ .**

| Uniprot code            | Protein names   | P-Site    | FC       | Relevant Domain |
|-------------------------|---|-----------|----------|-----------------|
| <b>Adaptor/Scaffold</b> |   |           |          |                 |
| ABI1_HUMAN*             | Abl interactor 1 (ABI1)   | Y213      | $\infty$ |                 |
|                         |   | Y455      | $\infty$ | SH3             |
| BANK1_HUMAN*            | B-cell scaffold protein with ankyrin repeats (BANK1)                                  | Y443      | $\infty$ |                 |
|                         |   | Y737      | 3.00     |                 |
| CASS4_HUMAN*            | Cas scaffolding protein family member 4 (CASS4)                                       | Y350      | $\infty$ |                 |
|                         |   | Y174      | $\infty$ |                 |
|                         |   | Y195      | $\infty$ |                 |
| CRKL_HUMAN              | Crk-like protein (CRKL)   | Y207      | $\infty$ |                 |
|                         |   | Y132      | $\infty$ | SH3             |
|                         |   | Y127      | $\infty$ | SH3             |
|                         |   | Y198+Y207 | $\infty$ |                 |
|                         |   | Y251      | 4.00     | SH3             |
| DAPP1_HUMAN*            | Dual adapter for phosphotyrosine and 3-phosphotyrosine and 3-phosphoinositide (DAPP1) | Y139      | 3.00     |                 |
| DOK1_HUMAN*             | Docking protein 1 (DOK1)  | Y377      | $\infty$ | Pro rich        |
|                         |   | Y296      | $\infty$ | Pro rich        |
|                         |   | Y362+Y377 | $\infty$ | Pro rich        |
|                         |   | Y409      | 3.50     | Pro rich        |
|                         |   | Y449      | 1.50     |                 |
| DOK2_HUMAN              | Docking protein 2 (DOK2)  | Y139      | $\infty$ |                 |
|                         |   | Y402      | $\infty$ |                 |
| DOK3_HUMAN*             | Docking protein 3 (DOK3)  | Y398      | 2.44     | Pro rich        |
| EFS_HUMAN*              | Embryonal Fyn-associated substrate (EFS)  | Y163      | $\infty$ | Pro rich        |
| FYB1_HUMAN              | FYN-binding protein 1 (FYB1; ADAP; FYB; SLAP130)                                      | Y387      | 5.00     |                 |
|                         |   | Y757      | 3.00     | SH3             |
| GAB1_HUMAN*             | GRB2-associated-binding protein 1 (GAB1)  | Y659      | $\infty$ |                 |
| GRAP2_HUMAN             | GRB2-related adapter protein 2 (GRAP2; GADS)  | Y207      | $\infty$ |                 |
|                         |   | Y45       | 3.00     | SH3             |
| GRB2_HUMAN              | Growth factor receptor-bound protein 2 (GRB2)   | Y209      | $\infty$ | SH3             |
| LAT_HUMAN               | Linker for activation of T-cells family member 1 (LAT)                                | Y45       | 4.67     |                 |
|                         |   | Y220      | 3.40     |                 |
| LCP2_HUMAN              | Lymphocyte cytosolic protein 2 (LCP2; SLP-76; SLP76)                                  | Y532      | $\infty$ |                 |
|                         |   | Y39       | 2.00     |                 |
| NCK1_HUMAN*             | Cytoplasmic protein NCK1 (NCK1; NCK)  | Y105      | 1.56     |                 |

| Uniprot code            | Protein names  | P-Site    | FC        | Relevant Domain      |
|-------------------------|--|-----------|-----------|----------------------|
| <b>Adaptor/Scaffold</b> |  |           |           |                      |
| NCK2_HUMAN*             | Cytoplasmic protein NCK2 (NCK2)  | Y50       | $\infty$  | SH3                  |
|                         |  | Y251      | $\infty$  | SH3                  |
|                         |  | Y110      | 2.91      |                      |
| NTAL_HUMAN*             | Linker for activation of T-cells family member 2 (LAT2; NTAL)                        | Y136      | $\infty$  |                      |
|                         |  | Y58       | $\infty$  |                      |
| PARD3_HUMAN*            | Partitioning defective 3 homolog (PARD3; PAR3)                                       | Y719      | $\infty$  |                      |
|                         |  | Y1080     | 4.00      |                      |
| PHAG1_HUMAN*            | Phosphoprotein associated with glycosphingolipid-enriched microdomains 1 (PAG1; PAG) | Y317      | 2.50      | Interaction with CSK |
|                         |  | Y163      | 2.00      |                      |
|                         |  | Y341      | 1.50      |                      |
| REPS2_HUMAN*            | RalBP1-associated Eps domain-containing protein 2 (REPS2)                            | Y558      | $\infty$  |                      |
| SKAP2_HUMAN             | Src kinase-associated phosphoprotein 2 (SKAP2)                                       | Y169      | $\infty$  |                      |
|                         |  | Y334      | 2.00      | SH3                  |
|                         |  | Y151/Y152 | -2.00     |                      |
| SPN90_HUMAN*            | NCK-interacting protein with SH3 domain (NCKIPSD; SPIN90)                            | Y161      | $-\infty$ |                      |
| STAM2_HUMAN*            | Signal transducing adapter molecule 2 (STAM2)  | Y374      | 2.00      | ITAM                 |
| VAV_HUMAN               | Proto-oncogene vav (VAV1; VAV)   | Y844      | $\infty$  |                      |
|                         |  | Y791      | $\infty$  | SH3                  |
|                         |  | Y110      | 11.00     |                      |
| WASP_HUMAN*             | Wiskott-Aldrich syndrome protein (WAS)   | Y212      | $\infty$  |                      |
|                         |  | Y291      | 3.33      |                      |
| WIPF1_HUMAN*            | WAS/WASL-interacting protein family member 1 (WIPF1)                                 | Y455      | $\infty$  |                      |
| WIPF2_HUMAN*            | WAS/WASL-interacting protein family member 2 (WIPF2)                                 | Y400      | $\infty$  |                      |
| <b>Kinase</b>           |  |           |           |                      |
| ABL2_HUMAN*             | Tyrosine-protein kinase ABL2 (ABL2)  | Y439      | $\infty$  | Tyr- kinase          |
| ACK1_HUMAN*             | Activated CDC42 kinase 1 (TNK2; ACK1)  | Y518      | 2.00      |                      |
|                         |  | Y827      | -1.67     |                      |
| BTK_HUMAN               | Tyrosine-protein kinase BTK (BTK)  | Y334      | $\infty$  | SH2                  |
|                         |  | Y223      | 5.27      | SH3                  |
|                         |  | Y361      | 3.00      | SH2                  |
|                         |  | Y315      | 2.00      | SH2                  |
| CSK_HUMAN*              | Tyrosine-protein kinase CSK (CSK)  | Y18       | $\infty$  | SH3                  |

| Uniprot code  | Protein names   | P-Site      | FC    | Relevant Domain |
|---------------|---|-------------|-------|-----------------|
| <b>Kinase</b> |   |             |       |                 |
| FAK1_HUMAN*   | Focal adhesion kinase 1 (PTK2; FAK; FAK1)                             | Y925        | 2.50  |                 |
| FER_HUMAN     | Tyrosine-protein kinase Fer (FER)                                     | Y402        | ∞     |                 |
|               |   | Y714        | 3.33  | Tyr- kinase     |
| FES_HUMAN*    | Tyrosine-protein kinase Fes/Fps (FES; FPS)                            | Y513        | ∞     |                 |
|               |   | Y713        | 5.00  | Tyr- kinase     |
| FGR_HUMAN*    | Tyrosine-protein kinase Fgr (FGR; SRC2)                               | Y208/Y209   | ∞     | SH2             |
| FYN_HUMAN     | Tyrosine-protein kinase Fyn (FYN)                                     | Y213        | ∞     | SH2             |
| JAK2_HUMAN*   | Tyrosine-protein kinase JAK2 (JAK2)                                   | Y570        | 1.83  | Tyr- kinase     |
| KALRN_HUMAN*  | Kalirin (KALRN)   | Y2265       | 4.00  |                 |
| KGP1_HUMAN*   | cGMP-dependent protein kinase 1 (PRKG1)                               | Y336        | ∞     |                 |
| KPCD_HUMAN    | Protein kinase C delta type (PRKCD)                                   | Y64         | ∞     |                 |
|               |   | Y313        | 3.00  |                 |
|               |   | Y334        | 2.40  |                 |
| KSYK_HUMAN    | Tyrosine-protein kinase SYK (SYK)                                     | Y296        | ∞     |                 |
|               |   | Y323        | 19.00 |                 |
|               |   | Y352        | 15.00 |                 |
|               |   | Y348+Y352   | 10.00 |                 |
|               |   | Y525+Y526   | 3.50  | Tyr- kinase     |
| Y526          | 1.50  | Tyr- kinase |       |                 |
| LYN_HUMAN     | Tyrosine-protein kinase Lyn (LYN)                                     | Y306        | 4.00  | Kinase          |
| MK01_HUMAN    | Mitogen-activated protein kinase 1 (MAPK1; ERK2)                      | Y187        | 6.00  | Tyr- kinase     |
| MK03_HUMAN    | Mitogen-activated protein kinase 3 (MAPK3; ERK1)                      | Y204        | ∞     | Kinase          |
| MK08_HUMAN    | Mitogen-activated protein kinase 8 (MAPK8; JNK1)                      | Y185        | ∞     | Kinase          |
| MK09_HUMAN    | Mitogen-activated protein kinase 9 (MAPK9; JNK2)                      | Y185        | 1.82  | Kinase          |
| MK12_HUMAN    | Mitogen-activated protein kinase 12 (MAPK12; ERK6)                    | Y185        | 6.00  | Kinase          |
| MK14_HUMAN    | Mitogen-activated protein kinase 14 (MAPK14; CSBP)                    | Y182        | 2.15  | Kinase          |
| MRCKB_HUMAN*  | Serine/threonine-protein kinase MRCK beta (CDC42BPB)                  | Y1638       | 3.00  |                 |
| P85A_HUMAN    | Phosphatidylinositol 3-kinase regulatory subunit alpha (PIK3R1; GRB1) | Y467        | 2.00  |                 |
| PI4KA_HUMAN*  | Phosphatidylinositol 4-kinase alpha (PI4KA; PIK4)                     | Y1154       | -4.00 |                 |
| ROCK2_HUMAN*  | Rho-associated protein kinase 2 (ROCK2)                               | Y722        | 5.00  |                 |
|               |   | Y450        | 5.00  |                 |
| SRC_HUMAN     | Proto-oncogene tyrosine-protein kinase Src (SRC; SRC1)                | Y187        | -2.00 | SH2             |

| Uniprot code         | Protein names  | P-Site      | FC    | Domain      |
|----------------------|--|-------------|-------|-------------|
| <b>Phosphatase</b>   |  |             |       |             |
| PTN1_HUMAN           | Tyrosine-protein phosphatase non-receptor type 1 (PTPN1; PTP1B)                | Y46         | 4.00  | Phosphatase |
| PTN11_HUMAN          | Tyrosine-protein phosphatase non-receptor type 11 (PTPN11; SHP-2)              | Y584        | 1.57  |             |
| PTN12_HUMAN*         | Tyrosine-protein phosphatase non-receptor type 12 (PTPN12; PTPG1)              | Y64         | ∞     | Phosphatase |
| PTN18_HUMAN*         | Tyrosine-protein phosphatase non-receptor type 18 (PTPN18)                     | Y426        | ∞     |             |
|                      |  | Y389        | 2.50  |             |
| PTN6_HUMAN           | Tyrosine-protein phosphatase non-receptor type 6 (PTPN6; SHP-1)                | Y64         | 3.00  | SH2         |
|                      |  | Y564        | 9.00  |             |
|                      |  | Y98         | 2.00  | SH2         |
|                      |  | Y536        | 1.78  |             |
| SHIP1_HUMAN          | Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1 (INPP5D; SHIP; SHIP1) | Y1022       | ∞     | Pro rich    |
| SHIP2_HUMAN*         | Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2 (INPPL1; SHIP2)       | Y986        | ∞     | Pro rich    |
|                      |  | Y1135       | ∞     |             |
|                      |  | Y886        | ∞     |             |
| <b>Phospholipase</b> |  |             |       |             |
| PLCG2_HUMAN          | 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-2 (PLCG2)      | Y1217       | ∞     |             |
|                      |  | Y1245       | ∞     |             |
|                      |  | Y753        | ∞     |             |
|                      |  | Y495        | ∞     |             |
|                      |  | Y759        | 23.50 |             |
| <b>Receptor</b>      |  |             |       |             |
| CLC1B_HUMAN          | C-type lectin domain family 1 member B (CLEC1B; CLEC2)                         | Y7          | ∞     | ITAM        |
| FCERG_HUMAN*         | High affinity immunoglobulin epsilon receptor subunit gamma (FCER1G)           | Y58         | 2.00  | ITAM        |
| FCG2A_HUMAN*         | Low affinity immunoglobulin gamma Fc region receptor II-a (FCGR2A)             | Y304        | 3.00  |             |
| G6B_HUMAN            | Megakaryocyte and platelet inhibitory receptor G6b (MPIG6B; G6B; G6B-B)        | Y211        | 4.33  | ITIM        |
|                      |  | Y237        | 2.00  | ITIM        |
| PTPRA_HUMAN*         | Receptor-type tyrosine-protein phosphatase alpha (PTPRA; PTPA)                 | Y798        | 2.00  |             |
| PTPRJ_HUMAN          | Receptor-type tyrosine-protein phosphatase eta (PTPRJ; CD148)                  | Y1311+Y1320 | ∞     |             |
|                      |  | Y1311       | 9.00  |             |
| JAM1_HUMAN*          | Junctional adhesion molecule A (JAM-A)   | Y280        | -3.00 |             |
| TRML1_HUMAN*         | Trem-like transcript 1 protein (TLT-1)   | Y245        | 3.06  | Pro rich    |



| Uniprot code        | Protein names  | P-Site | FC        | Domain |
|---------------------|--|--------|-----------|--------|
| <b>Small GTPase</b> |  |        |           |        |
| RAB13_HUMAN*        | Ras-related protein Rab-13 (RAB13)   | Y5     | $\infty$  |        |
| RAB1B_HUMAN*        | Ras-related protein Rab-1B (RAB1B)   | Y5     | $\infty$  |        |
| RAN_HUMAN*          | GTP-binding nuclear protein Ran (RAN)  | Y39    | 1.50      |        |
| <b>GAP</b>          |  |        |           |        |
| ARAP1_HUMAN*        | Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 1 (ARAP1) | Y737   | 3.50      |        |
| IQGA2_HUMAN*        | Ras GTPase-activating-like protein IQGAP2 (IQGAP2)                                 | Y93    | $\infty$  |        |
| RASA1_HUMAN*        | Ras GTPase-activating protein 1 (RASA1)  | Y615   | 2.00      |        |
| RASA3_HUMAN*        | Ras GTPase-activating protein 3 (RASA3)  | Y66    | $\infty$  |        |
| RHG18_HUMAN*        | Rho GTPase-activating protein 18 (ARHGAP18)  | Y41    | 4.00      |        |
| RHG35_HUMAN*        | Rho GTPase-activating protein 35 (ARHGAP35)  | Y1087  | $\infty$  |        |
|                     |  | Y1105  | 1.75      |        |
| <b>GDI</b>          |  |        |           |        |
| GDIR2_HUMAN*        | Rho GDP-dissociation inhibitor 2 (ARHGDI2)   | Y130   | $-\infty$ |        |
|                     |  | Y24    | $\infty$  |        |
| <b>GEF</b>          |  |        |           |        |
| DEN2C_HUMAN         | DENN domain-containing protein 2C (DENND2C)  | Y322   | $\infty$  |        |
|                     |  | Y237   | $\infty$  |        |
|                     |  | Y475   | $\infty$  |        |
|                     |  | Y195   | $\infty$  |        |
|                     |  | Y102   | $\infty$  |        |
|                     |  | Y310   | $\infty$  |        |
|                     |  | Y50    | $\infty$  |        |
|                     |  | Y219   | $\infty$  |        |
|                     |  | Y228   | $\infty$  |        |
|                     | Y185   | 2.75   |           |        |
| GRP2_HUMAN*         | RAS guanyl-releasing protein 2 (RASGRP2)   | Y523   | -2.00     |        |
| <b>Other</b>        |  |        |           |        |
| LY66F_HUMAN*        | Lymphocyte antigen 6 complex locus protein G6f (LY6G6F; G6F)                       | Y281   | 4.50      |        |
| PECA1_HUMAN*        | Platelet endothelial cell adhesion molecule (PECAM-1)                              | Y663   | 3.67      |        |
|                     |  | Y690   | $\infty$  | ITIM   |

A positive fold change indicates that the variation is favourable to “Rhod” samples (rhodocytin- activated in absence of secondary mediators’ inhibitors), and a negative fold change indicates that the variation is favourable to basal samples. Fold changes were calculated as the quotient of the total number of PSMs that led to the identification of the protein p-site. GAP: GTPase-activating protein; GDI: GDP dissociation inhibitor; GEF: Guanine nucleotide exchange factor.  $\infty$  means infinity.

\* Means that the protein was not previously reported to participate in CLEC-2 signalling pathway.

## Figure legends

**Figure 1. Experimental workflow.** Schematic representation of the methodology applied and the equipment employed in this study.

**Figure 2. P-Tyr peptides, and p-Tyr sites identified by LC-MS/MS. Function of proteins with differentially regulated p-Tyr sites in CLEC-2-activated platelets.**

Venn diagrams represent the total number of p-Tyr peptides (**A**), and the corresponding p-Tyr sites (**B**), that were identified in each analysed condition. **C**) Functions of proteins that were identified with differentially regulated p-Tyr sites in CLEC-2-activated vs. basal platelets. Charts represent protein functions distribution ( $FC \geq 1.5$ ).

**Figure 3.  $Ca^{2+}$  mobilization following CLEC-2 activation is dependent on secondary mediators.**

**A)** Secondary mediators play a fundamental role in CLEC-2-mediated  $Ca^{2+}$  release induced by platelet activation with Rhodocytin. **B)** Secondary mediators are needed for a full platelet activation induced by Rhodocytin, in contrast with what occurs in the case of platelet activation with CRP or thrombin. **C)** Both ADP and TXA2 together are needed for full CLEC-2 activation. Rhod, Rhodocytin; CRP, collagen-related peptide; THR, thrombin. All measurements were done in triplicate (biological replicates). For further experimental details please see section 2.6.

**Figure 4. Contribution of ADP and TXA2 to phosphorylation of relevant tyrosine residues in Src, Syk, and PLC $\gamma$ 2 downstream of CLEC-2.**

**A)** Aggregation profile of platelets stimulated with 200nM rhodocytin in absence and presence of secondary mediators inhibitors (2U/mL apyrase and indomethacin 10 $\mu$ M). Representative western blot images (**B**), and densitometry graphs (**C**), representing tyrosine phosphorylation levels of Src p-Tyr<sup>419</sup>, Syk p-Tyr<sup>525+526</sup>, and PLC $\gamma$ 2 p-Tyr<sup>759</sup>. \*\*  $p < 0.01$  (RM one way

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2  
3 Anova with Tukey's post-hoc multiple comparisons). Experiments were done with  
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5 samples obtained from 4 different healthy donors (biological replicates).  
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8 **Figure 5. ADP and arachidonic acid do not induce tyrosine phosphorylation of Src,**  
9 **Syk, and PLC $\gamma$ 2 in the same residues CLEC-2 activation does.** A) Aggregation  
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11 profile of platelets stimulated with 200nM rhodocytin, 10  $\mu$ M ADP, and 100  $\mu$ M AA.  
12  
13 Representative western blot images (B) and densitometry graphs (C) showing tyrosine  
14  
15 phosphorylation levels of Src p-Tyr<sup>419</sup>, Syk p-Tyr<sup>525+526</sup>, and PLC $\gamma$ 2 p-Tyr<sup>759</sup> in  
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17 response to Rhodocytin and AA+ADP. Comparisons were between each stimulus and  
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19 its respective control. \*\* p < 0.01 (paired Student's t-test). Experiments were done with  
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21 samples obtained from 4 different healthy donors (biological replicates).  
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28 **Figure 6. Kinetic studies on essential tyrosine residues in Src, Syk, and PLC $\gamma$ 2**  
29 **downstream of CLEC-2 point out differences in the phosphorylation pattern.**  
30  
31 Representative western blot images (A), and densitometry graphs (B) showing tyrosine  
32  
33 phosphorylation profiles of Src p-Tyr<sup>419</sup>, Syk p-Tyr<sup>525+526</sup>, and PLC $\gamma$ 2 p-Tyr<sup>759</sup>  
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35 following platelet activation with rhodocytin for 30s, 60s, 120s, 180s and 300s. For  
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37 statistics, comparisons were always between each activation time point and basal  
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39 (control platelets). \*p < 0.05, \*\* p < 0.01, and \*\*\* p < 0.001 (paired Student's t-test).  
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42 Experiments were done with samples obtained from 4 different healthy donors  
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44 (biological replicates).  
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49 **Figure 7. The CLEC-2 signalling pathway in platelets: solving the puzzle.** New  
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51 proposal for the platelet CLEC-2 signalling cascade in which relevant signalling  
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53 proteins and their corresponding differentially regulated p-Tyr sites identified in the  
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55 present study are highlighted. This figure only includes those Tyr residues that were  
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57 identified as differentially regulated in the present proteomic study (Fold change  $\geq$  1.5).  
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3 Further information on how the information to build the figure was retrieved can be  
4  
5 found in section 2.4.  
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