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# Synthetic glycopolymers and natural fucoidans cause human platelet aggregation via PEAR1 and GPIb $\alpha$

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## Key Points

- Synthetic sulfated glycopolymers bind to EGF-like repeat 13 of PEAR1, which leads to Src- and PI3K-dependent aggregation of human platelets.
- GPIb $\alpha$  supports synthetic glycopolymer activation of human and mouse platelets, but the predominant signaling receptor in mice is CLEC-2.

Fucoidans are sulfated fucose-based polysaccharides that activate platelets and have pro- and anticoagulant effects; thus, they may have therapeutic value. In the present study, we show that 2 synthetic sulfated  $\alpha$ -L-fucoside-pendant glycopolymers (with average monomeric units of 13 and 329) and natural fucoidans activate human platelets through a Src- and phosphatidylinositol 3-kinase (PI3K)-dependent and Syk-independent signaling cascade downstream of the platelet endothelial aggregation receptor 1 (PEAR1). Synthetic glycopolymers and natural fucoidan stimulate marked phosphorylation of PEAR1 and Akt, but not Syk. Platelet aggregation and Akt phosphorylation induced by natural fucoidan and synthetic glycopolymers are blocked by a monoclonal antibody to PEAR1. Direct binding of sulfated glycopolymers to epidermal like growth factor (EGF)-like repeat 13 of PEAR1 was shown by avidity-based extracellular protein interaction screen technology. In contrast, synthetic glycopolymers and natural fucoidans activate mouse platelets through a Src- and Syk-dependent pathway regulated by C-type lectin-like receptor 2 (CLEC-2) with only a minor role for PEAR1. Mouse platelets lacking the extracellular domain of GPIb $\alpha$  and human platelets treated with GPIb $\alpha$ -blocking antibodies display a reduced aggregation response to synthetic glycopolymers. We found that synthetic sulfated glycopolymers bind directly to GPIb $\alpha$ , substantiating that GPIb $\alpha$  facilitates the interaction of synthetic glycopolymers with CLEC-2 or PEAR1. Our results establish PEAR1 as the major signaling receptor for natural fucose-based polysaccharides and synthetic glycopolymers in human, but not in mouse, platelets. Sulfated  $\alpha$ -L-fucoside-pendant glycopolymers are unique tools for further investigation of the physiological role of PEAR1 in platelets and beyond.

## Introduction

Marine fucoidans are heterogeneous fucose-rich sulfated polysaccharides derived from brown seaweed and echinoderms. Fucoidans have a wide range of biological functions and potential medical applications due to their anticancer, antimicrobial, and anti-inflammatory properties.<sup>1</sup> In addition, fucoidan polysaccharides have been shown to affect hemostasis,<sup>1</sup> having both procoagulant<sup>2,3</sup> and anticoagulant<sup>4,5</sup> actions. Fucoidans also have platelet-activating and -inhibiting properties, depending on

their origin.<sup>5</sup> The conflicting observations are explained by varying chain length, branching, and degree of sulfation.<sup>5-8</sup>

The activation of platelets by sulfated polysaccharides of different monosaccharide backbones has been investigated. Manne et al reported that fucoidan from *Fucus vesiculosus* activates human and mouse platelets through C-type lectin-like receptor 2 (CLEC-2),<sup>9</sup> whereas a later study showed that higher concentrations also activate GPVI in mouse platelets.<sup>10</sup> On the other hand, the highly sulfated heparin-like polysaccharide dextran sulfate, which has a glucoside backbone, stimulates platelet aggregation simultaneously through PEAR1 and CLEC-2.<sup>11</sup>

The multivalent nature of polysaccharides, in combination with their complex and heterogenic structure, gives rise to multiple protein interactions and complex mechanisms of platelet activation.<sup>7,8,12</sup> For these reasons, we synthesized highly sulfated and unbranched glycopolymers of different average chain lengths with fucoidan-mimetic properties and compared these with natural fucoidan from *F vesiculosus*.<sup>6,13,14</sup> We show that, unexpectedly, these fucoidan-like constructs induce human platelet activation predominantly via PEAR1, facilitated by GPIIb $\alpha$ . In contrast, in mouse platelets, the synthetic compounds induce platelet activation via CLEC-2, again facilitated by GPIIb $\alpha$ .

## Materials and methods

### Chemicals and antibodies

Sulfated  $\alpha$ -L-fucoside-pendant glycopolymers C329 ( $n = 329$  average monomeric units) and C13 ( $n = 13$  average monomeric units), as well as biotinylated glycopolymers (average monomeric units  $n = 27$ ), were synthesized as described.<sup>6,14</sup> The sulfated glycopolymers are described in more detail in supplemental Materials and methods and supplemental Table 1. Fucoidan from *F vesiculosus*  $\geq 95\%$  (#F8190) and dextran sulfate (#D8906) were purchased from Sigma-Aldrich. Rhodocytin was purified from *Calloselasma rhodostoma* venom, as described,<sup>15</sup> and convulxin was obtained from PENTAPHARM. The monoclonal antibody (mAb) against CLEC-2 was raised as described.<sup>16</sup> For a complete list of reagents, see supplemental Materials and methods.

### Human platelet preparation

This study was conducted in accordance with the Declaration of Helsinki. Blood sampling was approved by the Regional Ethical Review Board in Uppsala, Sweden (Dnr 2015/543). Heparinized blood (10 IU/mL; LEO Pharma) was drawn by venipuncture from healthy volunteers and treated with acid-citrate-dextrose (71 mM citric acid, 85 mM sodium citrate, 111 mM glucose) at a 1:5 ratio. Platelets were isolated using 2-step centrifugation and resuspended in Krebs-Ringer glucose buffer with 0.05 U/mL apyrase and 1 mM CaCl<sub>2</sub>, as described.<sup>6</sup>

### Mouse platelet preparation

Platelet-specific CLEC-2-knockout mice (*Clec1b<sup>fl/fl</sup>;PF4-Cre*)<sup>17</sup> and hIL-4R $\alpha$ /GPIIb $\alpha$ -Tg mice were bred as described.<sup>18</sup> All procedures were performed following United Kingdom Home Office approval (PPL P0E98D513). Washed mouse platelets were pelleted from harvested platelet-rich plasma by centrifugation at 500g for 9 minutes and otherwise prepared as described.<sup>19</sup> *Pear1<sup>-/-</sup>* mice were produced, and mouse platelets were isolated as previously described.<sup>11</sup> Animal experimental procedures were approved by the local Ethics Committee of KU Leuven.

## Platelet aggregation

Platelet aggregation was measured using a lumi-aggregometer (Model 700; CHRONO-LOG) under stirring conditions at 37°C. Human and mouse platelets were used at concentrations of  $2.5 \times 10^8$ /mL and  $2 \times 10^8$ /mL, respectively.

## Intracellular Ca<sup>2+</sup> mobilization

Platelet-rich plasma was incubated with 4  $\mu$ M Fura-2, AM (#F0888; Sigma-Aldrich),<sup>20</sup> and Ca<sup>2+</sup> was measured as described in supplemental Materials and methods.

## Western blotting

Stimulation of washed human platelets ( $2.5 \times 10^8$ /mL) and murine platelets ( $5 \times 10^8$ /mL) was performed at 37°C. Reactions were stopped using lysis buffer.<sup>6,21</sup> Immunoprecipitation was performed according to the manufacturer's instructions using a Pierce Classic IP Kit (#26146; Thermo Fisher Scientific) in the presence of protease and phosphatase inhibitor cocktails. The level of chemiluminescence and fluorescence was registered using an Odyssey Fc imaging system (LI-COR). Densitometry of the bands was performed using Image Studio software version 3.1 (LI-COR). Results are presented in arbitrary units.

## Recombinant protein expression

Plasmid design and expression of pentameric  $\beta$ -lactamase-containing proteins with C-terminal His6 tag were performed.<sup>22</sup> Further details are provided in supplemental Materials and methods.

## AVEXIS

An avidity-based extracellular protein interaction screen (AVEXIS) was performed.<sup>22</sup> Further details are provided in supplemental Materials and methods.

## Statistics

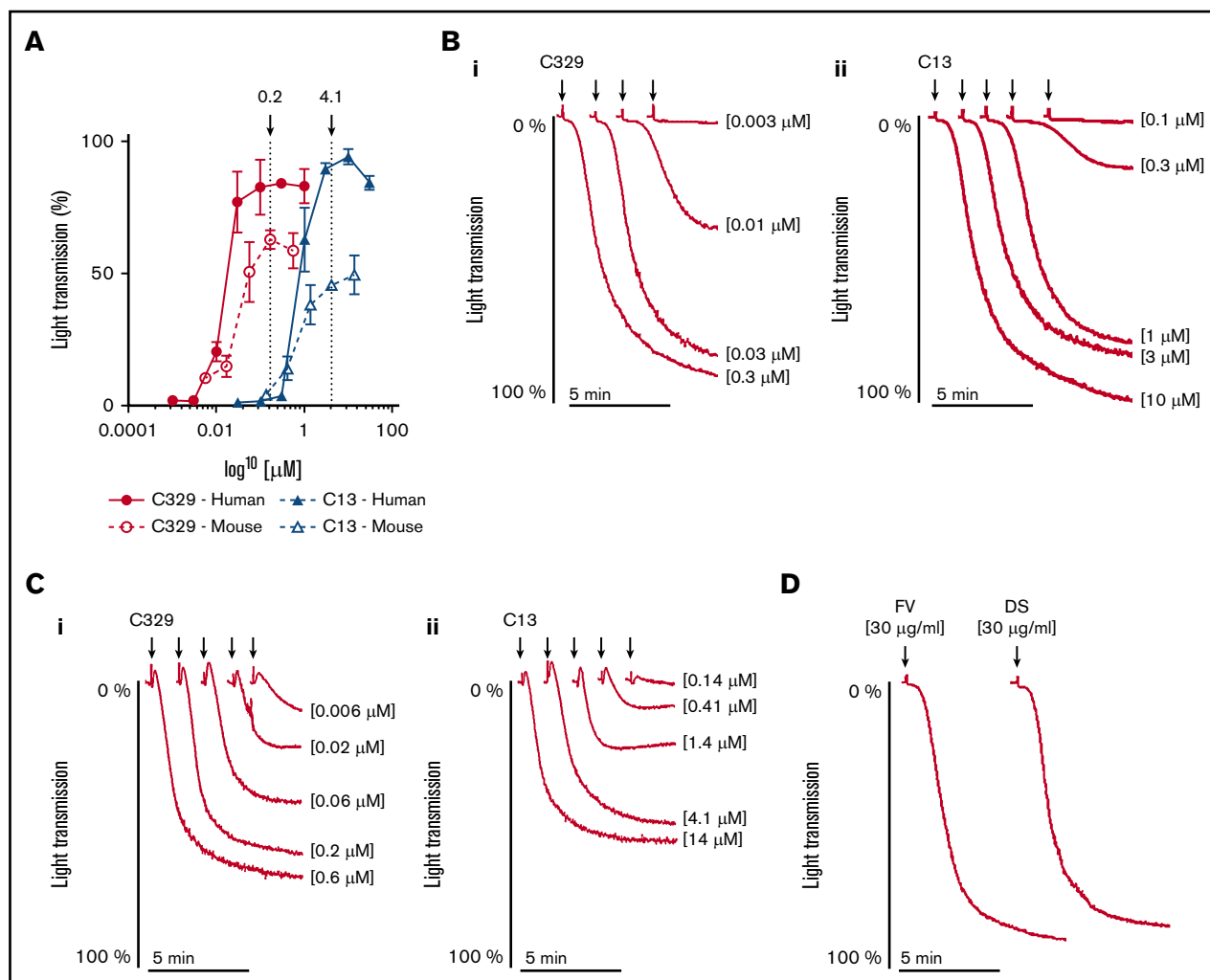
Data analysis was performed using GraphPad Prism 6. Significance was calculated using 1-way ANOVA with the Bonferroni correction for multiple comparisons.  $P < .05$  was considered significant. The results are presented as mean  $\pm$  standard error of the mean (SEM).

## Results

### Synthetic glycopolymers cause concentration-dependent aggregation of human and mouse platelets

In this study, the sulfated  $\alpha$ -L-fucoside-pendant glycopolymers C329 (average monomeric units  $n = 329$ ) and C13 (average monomeric units  $n = 13$ ) induce concentration-dependent aggregation (Figure 1). Maximal magnitude of aggregation of human and mouse platelets took place at  $\sim 0.2 \mu$ M C329 and  $\sim 4.1 \mu$ M C13, which represents a weight dose of 30  $\mu$ g/mL in both cases. Molar concentrations were calculated based on the average  $M_n$  calc, as described in supplemental Table 1. Natural fucoidan has a much larger molecular weight spread and unpredictable branching compared with the synthetic compounds<sup>6,8,13</sup> and was used at a comparable weight dose (30  $\mu$ g/mL) in this study.

The concentration-effect curves in Figure 1A reveal that C329 is more potent at causing aggregation in human (Figure 1B) and mouse (Figure 1C) platelets when compared on a molar basis. This



**Figure 1. Potency of synthetic glycopolymer-induced platelet aggregation is chain length dependent.** (A) Isolated human and mouse platelet aggregation was measured in response to increasing molar concentrations of synthetic glycopolymers C329 and C13. Maximal increase in light transmission is presented as mean  $\pm$  SEM ( $n = 3$  experiments). Original traces of C329-induced (Bi) and C13-induced (Bii) human platelet aggregation and C329-induced (Ci) and C13-induced (Cii) mouse platelet aggregation. (D) Comparison of equal weight doses (30  $\mu$ g/mL) of fucoidan from *F vesiculosus* (FV) and dextran sulfate (DS).

can be explained by the almost 30 times greater number of average monomeric units per molecule in C329 relative to C13. Platelet aggregation in mice (Figure 1C) is initiated after a prominent shape change that is not present in human platelets (Figure 1B). Figure 1D shows a comparison of aggregation in human platelets caused by 30  $\mu$ g/mL fucoidan from *F vesiculosus* and dextran sulfate. The fucoidan dose used in this study (30  $\mu$ g/mL) was selected based on dose-response experiments shown in supplemental Figure 1A. A dose of 30  $\mu$ g/mL is 10-fold higher than the lowest dose that induces maximal platelet aggregation. Fucoidan from *F vesiculosus* (30  $\mu$ g/mL) causes time-dependent intracellular phosphorylation in isolated platelets, as shown in supplemental Figure 1B.

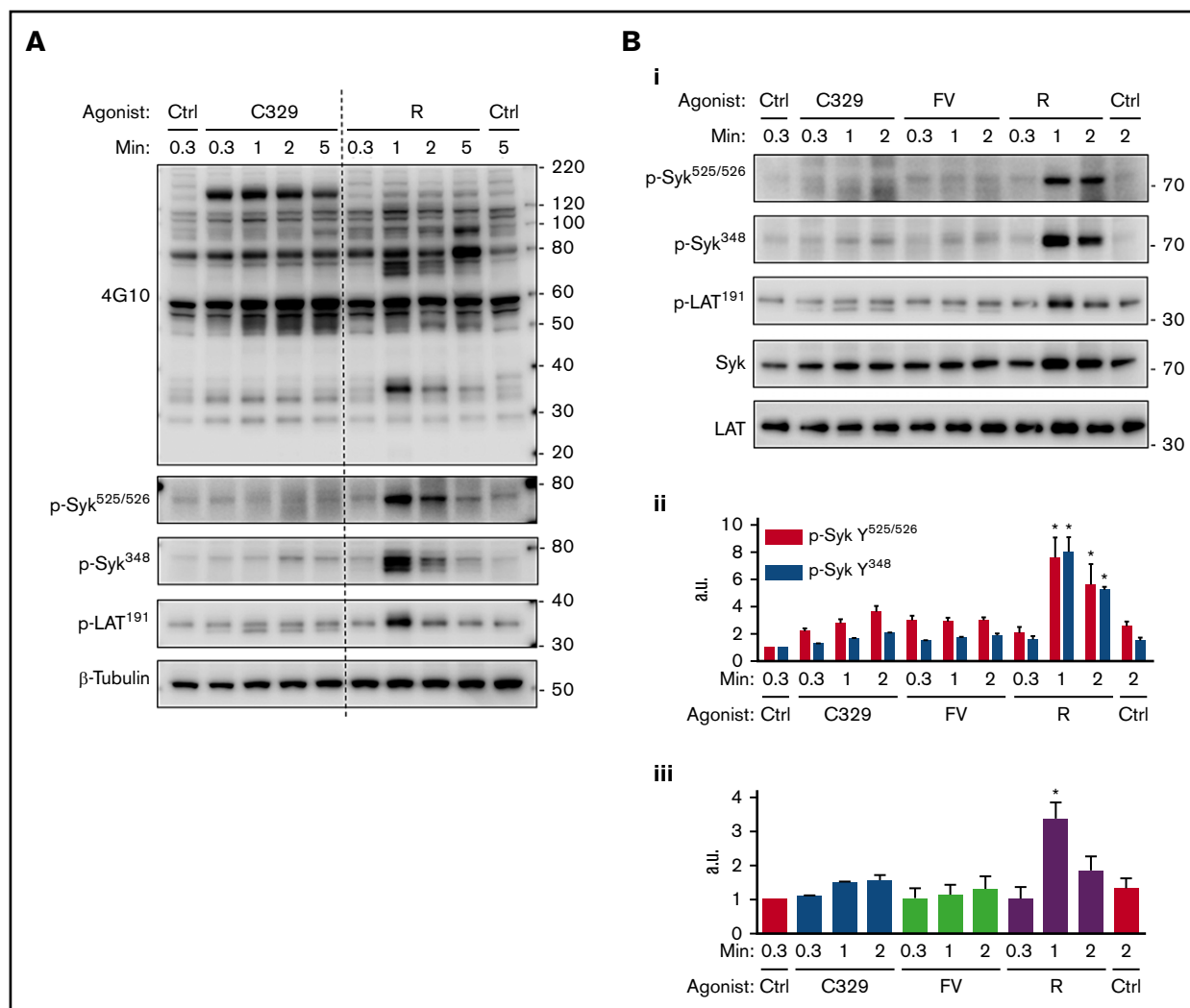
### Synthetic glycopolymers and natural fucoidan do not activate Syk in human platelets

In human platelets, the synthetic glycopolymer C329 (0.2  $\mu$ M), at a concentration that gives rise to maximal aggregation and the CLEC-2 agonist rhodocytin (50 nM), stimulates distinct patterns

and time courses of protein tyrosine phosphorylation (Figure 2A). Rhodocytin stimulated tyrosine phosphorylation of multiple proteins with a delay of 20 seconds, including prominent bands of 36, 70-80, and 120 kDa. The increase in phosphorylation was transient. In contrast, C329 stimulated a distinct pattern of tyrosine phosphorylation at 20 seconds that was sustained for up to 5 minutes, including a prominent band  $\sim$  150 kDa.

We used phosphospecific antibodies to further probe the increase in phosphorylation. Rhodocytin induced phosphorylation of Syk on tyrosines 348 and 525/526 and LAT on tyrosine 191 (Figure 2A). In contrast, neither synthetic glycopolymer C329 nor natural fucoidan stimulated a significant increase in phosphorylation of Syk on these residues (Figure 2Bi-iii). In addition, we were unable to detect an increase in tyrosine phosphorylation of CLEC-2 in human platelets in response to synthetic glycopolymers and natural fucoidans (data not shown).

These results show that the synthetic glycopolymers stimulate marked phosphorylation of a major band of 150 kDa in human platelets independent of CLEC-2.



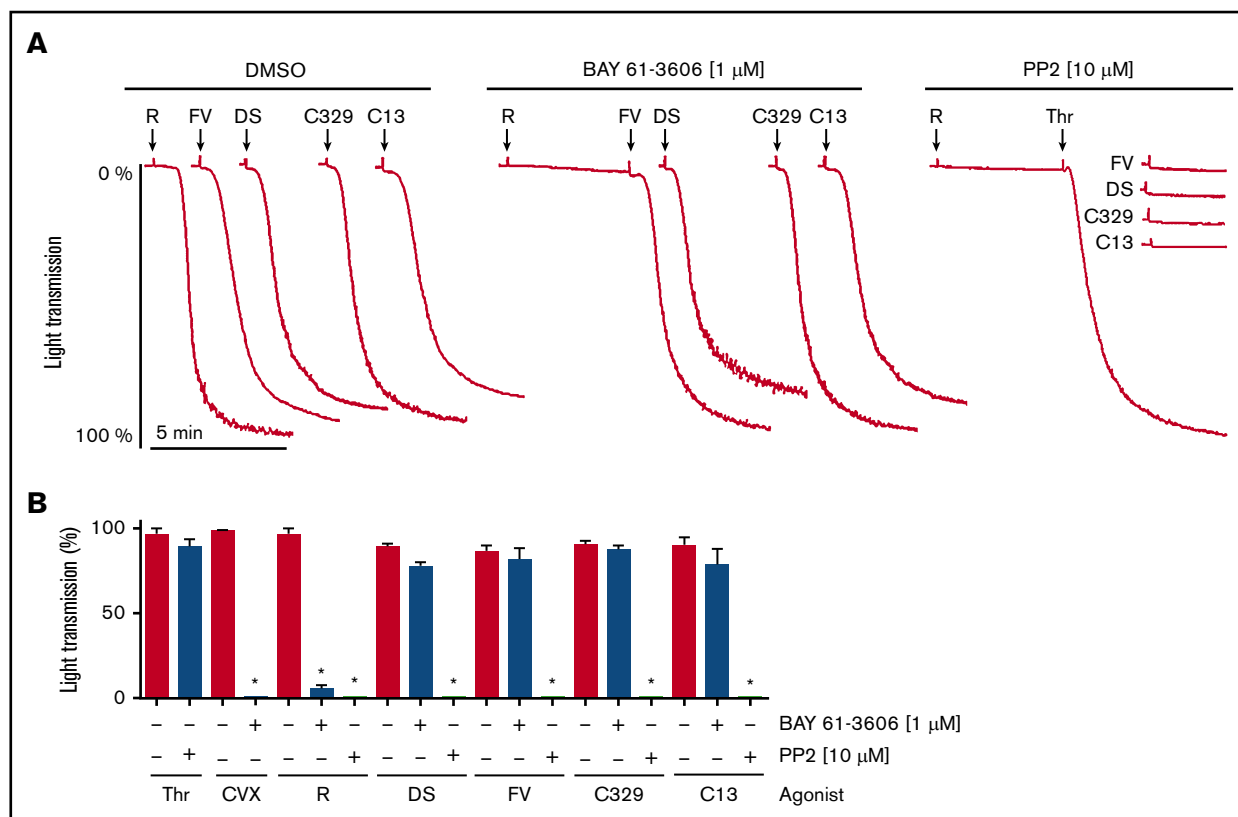
**Figure 2. Differences in human platelet tyrosine phosphorylation patterns comparing synthetic glycopolymers with rhodocytin, the snake venom agonist for CLEC-2.** (A) Isolated platelets were stimulated using the synthetic glycopolymer C329 (0.2  $\mu$ M) or the CLEC-2 agonist rhodocytin (R; 50 nM) for 0.3, 1, 2, or 5 minutes. Control (Ctrl) sample was treated with an equal volume of H<sub>2</sub>O. Samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by western blot. Membranes were stained for tyrosine phosphorylation (4G10), p-Syk Tyr<sup>525/526</sup>, p-Syk Tyr<sup>348</sup>, and LAT Tyr<sup>191</sup>. (B) Phosphorylation patterns of p-Syk Tyr<sup>525/526</sup>, p-Syk Tyr<sup>348</sup>, and LAT Tyr<sup>191</sup> were also compared for fucoidan from *F vesiculosus* (FV; 30  $\mu$ g/mL). Quantification of Syk (Bii) and LAT (Biii) phosphorylation. Graphs represent data from 3 or 4 experiments, and densitometry quantification is presented in arbitrary units (a.u.) as mean  $\pm$  SEM. Control = 1 a.u. \* $P$  < .05 vs 0.3-minute control.

### Synthetic glycopolymers and natural fucoidan activate human platelets via a Src-dependent and Syk-independent pathway

It has previously been described that signaling by CLEC-2 is dependent on Src and Syk tyrosine kinases.<sup>9,23</sup> The pan Src family kinase inhibitor PP2, its inactive analog PP3, and the Syk inhibitor BAY 61-3606 were used to investigate the role of Src and Syk tyrosine kinases in platelet activation. PP2 (10  $\mu$ M), but not its inactive analog PP3 (10  $\mu$ M) or BAY 61-3606 (1  $\mu$ M), blocked aggregation to C13, C329, fucoidan from *F vesiculosus*, and dextran sulfate, whereas BAY 61-3606 (1  $\mu$ M) blocked aggregation to rhodocytin and convulxin (Figure 3). The Src inhibitor PP2 did not block aggregation by thrombin (0.1 U/mL). Likewise, synthetic glycopolymer-induced human platelet aggregation was not

inhibited in control experiments by a second Syk inhibitor (PRT-060318, 10  $\mu$ M) (data not shown).

Platelet activation by CLEC-2 and GPVI gives rise to robust elevation of intracellular Ca<sup>2+</sup>. In contrast, the activation of platelets by dextran sulfate is associated with only a small change in intracellular Ca<sup>2+</sup>,<sup>24</sup> which was confirmed in this study (supplemental Figure 2A). Similarly, C13, C329, and fucoidan from *F vesiculosus* induced a minimal increase in intracellular Ca<sup>2+</sup> in contrast to the marked increase induced by convulxin (supplemental Figure 2A). The convulxin-induced peak rise in cytosolic Ca<sup>2+</sup> was not significantly altered by preincubation of synthetic glycopolymers or sulfated polysaccharides (supplemental Figure 2Aii), confirming platelet viability. Neither synthetic glycopolymer C329 nor natural fucoidan caused phosphorylation of PLC- $\gamma$ 2 on tyrosine



**Figure 3. Functional consequence of Syk and Src inhibition in synthetic glycopolymer- and fucoidan-stimulated human platelets.** Isolated platelets were incubated for 5 minutes with the Syk inhibitor BAY 61-3606 (1  $\mu$ M) or the Src inhibitor PP2 (10  $\mu$ M). Samples were stimulated using the CLEC-2 agonist rhodocytin (R; 50 nM), dextran sulfate (DS; 30  $\mu$ g/mL), fucoidan from *F vesiculosus* (FV; 30  $\mu$ g/mL) or the glycopolymers C329 (0.2  $\mu$ M) or C13 (4.1  $\mu$ M) and analyzed by aggregometry. The GPVI agonist convulxin (CVX; 10 ng/mL) and thrombin (Thr; 0.1 U/mL) were used as controls. (A) Original traces. (B) Quantification of maximal aggregation. The inert analog PP3 (10  $\mu$ M) was used as a control against PP2. Aggregation was measured as increases in light transmission. All samples contained <0.05% dimethyl sulfoxide. Representation of  $n = 3$  experiments. \* $P < .05$ , treatment vs control.

1217. On the other hand, rhodocytin induced prominent phosphorylation after 1 minute (supplemental Figure 2B).

These results show that the synthetic glycopolymers and natural fucoidan activate human platelets via a Src-dependent and Syk-independent pathway that is similar to that described for the sulfated glucoside polysaccharide dextran sulfate.<sup>24</sup>

### Synthetic glycopolymers and natural fucoidan stimulate PI3K/Akt signaling in human platelets

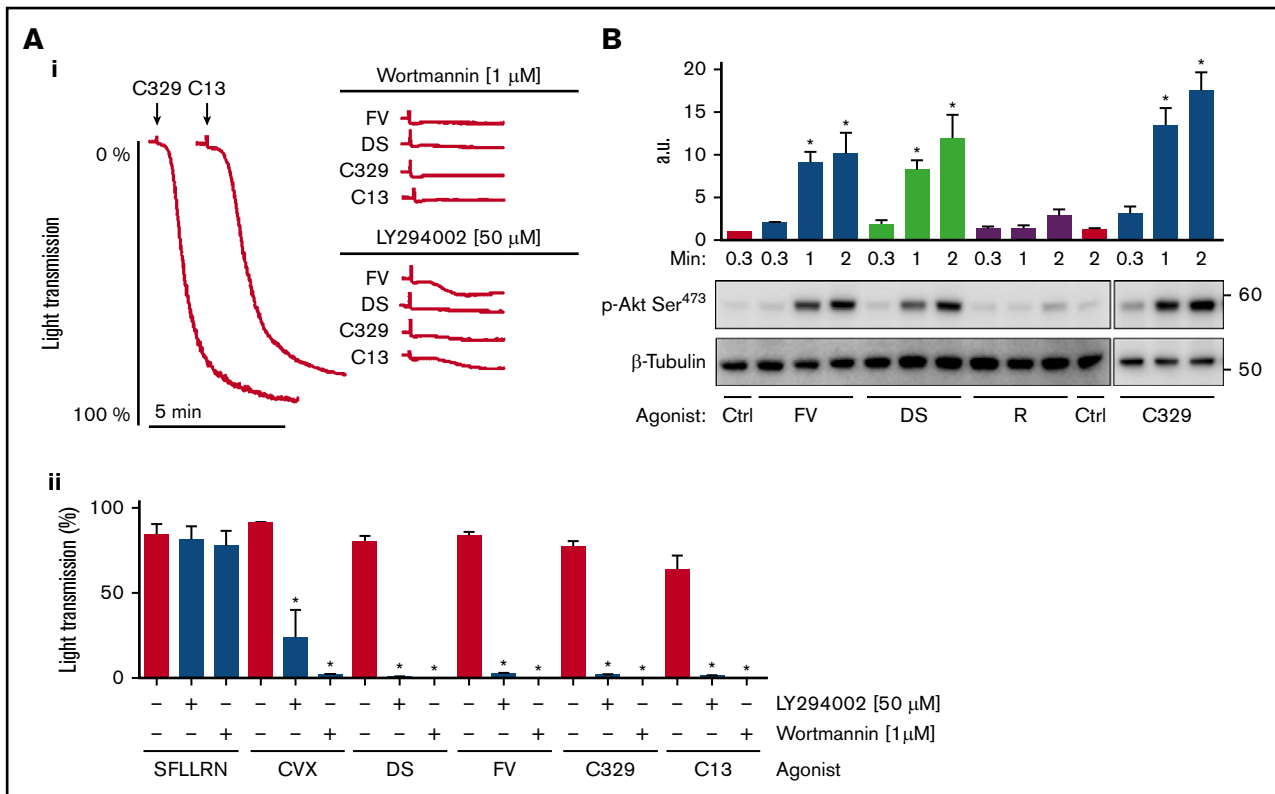
The sulfated glucoside polysaccharide dextran sulfate has been reported to stimulate aggregation of human platelets via PI3K-dependent phosphorylation of Akt,<sup>11</sup> which was confirmed in this study. Similarly, aggregation induced by synthetic glycopolymers C329 and C13 and natural fucoidan was abolished by the structurally distinct PI3K inhibitors, wortmannin and LY294002, as shown in the example traces (Figure 4Ai) and in the bar graph (Figure 4Aii). Both inhibitors also reduced the response to convulxin (Figure 4Aii), a well-described inducer of GPVI-Fc receptor  $\gamma$ -chain and Syk signaling.<sup>25,26</sup> In contrast, the maximal level of aggregation induced by the G protein-coupled PAR1 agonist peptide SFLLRN (single amino acid code) was unaltered in the presence of either inhibitor (Figure 4Aii). The selected concentrations of wortmannin (1  $\mu$ M) and LY294002 (50  $\mu$ M) used in this study abolish PI3K-induced Akt phosphorylation.<sup>27-29</sup> As shown in Figure 4B, synthetic glycopolymer

C329, natural fucoidan, and dextran sulfate, but not rhodocytin, stimulated marked Akt phosphorylation on serine 473 after 1 minute.

These results demonstrate that synthetic glycopolymers, as well as sulfated fucoside and glucoside polysaccharides, stimulate human platelet aggregation through a PI3K-dependent pathway that involves phosphorylation of Akt.

### Synthetic glycopolymers and natural fucoidans activate human platelets via PEAR1

Dextran sulfate has been reported to stimulate platelet activation via the surface glycoprotein receptor PEAR1.<sup>11</sup> Therefore, studies were designed to investigate whether PEAR1 is a receptor for synthetic glycopolymers and natural fucoidans. C13, C329, fucoidan from *F vesiculosus*, and dextran sulfate all stimulated marked phosphorylation of PEAR1, whereas neither convulxin nor rhodocytin induced phosphorylation (Figure 5A). Collagen has previously been shown to induce PEAR1 phosphorylation, but only after platelet-platelet contact has been established.<sup>30,31</sup> In contrast, in the present study we measured phosphorylation after 20 seconds, which is too early for secondary aggregation-dependent PEAR1 phosphorylation. Hence, neither rhodocytin nor convulxin triggered PEAR1 phosphorylation at 20 seconds, whereas the sulfated glycopolymers and polysaccharides did so substantially, as a result of direct PEAR1 activation. Furthermore, as shown in Figure 5B, a



**Figure 4. The PI3K/Akt signaling pathway is essential for human platelet activation by synthetic glycopolymers and natural fucoidan.** (Ai) Original traces of platelet aggregation by C329 (0.2  $\mu$ M), C13 (4.1  $\mu$ M), fucoidan from *F vesiculosus* (FV; 30  $\mu$ g/mL), and dextran sulfate (DS; 30  $\mu$ g/mL) in the presence or absence of wortmannin (1  $\mu$ M) or LY294002 (50  $\mu$ M). (Aii) Quantification of human platelet aggregation. PAR1 activating peptide SFLLRN (10  $\mu$ M) and GPVI agonist convulxin (CVX; 10 ng/mL) were used as controls. All samples contained <0.05% dimethyl sulfoxide. Maximal increase in light transmission is presented as mean  $\pm$  SEM (n = 3 or 4 experiments). \**P* < .05, PI3K inhibitors vs control. (B) Isolated platelets were stimulated using synthetic glycopolymer C329 (0.2  $\mu$ M), FV (30  $\mu$ g/mL), DS (30  $\mu$ g/mL), or the CLEC-2 agonist rhodocytin (R; 50 nM). Control (Ctrl) sample was treated with an equal volume of H<sub>2</sub>O. Reactions were stopped at 0.3, 1, or 2 minutes, and samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by western blot. Graph represents n = 3 experiments; densitometry quantification is presented in arbitrary units (a.u.) as mean  $\pm$  SEM. Control = 1 a.u. \**P* < .05, treatment vs 0.3-minute control.

mAb to PEAR1 inhibited platelet aggregation by C13, C329, *F vesiculosus* fucoidan, and dextran sulfate but had no effect on platelet aggregation to rhodocytin. In addition, preincubation with the PEAR1 mAb significantly reduced Akt phosphorylation induced by C13, C329, and *F vesiculosus* fucoidan (Figure 5C).

AVEXIS, a screening method for protein interactions, was used to investigate potential PEAR1 binding sites of synthetic sulfated glycopolymers. We have previously shown that high-affinity immunoglobulin E receptor  $\alpha$  (Fc $\epsilon$ R1 $\alpha$ ), the natural ligand to PEAR1, binds directly to epidermal-like growth factor (EGF) repeat 13 of the receptor.<sup>22</sup> We investigated whether this was also the case for sulfated glycopolymers. Binding was compared for EGF-like repeats 1 to 12, 13, and 12 to 14 of PEAR1. In Figure 5D, we show that sulfated biotinylated glycopolymers (SPs) bind to EGF-like repeats 13 and 12 to 14, but not to 1 to 12. This indicates that the PEAR1 EGF-like repeat 13 is sufficient to bind sulfated glycopolymers. The nonsulfated biotinylated glycopolymers (NSPs) followed a similar, but weaker, binding pattern. An irrelevant protein, JAM1, was used as negative-control prey.

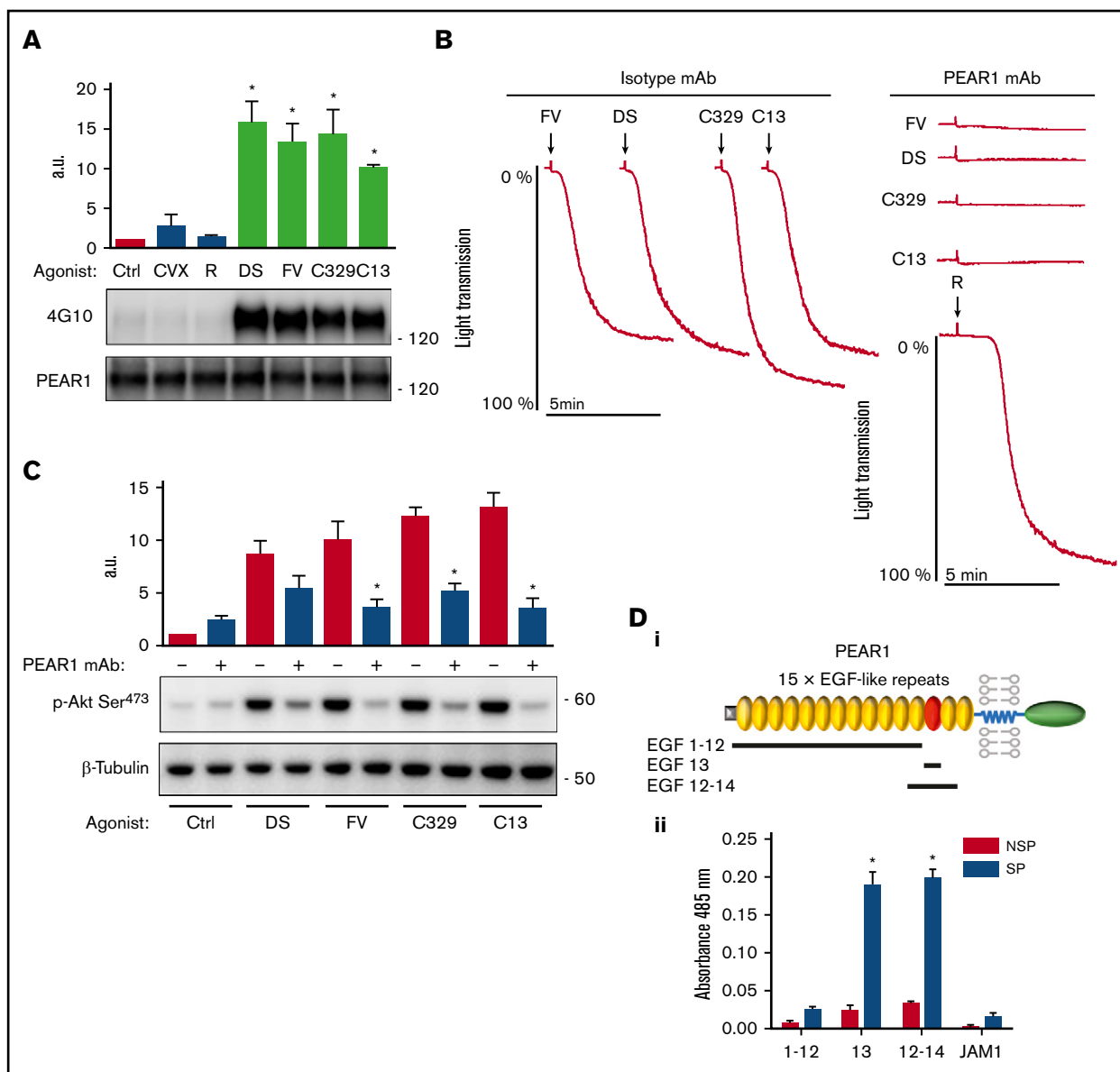
Taken together, these results confirm that synthetic sulfated  $\alpha$ -L-fucoside-pendant glycopolymers bind to and activate human platelets through PEAR1.

## PEAR1 is activated by synthetic glycopolymers in murine platelets but is not essential for aggregation

Murine platelet aggregation by dextran sulfate has been described to involve PEAR1 and CLEC-2.<sup>11</sup> In line with this, aggregation induced by synthetic glycopolymers is inhibited at lower concentrations in PEAR1-deficient mice (Figure 6A). However, higher concentrations of synthetic glycopolymers can overcome the lack of PEAR1 and induce platelet aggregation through a pathway that is blocked by the Syk inhibitor BAY 61-3606 (data not shown). This indicates that synthetic glycopolymers can activate PEAR1 in murine platelets but that the receptor is not essential for synthetic glycopolymer-induced aggregation, because the response is overcome at higher concentrations. Furthermore, the 2 synthetic glycopolymers induce PEAR1 phosphorylation in murine platelets (data not shown).

## Synthetic glycopolymer-induced platelet aggregation is inhibited in CLEC-2-deficient mice

It has been reported that fucoidan activates mouse platelets through CLEC-2 and GPVI, as shown by abolition of activation in mice deficient in both receptors.<sup>9,10</sup> At low concentrations, the response is abolished in mice deficient in CLEC-2.<sup>9</sup> Therefore, the activation of



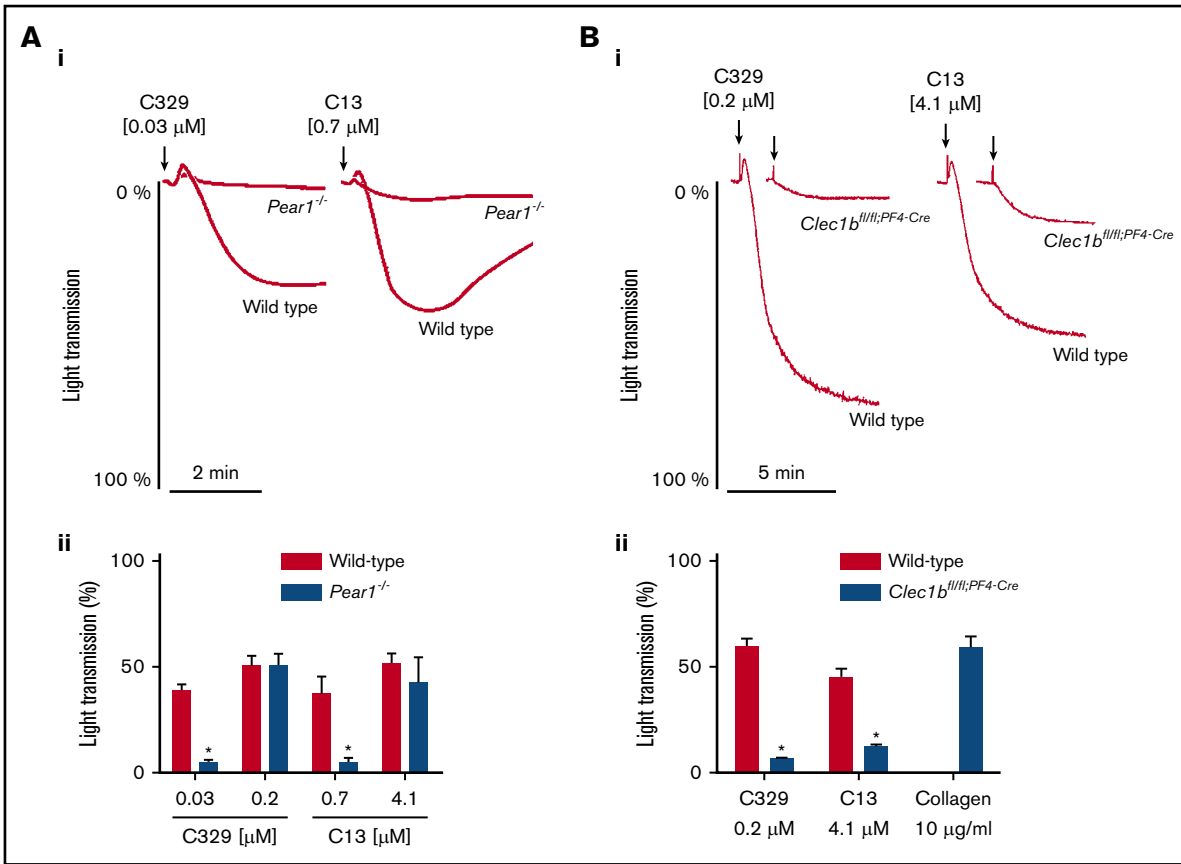
**Figure 5. Synthetic glycopolymers and sulfated polysaccharides cause human platelet aggregation via PEAR1 by direct binding to EGF-like repeats 13 and 12 to 14 of PEAR1.** (A) Aliquots of platelet suspension were stimulated with convulxin (CVX; 10 ng/mL), rhodocytin (R; 50 nM), dextran sulfate (DS; 30  $\mu$ g/mL), fucoidan from *F vesiculosus* (FV; 30  $\mu$ g/mL), C329 (0.2  $\mu$ M), or C13 (4.1  $\mu$ M) for 20 seconds, after which immunoprecipitation for PEAR1 was performed. Samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by western blot. Membranes were probed with the pan tyrosine phosphorylation antibody 4G10. Control sample was treated with an equal volume of H<sub>2</sub>O instead of agonist. \**P* < .05, treatment vs control. (B) Aggregation was measured as the increase in light transmission using isolated platelets. Isolated platelets were incubated for 2 minutes with anti-PEAR1 antibody (0.3  $\mu$ g/mL) or matching isotype control (0.3  $\mu$ g/mL) and subsequently stimulated with C329, C13, FV, or DS. R was used as control. All samples contain <0.0002% sodium azide. (C) Isolated platelets were preincubated with anti-PEAR1 antibody (0.3  $\mu$ g/mL) or corresponding isotype control (0.3  $\mu$ g/mL) for 2 minutes. Samples were stimulated with DS, FV, C329, or C13 for 1 minute. Samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by western blot. Representation of *n* = 3 experiments. \**P* < .05, isotype vs PEAR1 antibody. (D) Illustration of PEAR1 and schematic overview of the EGF-like repeats that were analyzed by AVEXIS. The red EGF-like repeat is number 13, which is sufficient to bind sulfated synthetic glycopolymers. (Dii) Detection of PEAR1–glycopolymer interaction by AVEXIS. SPs and NSPs were plate immobilized, acting as a bait for EGF-like repeats 1 to 12, 13, and 12 to 14. Absorbance for  $\beta$ -lactamase activity was measured at 485 nm. Data are mean  $\pm$  SEM (*n* = 3 experiments). \**P* < .05, SPs vs NSPs.

CLEC-2 or GPVI could explain the inhibitory effect of the Syk inhibitor BAY 61-3606 during aggregation of wild-type or *Pear1*<sup>-/-</sup> platelets.

C13 and C329 induce a prominent platelet shape change in mouse platelets (in contrast to human platelets), as indicated by the decrease in light transmission prior to the primary aggregation

phase (Figure 6). In CLEC-2–deficient mice, platelet aggregation to C13 and C329 was markedly inhibited but not abolished (Figure 6B). Platelets deficient for CLEC-2 were still responsive to collagen (10  $\mu$ g/mL), indicating platelet functionality (Figure 6Bii). The rapid shape change observed in wild type platelets was absent in





**Figure 6. Mouse platelets deficient in PEAR1 (*Pear1*<sup>-/-</sup>) still aggregate to high concentrations of synthetic glycopolymers, whereas CLEC-2-deficient platelets (*Clec1b*<sup>fl/fl</sup>;PF4-Cre) do not.** Murine platelets were analyzed for platelet aggregation upon stimulation by C329 and C13. (A) Original traces of PEAR1-deficient platelets compared with wild-type platelets stimulated with C329 (0.03 μM) and C13 (0.7 μM). (Aii) Quantification of maximal aggregation. Representation of 3 to 5 experiments. \**P* < .05, wild-type vs *Pear1*<sup>-/-</sup> mice. (B) Original traces of CLEC-2-deficient platelets compared with wild-type platelets stimulated by C329 (0.2 μM) and C13 (4.1 μM). (Bii) Quantification of maximal aggregation within 10 minutes. Collagen (10 μg/mL) was used as a control for *Clec1b*<sup>fl/fl</sup>;PF4-Cre platelet reactivity. Representation of 3 experiments. \**P* < .05, wild-type vs *Clec1b*<sup>fl/fl</sup>;PF4-Cre mice.

CLEC-2-deficient platelets (Figure 6Bi), resembling aggregation in human platelets (Figures 1B, 3A, 4Ai, and 5B). The synthetic glycopolymer C329 stimulated tyrosine phosphorylation of CLEC-2 in wild-type and *Pear1*<sup>-/-</sup> mouse platelets (data not shown).

The results show that, in contrast to human platelets, CLEC-2 (and not PEAR1) is the major signaling receptor for synthetic fucidans in mouse platelets. The observation that the response to a high concentration of the synthetic fucidans is blocked by BAY 61-3606 suggests that the CLEC-2-independent aggregation is mediated by GPVI, as previously shown,<sup>10</sup> rather than by PEAR1. The loss of response to low concentrations of the synthetic fucidans in PEAR1-deficient mice is most likely due to synergy with Syk activation or an adhesive effect.

### Synthetic glycopolymer-induced platelet aggregation is supported by GPIbα

The von Willebrand factor receptor GPIbα has previously been hypothesized as a possible binding target for the sulfated polysaccharide dextran sulfate.<sup>24</sup> In addition, it has been shown that O-linked carbohydrates are of importance in von Willebrand factor-GPIbα binding.<sup>32</sup> In the current study, we show that sulfated synthetic glycopolymers bind to the full-length extracellular domain of GPIbα,

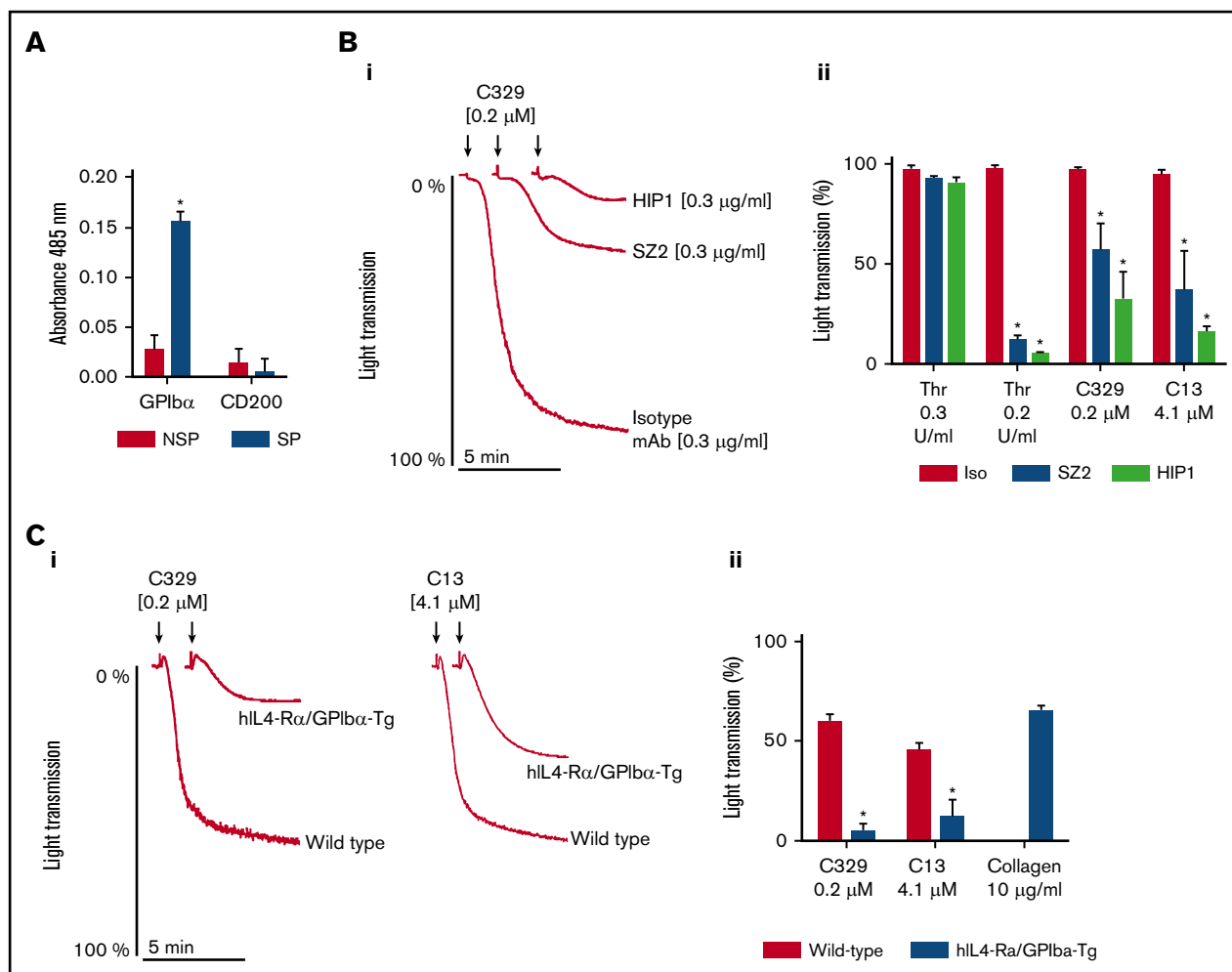
whereas nonsulfated glycopolymers do not (Figure 7A). The contribution of GPIbα to human platelet activation by synthetic glycopolymers was evaluated using GPIbα-blocking antibodies. This led to the demonstration that 2 antibody clones to GPIbα reduced, but did not block, aggregation to synthetic glycopolymers (Figure 7Bi-ii). Clone SZ2 binds the anionic region of the receptor, and HIP1 binds the leucine-rich repeat (LRR) region.<sup>33</sup> Thrombin was used as a control, because it has been shown to be inhibited, in part, by anti-GPIbα antibodies.<sup>34</sup>

The effect of glycopolymer-induced platelet aggregation was further assessed in hIL-4Rα/GPIbα-Tg mice, which lack the extracellular domain of GPIbα.<sup>18</sup> As shown in Figure 7Ci and 7Cii, platelet aggregation was significantly reduced in mice platelets without the extracellular domain of GPIbα. On the other hand, shape change occurred prior to aggregation of hIL-4Rα/GPIbα-Tg mouse platelets (Figure 7Ci-ii).

These results indicate a role for GPIbα in human and mouse platelet aggregation by synthetic glycopolymers.

### Discussion

This study shows that synthetic sulfated α-L-fucoside-pendant glycopolymers and natural highly purified fucoidan polysaccharides



**Figure 7. Synthetic glycopolymers require GPIIb/IIIa to induce full aggregation in murine and human platelets.** (A) Detection of GPIIb/IIIa-glycopolymer interaction by AVEIXIS. SPs and NSPs were plate immobilized, acting as a bait for the full-length extracellular domain of GPIIb/IIIa. CD200 was used as a negative control protein. Absorbance for  $\beta$ -lactamase activity was measured at 485 nm, presented as mean  $\pm$  SEM ( $n = 3$  experiments). \* $P < .05$ . (Bi) Human platelet aggregation. Platelet aliquots were treated with anti-GPIIb/IIIa antibody SZ2 (0.3  $\mu$ g/mL), HIP2 (0.3  $\mu$ g/mL), or isotype control (0.3  $\mu$ g/mL) for 2 minutes before stimulation by C329 or C13. (Bii) Quantification of maximal aggregation within 10 minutes. Thrombin was used as a control at 0.3 and 0.2 U/mL. Increase in light transmission is presented as mean  $\pm$  SEM ( $n = 3$  experiments). \* $P < .05$ . (Ci) Mouse platelet aggregation. Murine wild-type platelets and transgenic mouse platelets deficient in GPIIb/IIIa extracellular domain (hIL4-Ra/GPIIb/IIIa-Tg) were stimulated using synthetic glycopolymers C329 (0.2  $\mu$ M) and C13 (4.1  $\mu$ M). (Cii) Quantification of maximal increase in light transmission within 10 minutes. Collagen (10  $\mu$ g/ml) was used as a control for platelet viability. \* $P < .05$ , wild-type vs hIL4-Ra/GPIIb/IIIa-Tg mice.

activate human platelets through PEAR1. Synthetic glycopolymers and natural fucoidan of high purity from *F. vesiculosus* give rise to tyrosine phosphorylation patterns that differ significantly from those of the CLEC-2 agonist rhodocytin. Naturally occurring fucoidans from seaweed primarily consist of highly sulfated fucose polysaccharides but may also contain other types of sugars and contaminants. Even the season of harvest can affect the composition.<sup>1,7,8,12,35</sup> These factors may contribute to previously published observations showing a role for CLEC-2 in activation of human platelets by fucoidan of 80% purity from *F. vesiculosus*.<sup>9</sup>

Our data show that PEAR1 is the critical receptor causing human platelet activation by synthetic sulfated glycopolymers and natural fucoidan polysaccharides. PEAR1 is a transmembrane receptor of the EGF-like domain family. It has 15 EGF-like repeats and is highly expressed on endothelial cells and platelets. In endothelial

cells, PEAR1 is a modulator of cell proliferation during neoangiogenesis.<sup>36</sup> With respect to its role in platelets, genome-wide analysis has identified genetic variations in PEAR1 associated with altered responses during platelet aggregation by various agonists.<sup>37</sup> Certain PEAR1 gene haplotypes were strongly associated with human platelet integrin  $\alpha_{IIb}\beta_3$  activation and P-selectin expression upon GPVI stimulation, and, in various ethnicities, several PEAR1 single-nucleotide polymorphisms have been associated with increased platelet aggregation for 1 allele, coupled to a higher platelet PEAR1 expression.<sup>38-40</sup> Also in platelets, PEAR1 clustering triggers its phosphorylation via cSrc, recruitment of the p85 PI3K chain, and PI3K-mediated Akt phosphorylation.<sup>30</sup> Thus, PEAR1 facilitates platelet-platelet contact<sup>31</sup> and stabilizes platelet aggregates.<sup>30</sup> However, the absence of PEAR1 in mice did not significantly affect hemostasis, indicating

species differences.<sup>41</sup> Yet, the exact physiological role of PEAR1 in human hemostasis remains to be clarified. The synthetic glycopolymers in this study represent unique tools for further investigating the physiological role of PEAR1.

The high-affinity immunoglobulin E receptor  $\alpha$  (Fc $\epsilon$ R1 $\alpha$ ) has been identified as a natural ligand for PEAR1.<sup>22</sup> Fc $\epsilon$ R1 $\alpha$  is highly glycosylated<sup>42</sup> and, similarly to the sulfated glycopolymers, binds to EGF-like repeat 13 of PEAR1, which was shown using AVEIXIS technology. However, the significance of Fc $\epsilon$ R1 $\alpha$  glycosylation in the binding to, and activation of, PEAR1 remains to be ascertained. The full-length PEAR1 receptor has weaker binding to the sulfated glycopolymers compared with the shorter EGF-like repeats 13 and 12 to 14 (data not shown). This may be caused by steric hindrance due to the size of the full-length PEAR1 receptor, such that EGF-like repeat 13 may not be able to reach the sulfated glycopolymers.

The synthetic glycopolymers and natural fucoidan activate CLEC-2 in mouse platelets, inducing Src and Syk signaling in agreement with a previous study using an 80% pure preparation of fucoidan from *F vesiculosus*.<sup>9</sup> Dextran sulfate activates mouse platelets via PEAR1 and CLEC-2.<sup>11</sup> The sulfated synthetic glycopolymers used in this study cause murine platelet aggregation mainly via CLEC-2; however, the synthetic glycopolymers cause phosphorylation of PEAR1, but the receptor is not essential for aggregation at higher concentrations of the compounds. On the contrary, in human platelets, PEAR1 is essential for synthetic glycopolymer- and natural fucoidan-induced aggregation. The demonstration of a clear difference between man and mouse in terms of the mechanism of platelet activation may reflect the large difference in receptor density. Human platelets express ~2000 to 4000<sup>43</sup> copies of CLEC-2, whereas the mouse counterparts express ~41 000 copies.<sup>44</sup> Another difference between human and mouse platelet aggregation by synthetic glycopolymers is the lack of shape change in human platelets. Shape change is primarily regulated by increases in cytosolic calcium or activation of the G<sub>12/13</sub> family of heterotrimeric G proteins.<sup>45</sup> Hence, the lack of shape change upon synthetic glycopolymer stimulation in human platelets is most likely due to the absence of Syk/LAT/PLC- $\gamma$ 2 signaling and, subsequently, the minimal increase in cytosolic Ca<sup>2+</sup> or G protein activation (Figure 2; supplemental Figure 2).

Our study also shows that the extracellular domain of GPIb $\alpha$  is important in synthetic glycopolymer-induced aggregation of murine and human platelets. The extracellular N-terminal region of GPIb $\alpha$  contains several LRRs, which regulate binding to the von Willebrand factor A1 domain. By blocking the curved concave face of the LRRs, von Willebrand factor A1-GPIb $\alpha$  interaction can be inhibited.<sup>46</sup> Inhibiting binding to the LRR region of human GPIb $\alpha$  with the antibody clone HIP1, which binds to an epitope in the vicinity of the second LRR,<sup>47</sup> or through loss of the extracellular domain of GPIb $\alpha$  in mouse platelets reduces, but does not block, aggregation to synthetic glycopolymers. This indicates that activation of PEAR1 in human platelets or CLEC-2 in mouse platelets is potentiated by binding to GPIb $\alpha$  but is not dependent on this interaction, because the response is reduced but not blocked. This can be explained through an avidity effect in which binding of the synthetic glycopolymers to GPIb $\alpha$  facilitates the interaction with PEAR1 or CLEC-2. Binding to GPIb $\alpha$  may also potentially generate intracellular signals that synergize with those from PEAR1 or

CLEC-2; however, on their own, these are insufficient to mediate activation of human platelets, because an antibody to PEAR1 abrogates aggregation. Thus, we propose that the short and long synthetic glycopolymers used in this study induce platelet activation through clustering of GPIb $\alpha$  to PEAR1 (human) or CLEC-2 (mouse). The species difference can be explained by different levels of CLEC-2 in human and mouse platelets, as discussed above. The receptor density of PEAR1 is lower than CLEC-2 in both species. Mouse platelets express ~3100 receptor<sup>44</sup> copies of PEAR1, whereas human platelets contain ~1800 copies.<sup>43</sup> Therefore, the PEAR1 preference in humans may also be due to differences in glycopolymer-protein affinity between the 2 receptors.

We propose that electrostatic interactions mediated by binding of the negatively charged fucoidans and synthetic glycopolymers to positively charged residues in PEAR1 and CLEC-2 play a critical role in mediating receptor binding and activation. Patterns of basic amino acid (lysine, arginine, and histidine) clustered within protein sequences have been shown to affect strength and specificity of binding of glycosaminoglycans, such as heparin and heparan sulfate.<sup>48,49</sup> Our data show that an  $\alpha$ -L-fucoside-pendant glycopolymer of ~13 monosaccharide residues causes full platelet aggregation. For heparin binding to heparin cofactor II, a minimum of 13 monosaccharide residues is required,<sup>50</sup> and heparin binding of antithrombin III relies on a specific sequence of 5 monosaccharides.<sup>51</sup> Heparin also binds to positively charged amino acid regions within the heparin-binding EGF-like growth factor.<sup>52</sup> Variable lymphocyte receptors containing LRRs also recognize short trisaccharides on red blood cells.<sup>53</sup> Exactly how many fucose monosaccharide residues are required for inducing PEAR1/GPIb $\alpha$ /CLEC-2 aggregation or receptor clustering remains unexplored. The longer synthetic glycopolymer C329 can induce full platelet aggregation when used at much lower molarity than the short synthetic glycopolymer C13 (Figure 1). The length-dependent concentration-response curve shift may be attributed to the ability to cross-link multiple receptors.

In PEAR1, basic amino acids are spread throughout the EGF repeat sequence. However, EGF-like repeats 13 and 14 are the only repeats that contain 2 consecutive positively charged amino acids.<sup>31</sup> Electrostatic interactions have previously been shown to play a critical role in the activation of CLEC-2 by podoplanin. The podoplanin extracellular domain contains 3 platelet aggregation-stimulating (PLAG) domains with the sequence EDXXXT/S (single amino acid code). Sialylation of the serine or threonine is critical for platelet activation.<sup>54,55</sup> Crystallization of a glycopeptide encompassing PLAG2 and PLAG3 with CLEC-2 has identified 2 sites of electrostatic interaction between the DXXXT/S sequence and 4 arginines in CLEC-2.<sup>56</sup> Future studies using advanced computer modeling and crystallography may provide deeper insights on carbohydrate-protein interactions.

In conclusion, we propose that synthetic glycopolymers and natural fucoidan activate human platelets through cross-linking of PEAR1 and GPIb $\alpha$ , which is largely governed through an electrostatic interaction. In mouse platelets, fucoidans induce activation mainly through CLEC-2 and GPIb $\alpha$ , most likely due to the much greater level of expression compared with human platelets. Synthetic sulfated  $\alpha$ -L-fucoside-pendant glycopolymers represent novel reagents for studying the functional role of PEAR1 in platelets.

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## Authorship

Contribution: C.K., M.G., K.F., E.J.H., Y.S., and S.P.W. designed experiments; M.T. and P.K. designed and synthesized glycopolymers;

C.K., K.F., E.J.H., S.W., M.L., M.C., R.B., and Y.S. performed research; C.K., K.F., M.G., and Y.S. analyzed data; C.K., M.G., K.F., E.J.H., Y.S., S.P.W., and P.P. interpreted research results; C.K., M.G., and S.P.W. wrote the manuscript; and C.K., K.F., E.J.H., M.L., P.P., L.U.L., M.T., G.E.R., S.W., M.F.H., J.A.E., J.E., P.K., S.P.W., Y.S., and M.G. provided scientific input and reviewed the manuscript.

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