

Canonical Wnt signaling negatively regulates platelet function

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Wnts regulate important intracellular signaling events, and dysregulation of the Wnt pathway has been linked to human disease. Here, we uncover numerous Wnt canonical effectors in human platelets where Wnts, their receptors, and downstream signaling components have not been previously described. We demonstrate that the Wnt3a ligand inhibits platelet adhesion, activation, dense granule secretion, and aggregation. Wnt3a also altered platelet shape change and inhibited the activation of the small GTPase RhoA. In addition, we found the Wnt- β -catenin signaling pathway to be functional in platelets. Finally, disruption of the Wnt Frizzled 6 receptor in the mouse resulted in a hyperactive platelet phenotype and a reduced sensitivity to Wnt3a. Taken together our studies reveal a novel functional role for Wnt signaling in regulating anucleate platelet function and may provide a tractable target for future antiplatelet therapy.

Wnt- β -catenin pathway | integrin α IIb β 3 | frizzled 6 knockout mice

Anucleate platelets are the principle effectors of haemostasis and are found circulating in a nonadhesive, quiescent state. At sites of vascular damage, platelets adhere to various exposed subendothelial matrix proteins and are activated, converting from a resting, discoid shape into larger, flattened structures with extended pseudopodia (1). Such activated platelets secrete and synthesize further agonists, inflammatory mediators, and vasoactive substances and through conformational changes in their major integrin receptor, α IIb β 3, aggregate to other platelets via fibrinogen (Fb) to form a haemostatic plug (2). Aberrant platelet activation can cause pathological thrombus formation, leading to thrombosis and ultimately vessel occlusion and tissue ischemia, the processes underlying myocardial infarction and stroke. Understanding the regulation of platelet activity is thus fundamental to comprehending thrombotic disorders and developing therapeutic strategies.

The mammalian Wnt gene family is comprised of 19 secreted Wnt glycoproteins, which play essential roles in cell proliferation, cell-fate determination, and cell-fate differentiation during embryonic development and adult homeostasis (3, 4). These Wnt ligands activate a number of different signaling pathways via distinct receptors and downstream effectors to mediate effects on gene transcription and cell adhesion/migration (5, 6). For the Wnt- β -catenin (β -cat) signaling pathway (Fig. 1A), traditionally referred to as the “canonical” pathway, Wnts bind to a surface receptor complex comprised of a Frizzled (Fzd) receptor and the Lipoprotein Receptor-related Protein 5/6 (LRP5/6) coreceptor (5, 7). The signal is then transduced to the cytoplasmic protein Dishevelled (Dvl) where downstream pathways regulate the stability of β -cat (5, 7). In the absence of Wnt, β -cat is phosphorylated by a destruction complex containing Casein Kinase 1 (CK1), Glycogen Synthase Kinase 3 β (GSK3 β), Axin-1, FRAT-1, and Adenomatous Polyposis Coli (APC), which targets β -cat for degradation via ubiquitination and subsequent proteosomal degradation (8). In the presence of Wnt, Dvl negatively

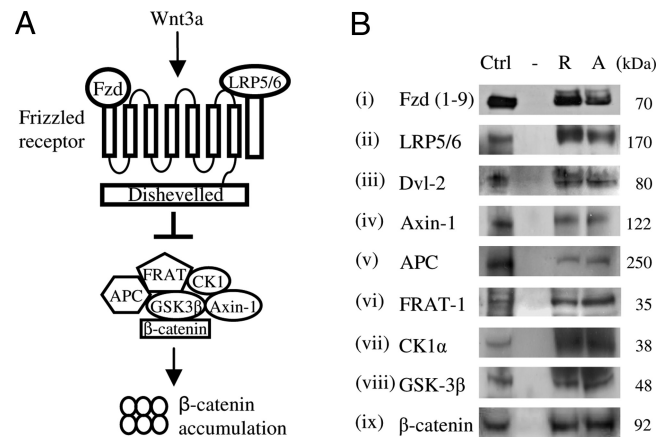


Fig. 1. The canonical Wnt- β -catenin pathway is present in platelets. (A) Wnt binds to a surface receptor complex comprising of the Fzd and LRP5/6 receptors. In the absence of Wnt, β -cat is phosphorylated by a destruction complex containing CK1, GSK3 β , Axin-1, FRAT-1, and APC, which target it for proteosomal degradation. In the presence of Wnt, β -cat is not phosphorylated and accumulates in the cytosol. Activatory signals are denoted by normal arrows, inhibitory signals by flat-headed arrows. (B) Positive control lysates (Ctrl), Resting (R) and 5 μ M TRAP-activated (A) platelets were resolved by SDS/PAGE and immunoblotted with antibodies to (i) Fzd isoforms 1–9, (ii) LRP5/6, (iii) Dvl-2, (iv) Axin-1, (v) APC, (vi) FRAT-1, (vii) CK1 α , (viii) GSK-3 β and (ix) β -cat. Representative blots are shown from 3 replicates.

regulates the phosphorylation of β -cat, preventing its degradation and leading to its cytosolic stabilization (8) (Fig. 1A).

Here, we demonstrate that components of the canonical Wnt- β -cat signaling pathway are present and functional in anucleate platelets and that the Wnt3a ligand inhibits platelet adhesion, shape change, dense granule secretion, RhoA activation, and aggregation. We also demonstrate that activation of the Wnt pathway through the Fzd6 receptor is functional in limiting platelet activation and is in part responsible for exogenous Wnt3a-mediated platelet inhibition. Our studies define a novel role for the Wnt signaling pathway in regulating platelet function.

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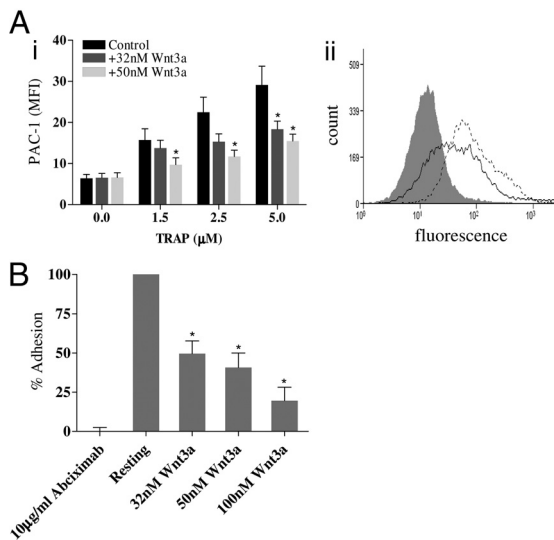


Fig. 4. Wnt3a inhibits integrin $\alpha\text{IIb}\beta_3$ activation. (A) (i) A FITC conjugated antibody for PAC-1 binding was used in flow cytometry to measure $\alpha\text{IIb}\beta_3$ activation to increasing concentrations of TRAP in the absence (black bars) and presence of 32 nM (dark gray bars) and 50 nM (light gray bars) Wnt3a. (ii) A representative histogram trace shows resting (solid gray curve) and 2.5 μM TRAP stimulated platelets in the absence (black dotted line) and presence of 50 nM Wnt3a (black solid line). (B) A static adhesion assay was performed to quantify the number of platelets adhering to Fb in the presence of increasing concentration of Wnt3a. Data are expressed as a % of the positive control (platelet-only sample, containing no Wnt3a). Abciximab (10 $\mu\text{g}/\text{ml}$) was used as a negative control. All results represented as a mean value \pm SEM from 3 independent platelet preparations (*, $P < 0.05$).

54). In control samples, precipitated Rho-GTP was found at all concentrations of thrombin (0.01–0.1U/ml) with lowest levels at 0.01U/ml and maximal levels at 0.025U/ml. In the presence of 50 nM Wnt3a, only basal levels of Rho-GTP were precipitated up to concentrations of 0.05U/ml thrombin, with low levels precipitated at 0.1U/ml. Thus, preincubation with 50 nM Wnt3a inhibited thrombin-induced platelet RhoA activation (Fig. 5A).

We further compared the effects of Wnt3a on platelet shape change to the calcium chelator (BAPTA-AM), which inhibits the “early” calcium-dependent shape change response and also to a

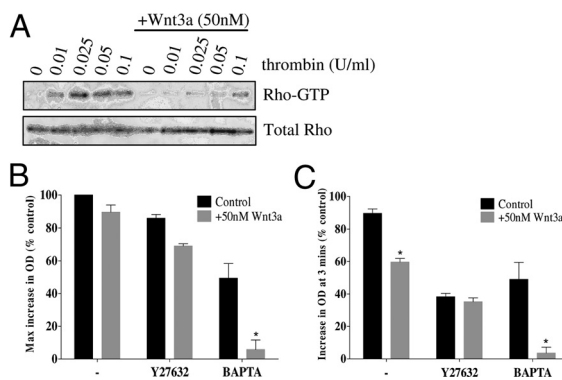


Fig. 5. Wnt3a inhibits RhoA activation and the rho-kinase dependant pathway of platelet shape change. (A) Inhibition of RhoA activation when 50 nM Wnt3a was added to platelets before thrombin activation (0–0.1U/ml). Anti-RhoA blot shows total RhoA in lysed samples. 5 μM ADP-induced platelet shape change in response to a variety of conditions was monitored by Optical Density (OD) in a ChronoLog aggregometer by measuring (B) maximum % increase in OD and (C) % increase in OD 3 min after stimulation, both versus vehicle-treated only. Data shown are representative of 3 separate donors and are presented as mean \pm SEM (*, $P < 0.05$).

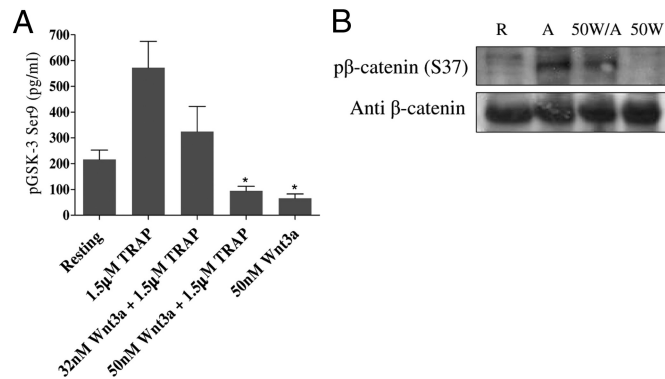


Fig. 6. Modification of Wnt canonical pathway events in platelets. (A) A phospho-GSK3 β (S9) ELISA was carried out using resting and 1.5 μM TRAP-activated washed platelet samples in the presence/absence of Wnt3a. The phospho-GSK3 concentration (pg/ml) of each sample is represented as a mean value \pm SEM from at least 3 separate platelet preparations. (B) Resting (R) and 1.5 μM TRAP-activated (A) platelet lysates, pretreated with 50 nM Wnt3a (50W and 50W/A respectively) were immunoblotted for phosphorylated (S37) β -cat. Blots were stripped and reprobed with anti- β -cat to demonstrate equal loading. Data shown are representative of 3 replicate experiments.

Rho kinase inhibitor (Y27632) which inhibits the “sustained” Rho-kinase-dependent response. Wnt3a 50 nM had little effect on the extent of platelet shape change induced by 5 μM ADP, but when Wnt3a was combined with BAPTA, shape change to ADP was blocked (Fig. 5B). Although Wnt3a did not influence the maximum platelet shape change, the response was less sustained at 3 min an effect that was also seen with Y27632 alone (Fig. 5C). Furthermore, when Wnt3a was combined with BAPTA, there was essentially no optical shape change to sustain (Fig. 5C). These results are consistent with Wnt3a inhibiting the Rho-kinase-dependent component of ADP-induced platelet shape change. Similar results were found for TRAP-induced shape change although as TRAP induces greater intracellular calcium mobilization, Y27632 had less of an inhibitory effect (Fig. S2 D and E and SI Text).

Modification of Canonical Wnt Signaling Events in Platelets. To demonstrate that Wnt/ β -cat signaling is active in platelets, we assessed the effects of Wnt3a on the phosphorylation status of two pivotal downstream effectors, GSK3 β and β -cat. Using an ELISA plate assay specific for the phospho-Ser 9 (S9) residue of GSK3 β (Fig. 6A), we found that GSK3 β was phosphorylated on S9 in resting platelets and phosphorylated S9 levels increased upon TRAP (1.5 μM) activation, as reported previously (10, 11). Wnt3a 32 nM decreased TRAP-induced phosphorylated S9 levels, while pretreatment with 50 nM Wnt3a reduced the levels of phosphorylated GSK3 β below baseline in both resting and TRAP-activated platelets. The effect seen is consistent with studies showing absence of GSK3 β phosphorylation at S9 upon Wnt signaling (12, 13).

Activation of the Wnt/ β -cat pathway leads to a decreased β -cat Ser-37 (S37) phosphorylation (8, 14). S37 phosphorylation of β -cat was slightly evident in resting (R) platelets (Fig. 6B) and these low levels were confirmed using Rho-GTP negative resting platelets (as in Fig. 5A). β -cat S37 phosphorylation increased upon TRAP (1.5 μM) activation (A) and this was attenuated by 50 nM Wnt3a (50W/A). 50 nM Wnt3a (50W) also abolished S37 phosphorylation in resting platelets.

Wnt3a Induced No Significant Change in the Extent of Ca^{2+} Signaling. Wnt signaling via the Wnt/ Ca^{2+} pathway results in a rise in intracellular Ca^{2+} release and subsequent activation of calcium calmodulin-dependent kinase 2 (CamK2) and protein kinase C (PKC) (15). We found no change in the extent of Ca^{2+} signaling

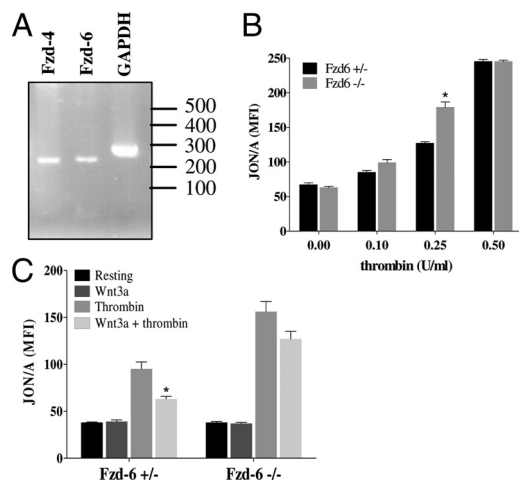


Fig. 7. *Fzd4* and *6* are present in platelets and *Fzd6*^{-/-} platelets have an increased activatory phenotype. (A) RT-PCR of platelet RNA using primers specific for *Fzd4*, *6* and *GAPDH* demonstrated their expression in platelets (predicted product sizes 211, 222, and 288 bp, respectively). (B) *Fzd6*^{-/-} mice have increased platelet activation. Platelets were isolated from *Fzd6*^{+/-} and *Fzd6*^{-/-} mice and activated with thrombin ($n = 4 \pm \text{SD}$; $P < 0.01$ vs. *Fzd6*^{+/-}). (C) Wnt3a does not antagonize *Fzd6*^{-/-} platelet activation. *Fzd6*^{+/-} (Left) and *Fzd6*^{-/-} (Right) platelets were incubated in the absence/presence of Wnt3a and stimulated with thrombin (0.25 U/ml).

at TRAP concentrations that were otherwise affected by Wnt3a (i.e., up to 5 μM TRAP), suggesting that the Wnt3a-mediated platelet effects were likely independent of altered Ca^{2+} (Fig. S2F and SI Text).

Fzd6 Is Present and Functional in Platelets. A recent megakaryocyte (MK) transcriptome study (16) reported that *Fzd4* and *6* were the most abundantly expressed *Fzd* isoforms in MKs, suggesting that these may also be present in platelets. Indeed, we detected *Fzd4* and *6* transcripts (Fig. 7A) and the *Fzd6* protein in platelets (Fig. S3A).

To further examine the functional role of Wnt signaling in platelets, we isolated washed platelets from *Fzd6*^{-/-} and *Fzd6*^{+/-} mice, and measured $\alpha\text{IIb}\beta_3$ activation to thrombin using JON/A antibody binding. *Fzd6*^{+/-} and *Fzd6*^{-/-} platelets had similar JON/A binding at low and high concentrations of thrombin stimulation (Fig. 7B). However, *Fzd6*^{-/-} platelets demonstrated significantly higher JON/A binding at moderate agonist stimulation (Fig. 7B, 0.25U/ml thrombin). Wnt3a reduced platelet activation in *Fzd6*^{+/-} (over 30%), ($P = 0.016$) in response to 0.25U/ml thrombin (Fig. 7C, Left) but had no significant effect on platelets from *Fzd6*^{-/-} mice ($P = 0.073$) (Fig. 7C, Right). Taken together these data demonstrate that *Fzd6* signaling blunts platelet activation and is responsible in part for Wnt-mediated inhibition of the platelet response to thrombin. However, as the response to Wnt3a was not abolished, other *Fzd* receptors may also be implicated.

Discussion

Our study shows the platelet expression of Frizzled receptors (including *Fzd4* and *6*), *Dvl-2*, *LRP5/6*, *Axin-1*, *Frat-1*, *CK1 α* , *GSK3 β* , and $\beta\text{-cat}$ and that Wnt3 and Soggy-1 are released upon platelet activation. In nucleated cells, the canonical Wnt pathway is widely regarded as a key regulator of gene expression through the stabilization and subsequent nuclear translocation of $\beta\text{-cat}$, which operates as a transcriptional coregulator (5, 7). The presence of such canonical Wnt signaling components in the anucleate and transcriptionally silent platelet was somewhat surprising. Yet our studies suggest a functional role for canonical Wnt signaling on platelet activity based on an array of functional

assays of platelet activation, secretion, aggregation, $\alpha\text{IIb}\beta_3$ activation, and adhesion.

We demonstrated that the Wnt ligand, Wnt3a, has a dose-dependent inhibitory effect (IC_{50} :32 nM) on human platelet aggregation in response to a variety of platelet agonists, suggesting that Wnt3a is having a general effect on platelet signaling, not limited to a specific surface receptor. There was no effect on calcium influx or α -granule secretion, but we did observe a decrease in dense granule ATP release in response to agonist stimulation, which may attenuate the secondary wave of platelet aggregation.

Furthermore, Wnt3a, which lacks an RGD sequence, modulated the activation of the major platelet integrin receptor $\alpha\text{IIb}\beta_3$ measured as the extent of Fb binding; the conformational change in the receptor that occurs with activation, which we detected as expression as the PAC-1 binding site; and the phosphorylation status of the intracellular tail of integrin β_3 subunit. Pretreatment with Wnt3a, therefore, inhibits the agonist-dependent transition of this integrin from a low- to a high-affinity state and thus reduces the extent of Fb binding across the platelet surface and subsequent platelet aggregation. A reduced static adhesion to Fb without prior agonist stimulation was also found as reported previously in platelets for certain other inhibitors, for example the Syk inhibitor piceannatol (17). Furthermore, although modulation of integrin activation is not a described response to canonical Wnt signaling, this was further confirmed in platelets lacking the *Fzd6* receptor, where $\alpha\text{IIb}\beta_3$ activation by thrombin was significantly attenuated.

Wnt3a also inhibited thrombin-induced RhoA activation and blocked ADP- and TRAP-induced Rho-kinase-dependent shape change. RhoA is a small GTPase, upstream of Rho-kinase, which plays an important role in cytoskeleton reorganization, $\alpha\text{IIb}\beta_3$ activation, and granule exocytosis and can do so in a calcium-independent manner (18). Interestingly, the noncanonical Wnt signaling pathway modulates cell migration via activation of the small G proteins RhoA and Rac (6, 19). Therefore, inactivation of RhoA could be due to Wnt3a-mediated cross-suppression of the noncanonical Wnt pathway (20, 21).

As platelets are anucleate and devoid of transcription, they do not lend themselves to analysis using reporter gene assays for canonical Wnt signaling. Furthermore, the time scale for platelet activation, and the observed inhibitory effect of Wnt (0–60 seconds), is relatively brief when compared to Wnt responses in nucleated cells. On the other hand, platelets have an extensive and dynamic phosphoproteome, which is central to the rapid reactivity of these cells and we found that Wnt3a induces a global reduction in tyrosine phosphorylation (Fig. S3B). Phosphorylation of two key components of the Wnt signaling pathway, *GSK3 β* and $\beta\text{-cat}$ was specifically assessed. *GSK3 β* is a negative regulator of platelet function (11). Pretreatment with Wnt3a in both resting and TRAP-activated platelets significantly inhibited the S9 phosphorylation of *GSK3 β* which is in agreement with other Wnt signaling studies (12, 13) and suggests that Wnt3a may influence the Akt-dependent platelet pathway leading to the phosphorylation of *GSK3 β* (S9) (11).

In the absence of Wnt, *GSK3 β* phosphorylates $\beta\text{-cat}$ at multiple sites including Ser-33 and S37 (8, 22), targeting this protein for degradation. In the presence of Wnt, the *GSK3 β* -associated destruction complex is inhibited, allowing cytosolic accumulation of $\beta\text{-cat}$ and its subsequent nuclear translocation (8). In the presence of Wnt3a, the phosphorylation of platelet $\beta\text{-cat}$ at S37 was inhibited even in resting platelets. However, as platelets are anucleate, unphosphorylated and thus stabilized $\beta\text{-catenin}$ cannot have a nuclear transcriptional role. Other roles, however, have been described. For example, studies in human monocytes and THP-1 monocyte cells show that Wnt3a activation of the canonical Wnt signaling pathway did not result in TCF-promoter activity or the expression of classical Wnt target genes (23, 24). Yet, induction of cytoplasmic $\beta\text{-cat}$ upon exposure of human monocytes to Wnt3a

resulted in a decrease in direct monocyte adherence to endothelial cells and subsequent transendothelial migration (24). A key role for cytosolic β -cat is to link adhesion receptors of the cadherin family to the actin cytoskeleton (25, 26). While adhesion through cadherins has not been observed in platelets, cadherins are expressed (9), and we find a number of cadherin/cadherin-like proteins present in our in-house human platelet proteomics datasets, which we are currently investigating.

In summary, we have demonstrated the expression of the major components of the canonical Wnt- β -cat signaling pathway in anucleate platelets and suppression of platelet activity by its ligand, Wnt3a. The Wnt3a effect on platelets is mediated in part through the Fzd6 receptor, suggesting that other Wnt signaling receptor pathways are probably involved. Our research, combined with other findings that include Wnt agonists found in plasma (27) and the Wnt pathway antagonist Dkk-1 present in and released from platelet α -granules (28), uncovers a new pathway of platelet regulation. Moreover, there is already evidence that circulating Wnt ligands levels may be modified in disease states (28, 29). There are many potential sources of circulating Wnt ligands including activated platelets, endothelial cells or possibly other circulating cells (30, 31). Further studies will be necessary to understand how the interplay of various Wnt ligands can modulate platelet function but one could hypothesize based on our genetic studies that Wnt agonists acting through Fzd6 may dampen platelet reactivity, akin to other endogenous regulators such as prostacyclin and nitric oxide. The canonical Wnt signaling pathway may thus represent a novel endogenous mechanism for regulating platelet activity.

Materials and Methods

Platelet Isolation. Human platelets were obtained from adult volunteers in accordance with approved guidelines from the University College Dublin research ethics committee. All animal procedures/protocols were approved by The John Hopkins Animal Care and Use Committee. Fzd6^{-/-} mice and Fzd6^{+/-} littermate controls were kindly provided by Dr. Jeremy Nathans and Dr. Yanshu Wang (The Johns Hopkins University) (32). Isolation of human and murine platelets was performed as described (33, 34). Platelet releasate was prepared as before (35).

Platelet Aggregation/ATP Release. Aggregation was measured using a Chrono-log 700 luminescent aggregometer (Chrono-log Co.). Aggregation experiments were carried out as described (35) with recombinant Wnt3a/Wnt5a (R&D Systems) added 30 seconds before agonist loading. For measurement of ATP release, 25 μ l of Chrono-Lume reagent was added before agonist loading and the maximum ATP release calculated according to the manufacturers' instructions.

SDS PAGE and Immunoblotting. Equal amounts of protein were analyzed by Western blotting and visualized as described (35). Antibodies used were as follows: Santa Cruz: Dvl-2, Fzd1-9, Wnt-3, LRP5/6, CK1 α , APC, RhoA, Fzd6; Abcam: Anti GSK-3 β , FRAT-1, Soggy-1; R&D Systems: Anti Axin-1, phospho- β -Catenin (S33/S37), phospho-GSK-3 (S9/S21), Anti β -Catenin; Invitrogen: Phos-

pho-Integrin β_3 (Y785); Millipore: 4G10. Lysates used as positive controls were as per manufacturers' instructions.

Flow Cytometry. Washed human platelets (1×10^8 /ml) were analyzed using a CyanADP flow cytometer by acquiring 15000 events of the gated platelet population (36). Isolated mouse platelets were prepared as described previously (34). All data are recorded as median fluorescent intensity (MFI) alongside representative histogram traces for each experiment. Antibodies used: BD Bioscience: conjugated FITC/PAC-1 and PE mouse antihuman CD62P; Dako: rabbit antihuman Fb-FITC; Emfret Analytix: JON/A antibody.

Static Platelet Adhesion Assay. Static adhesion was measured as described (37); 2×10^8 /ml gel filtered platelets were adhered to static fibrinogen (Fb) and lysed with Buffer A containing a substrate for acid phosphatases (0.1M sodium acetate, 0.1% Triton-X-100 and 10 mM *p*-nitrophenol phosphate). The reaction was stopped on addition of 1M NaOH and read at 405 nm in a plate reader (EL808, Bio-Tek Instruments).

Shape Change Assay. Indomethacin-treated (10 μ M) washed platelets (2×10^8 /ml) were treated with the Rho kinase inhibitor, Y27632 (10 μ M) (Sigma), and/or the intracellular Ca²⁺ chelator, BAPTA-AM (20 μ M) (Sigma), or vehicle control, for 10 min as before (38). Wnt3a (50 nM) or its carrier was added for 1 min before platelet stimulation with 5 μ M ADP or 1.5 μ M TRAP in a Chrono-log aggregometer under stirring in the presence of 1 mM EGTA. Optical shape change was recorded as the increase in optical density (OD)/height above basal. The maximum increase in OD and the increase in OD 3min after stimulation were measured and expressed as a percentage of control (vehicle-treated) platelets.

RhoA Activation Assay. Washed platelets (1×10^9 /ml) preincubated with 50 nM Wnt3a (30 seconds) were activated with thrombin for 1 min. Activation was terminated by addition of 500 μ l ice cold 2x lysis buffer (1% Nonidet P-40, 10% glycerol, 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 2.5 mM MgCl₂, 1 mM PMSF, 1X protease inhibitors, 1X phosphatase inhibitors). 20 μ l supernatant was kept for analysis of total Rho expression and Rho-GTP was precipitated using Rhotekin-RBD protein agarose beads (Cytoskeleton Inc.) according to the manufacturer's instructions. Total RhoA and precipitated Rho-GTP was measured by immunoblotting with anti-RhoA (Santa Cruz).

Measurement of GSK3 β Phosphorylation. Washed platelet samples, stimulated under described conditions, were prepared in an aggregometer; 3×10^8 plts/ml were lysed as above and the phospho-GSK3 β (S9) levels determined by ELISA (R&D Systems) in accordance with the manufacturer's instructions.

For additional methodology see *SI Text*.

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