Supporting Information

Steele et al. 10.1073/pnas.0906268106

SI Methods

MTT Toxicity Assay for Cell Viability. Washed platelets (3×10^8 plts/ml) were added to a 96-well plate along with varying concentrations of recombinant Wnt3a or Wnt5a; 10 μl of a 12 mM stock MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) was added and the samples incubated for 4 h at 37 °C. The reaction was stopped upon addition of 50 μl DMSO. Sample color was then quantified by reading the optical density (OD) at 540 nm using a multiwell scanning spectrophotometer (ELISA plate reader).

Calcium Flux Measurement. Platelet-rich plasma was loaded with the fluorescent dye Fura-2 AM (3 μM, 45 min). Washed platelets were then prepared as described (1×10^8 plts/ml). Changes in fluorescence emission at 510 nm following agonist stimulation were measured in the presence of various concentrations of Wnt3a using excitation wavelengths of 340 nm and 380 nm. The ratio of emission at 340 and 380 nm excitation was calibrated in terms of $[Ca^{2+}]$i using the method of Grynkiewicz et al. (1). The baseline-subtracted "area under the curve" (AUC) was calculated as a measure of the extent of intraplatelet Ca^{2+} signaling.

RT-PCR. RT-PCR was carried out on purified platelet RNA as previously described (2) using primers specific for *Fzd4*, *Fzd6* and *GAPDH* (see Table S1).

Statistical Analysis. Statistical analysis was performed using GraphPad Prism and Microsoft Excel software. All data are presented as mean value ± SEM from 3 or more independent experiments. Statistical comparisons between different treatment groups were conducted using the Student's Unpaired T-test. Differences in *p*-values of less than 0.05 were considered statistically significant.

SI Results

Wnt3a Inhibits Soluble Fibrinogen (Fb) Binding. Using flow cytometry, we examined Wnt3a effects on soluble Fb binding (Fig. S2*A*). Platelets were activated with TRAP at concentrations of 1.5, 2.5 and 5 μM in the absence (black bars) or presence of 32 nM (dark gray bars) or 50 nM (light gray bars) Wnt3a; 32 nM Wnt3a decreased Fb binding although this was only found to be significant at 1.5 μM TRAP ($P = 0.049$). At 50 nM Wnt3a, however, Fb binding to all concentrations of TRAP-stimulated platelets was significantly inhibited (1.5 μM ($P = 0.007$), 2.5 μM, ($P = 0.034$) and 5 μM TRAP ($P = 0.015$)) (Fig. S2*A*).

Wnt3a Induced No Significant Change in the Extent of Ca^{2+} Signaling. The Wnt/Ca^{2+} pathway, known to influence cell adhesion and cell movements during embryonic gastrulation signals through transmembrane Fzd receptors resulting in the release of intracellular Ca^{2+}, and subsequent activation of calcium calmodulin-dependent kinase 2 (CamK2) and protein kinase C (PKC), which play important roles in platelet activation and cell adhesion (3, 4). To examine whether the inhibitory effects of Wnt3a on platelet function may be also mediated by this Wnt/Ca^{2+} pathway, we measured Ca^{2+} flux in platelets after Wnt3a stimulation. We found no significant change in the extent of Ca^{2+} signaling (Fig. S2*F*), at TRAP (SFLLRN) concentrations that were otherwise affected by Wnt3a (i.e., up to 5 μM TRAP), suggesting that altered Ca^{2+} signaling is unlikely to mediate the effects of Wnt3a that we have described above. At much higher agonist concentrations (i.e., 10–30 μM TRAP), there was a significant inhibition of the extent of TRAP-induced Ca^{2+} signaling, which may be secondary to reduced granule secretion. Furthermore, Wnt3a did not itself induce a Ca^{2+} transient suggesting that Wnt3a does not activate the Wnt/Ca^{2+} pathway in platelets to account for our observed effects.

Wnt3a Inhibits Rho-Kinase Dependant TRAP-Induced Platelet Shape Change. 1.5 μM TRAP-induced platelet shape change (Fig. S2 *D* and *E*) was measured by optical density in a Chronolog aggregometer. Combined inhibition of the early calcium-dependent and the sustained Rho-kinase-dependent pathways (Y27632 & BAPTA-AM) completely blocks shape change, indicating that the underlying signaling involved in TRAP-induced platelet shape change is similar to ADP-induced shape change (Fig. 5 *B* and *C*). Y27632 had little effect on the maximum height unless the platelets were also treated with BAPTA; 50 nM Wnt3a alone has a similar (lack of) effect to Y27632, consistent with an inhibitory effect on the Rho/Rho kinase pathway. Furthermore, 50 nM Wnt3a alone had no significant effect on the extent to which shape change is sustained (Fig. S2*E*), possibly due to a more sustained Ca^{2+} signaling with TRAP when compared to ADP. However, when increases in $[Ca^{2+}]_i$ were blocked with BAPTA, Wnt3a significantly reduced TRAP-induced platelet shape change (Fig. S2 *D* and *E*).

1. Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440–3450.
2. Macaulay IC, et al. (2007) Comparative gene expression profiling of in vitro differentiated megakaryocytes and erythroblasts identifies novel activatory and inhibitory platelet membrane proteins. *Blood* 109:3260–3269.
3. Kohn AD, Moon RT (2005) Wnt and calcium signaling: beta-catenin-independent pathways. *Cell Calcium* 38:439–446.
4. Sheldahl LC, et al. (2003) Dishevelled activates Ca2+ flux, PKC, and CamKII in vertebrate embryos. *J Cell Biol* 161:769–777.

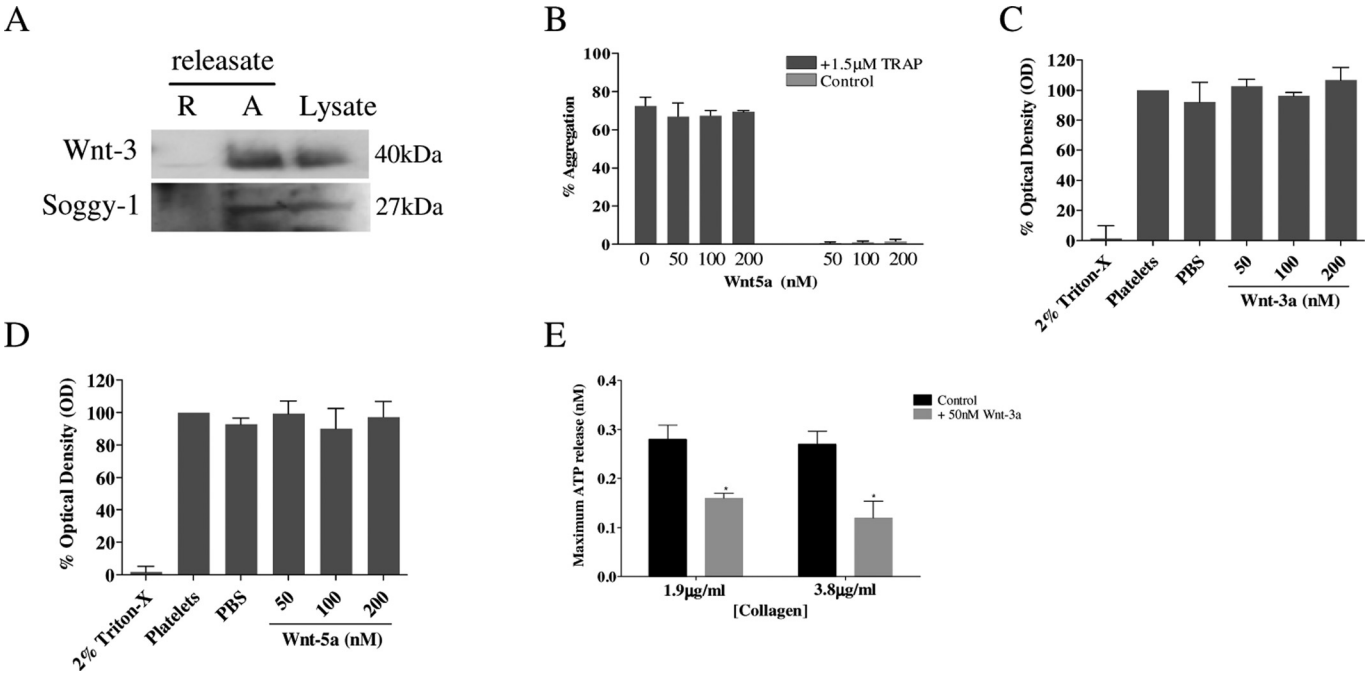

Fig. S1. (*A*) Resting (R) and 0.6U/ml Thrombin activated (A) platelet releasates were immunoblotted with antibodies against the Wnt agonist, Wnt-3 and the putative Wnt antagonist, Soggy-1. Resting whole platelet lysate (Lysate) was used to confirm the presence of these proteins in human platelets. (*B*) Wnt5a (up to [200 nM]) had no effect on control (resting platelets under constant stirring) (*Right*) or 1.5 μM TRAP-induced (*Left*) platelet aggregation. (*C* and *D*) MTT toxicity assays were carried out using washed platelets to ascertain that recombinant (*C*) Wnt3a and (*D*) Wnt5a proteins were not toxic to human platelets at concentrations of up to 200 nM. (*E*) Using a luminescent Chronolog-700 aggregometer, ATP secretion was measured in response to 1.9 μg/ml and 3.8 μg/ml collagen in the absence (black bars) and presence (gray bars) of 50 nM Wnt3a. All data are representative of 3 independent experiments and are presented as mean $\pm$ SEM (*, $P < 0.05$).

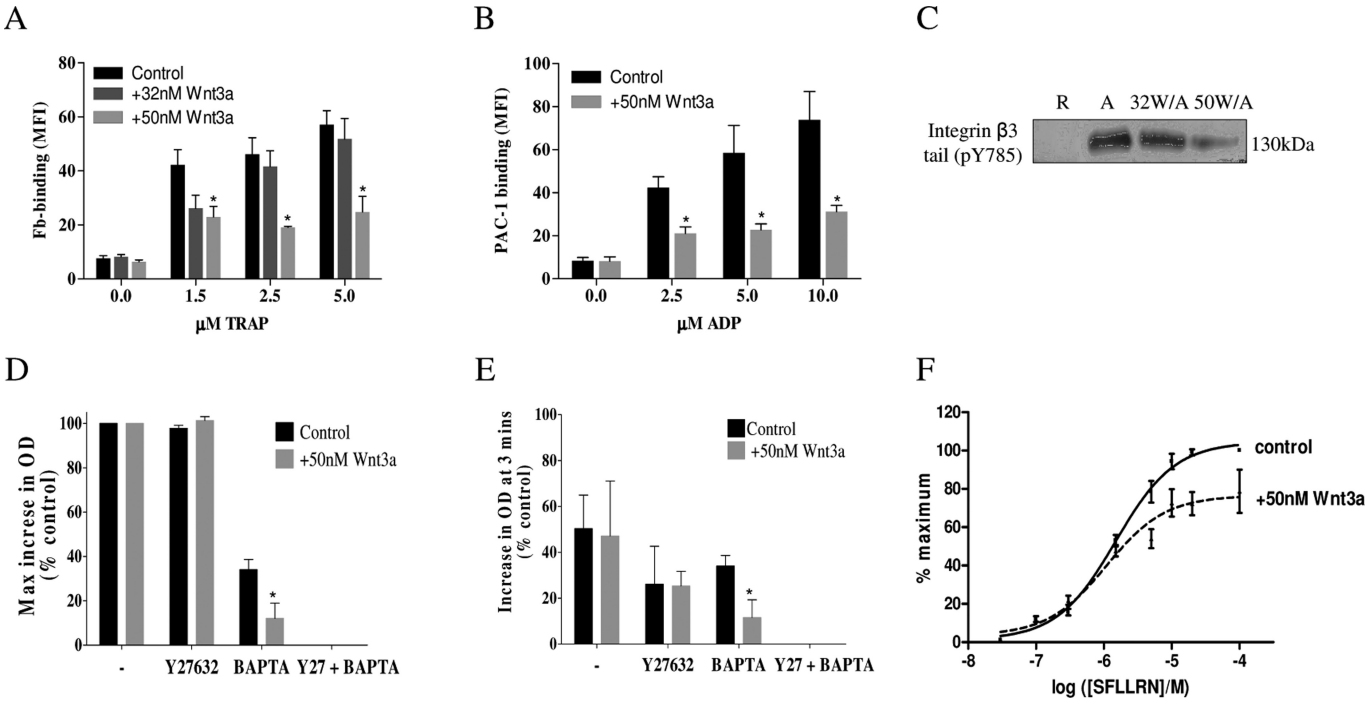

Fig. S2. (*A*) Soluble Fb binding (FITC-conjugated antibody toward fibrinogen) in TRAP-activated platelets was measured using flow cytometry in the absence (black bars) and presence of increasing concentrations of Wnt3a (32 nM is denoted by dark gray bars and 50 nM by light gray bars). (*B*) Flow cytometry analysis of PAC-1 binding in human platelets treated with ADP. The absence of Wnt3a is denoted by black bars and pretreatment with 50 nM Wnt3a by gray bars. (*C*) Resting (R), 1.5 μM TRAP (A), and 1.5 μM TRAP washed platelet lysates in the presence of 32 nM Wnt3a (32W/A) and 50 nM Wnt3a (50W/A) were blotted for phospho integrin β3 [pY785]. (*D* and *E*) TRAP (1.5 μM)-induced platelet shape change in response to a variety of conditions was monitored by Optical Density (OD) in a Chronolog aggregometer by measuring (*D*) the maximum % increase in OD and (*E*) % increase in OD 3 min after stimulation, both versus vehicle-treated only. (*F*) No change in calcium release was observed during Wnt3a signaling in platelets at TRAP concentrations that were otherwise affected by Wnt3a. The level of intracellular calcium release was determined using the fluorescent dye Fura-2 AM. All data are representative of 3 independent experiments and are presented as mean ± SEM (*, $P < 0.05$).

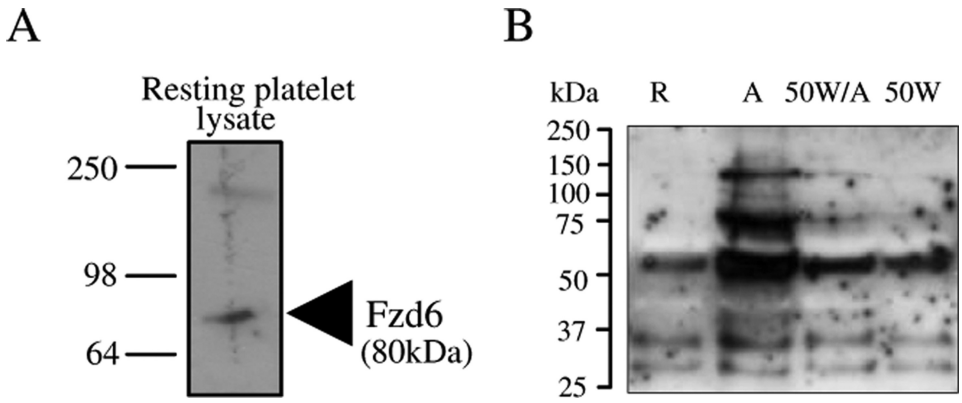

Fig. S3. (*A*) Detection of Fzd6 in platelet lysate by Western blotting. (*B*) Wnt signaling was found to reduce global tyrosine phosphotyrosine levels. Resting (R), 1.5 μM TRAP activated (A), 50 nM Wnt3a + 1.5 μM TRAP (50W/A) and 50 nM Wnt3a alone (50W) washed platelet lysates were blotted using the antiphosphotyrosine monoclonal 4G10 antibody. All data are representative of 3 independent experiments.

Table S1. RT-PCR primers utilized for the detection of Fzd4 and Fzd6 transcripts

Transcript	Forward primer	Reverse primer	Product length (bp)
Fzd4	GCCAATGTGCACAGAGAAGA	GGTTTTGTGAGGTAAGGGCA	211
Fzd6	TTGTTGGCATCTCTGCTGTC	CCATGGATTTGGAAATGACC	222
GAPDH	ACAGTCAGCCGCATCTTCTT	TGGAAGATGGTGATGGGATT	288