SUPPLEMENTARY APPENDIX

Mechanisms of increased mitochondria-dependent necrosis in Wiskott-Aldrich syndrome platelets

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Figure S1

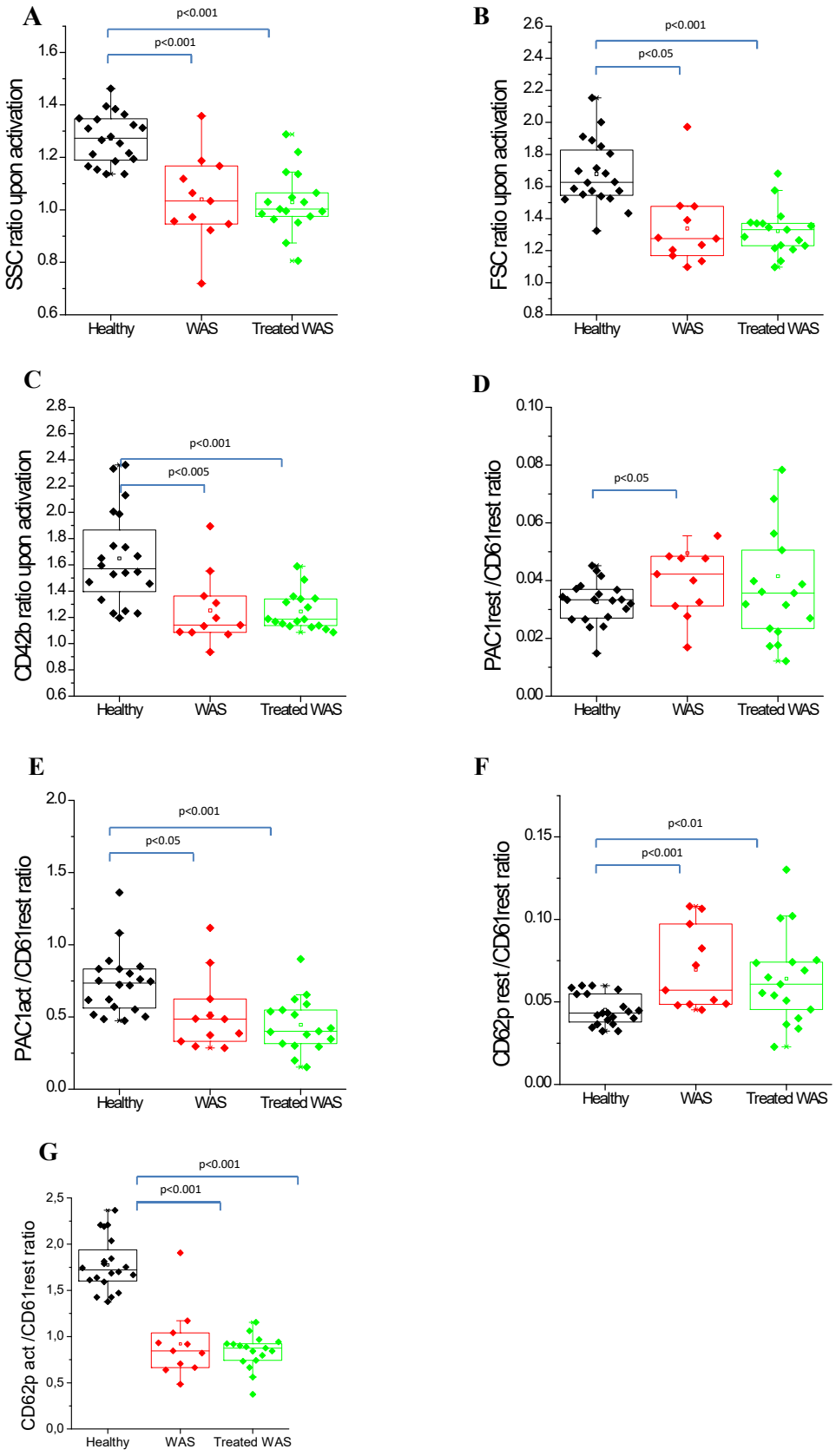

Fig. S1. Additional panels for Fig. 2. Comparison of healthy children (n=21), WAS patients with (n=17) or without treatment (n=11) by SSC/FSC/CD42b ratios upon activation (**A-C**). **D-G** normalization of the expression of activation markers PAC1 and CD62p (MFI) on the CD61 MFI. Mann Whitney U test was used. "act", activated platelets, "rest", resting platelets.

Figure S2

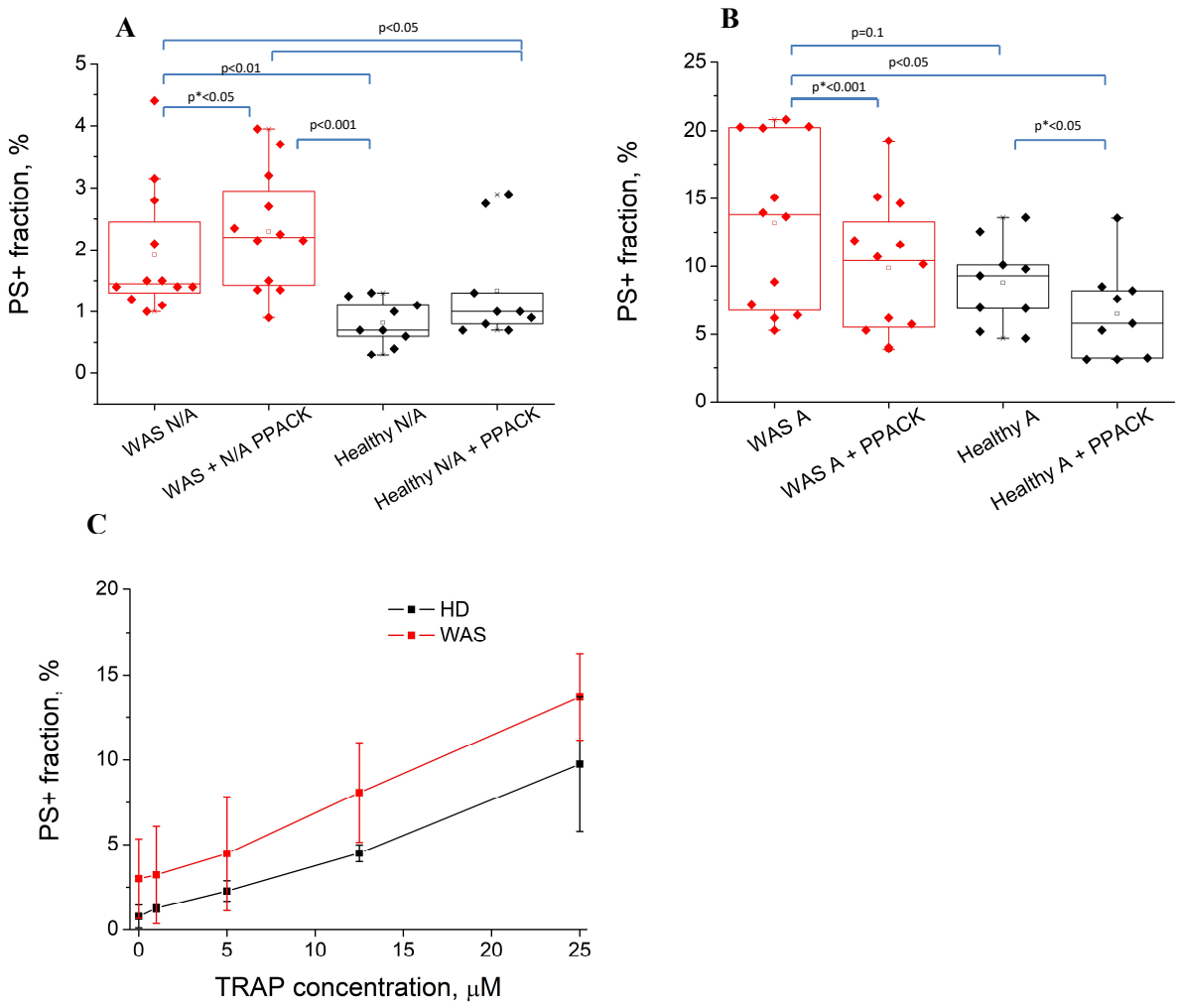

Fig. S2. The PS+ platelet fraction of WAS and healthy donors with/without 25 μM TRAP-6 activation. In order to investigate the activation status of WAS platelet in plasma (with minimal mechanical action at platelet isolation), we analyzed the PS-positive platelet subpopulation formation at low platelet activation by TRAP-6. (**A-B**) We used two different approach to obtain the data of platelet activation in plasma: in the absence of thrombin inhibitor PPACK and in the presence of high concentration of PPACK. We observed that the activation status of WAS platelets in plasma is increased, as compared with normal platelets. In the presence of PPACK is inhibited the activation-induced formation of PS-positive platelet subpopulation in WAS and HD, that suggest that the formation PS-positive platelets in plasma partly is due to additional thrombin generation at platelet activation. p – Mann–Whitney U-test, p* –Wilcoxon signed-rank test.

(C) - Dependence of PS+ fraction on TRAP concentration for HD and WAS (n=2).

Figure S3

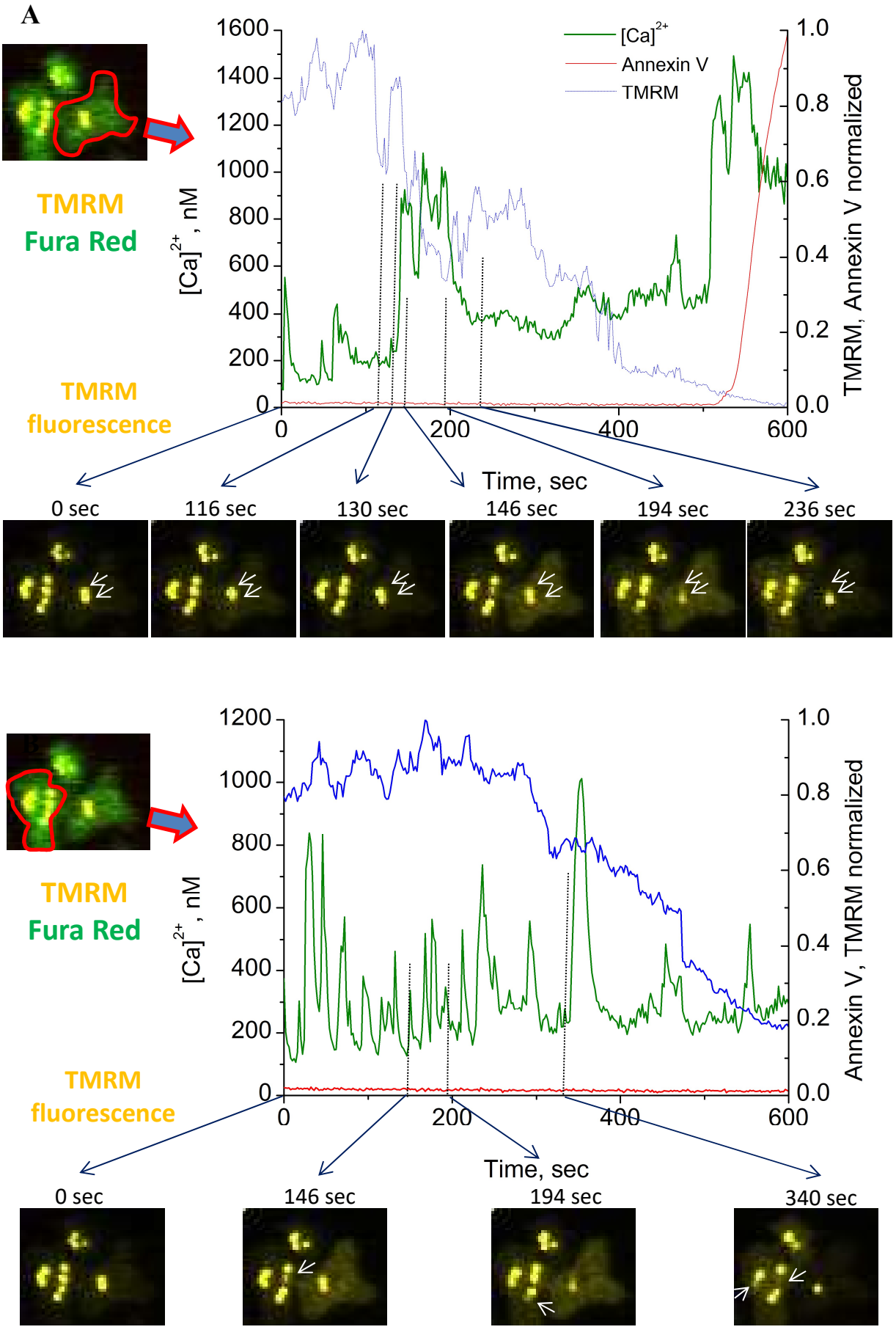

Fig. S3. Additional panel for Fig. 3. Calcium and mitochondrial membrane potentials dynamics of WAS PS+ platelet with 2 mitochondria (**A**) and PS- platelets with 6 mitochondria (**B**).

Figure S4

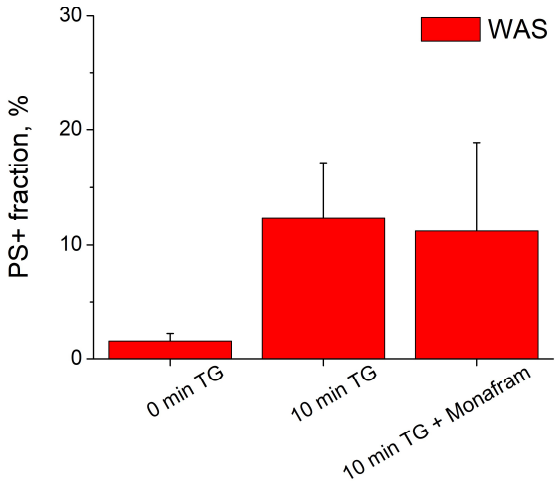

Fig S4. Effects of monafram on TG-induced necrosis in suspension (n=3).

Figure S5

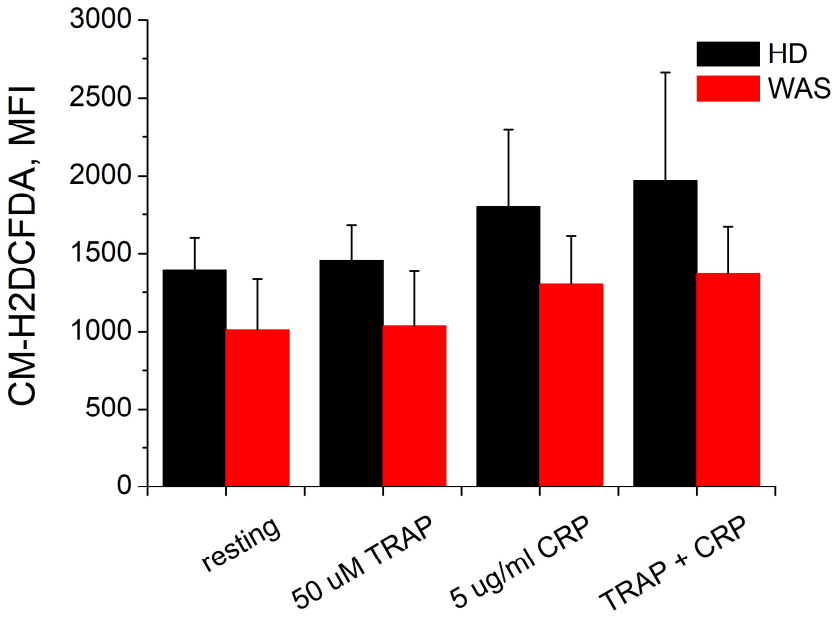

Fig S5. Reactive oxygen species detection. 2'7'-dichlorodihydrofluorescein diacetate (DCFDA) was used for analysis of ROS production upon platelet activation. ROS production was observed only at stimulation of platelets with CRP or dual agonist stimulation CRP+TRAP (n=3). At the same time ROS production was not detected at stimulation of platelets with TRAP or spreading on fibrinogen (data not shown). This suggest that ROS production is not involved in PS exposure at spreading on fibrinogen.

Figure S6

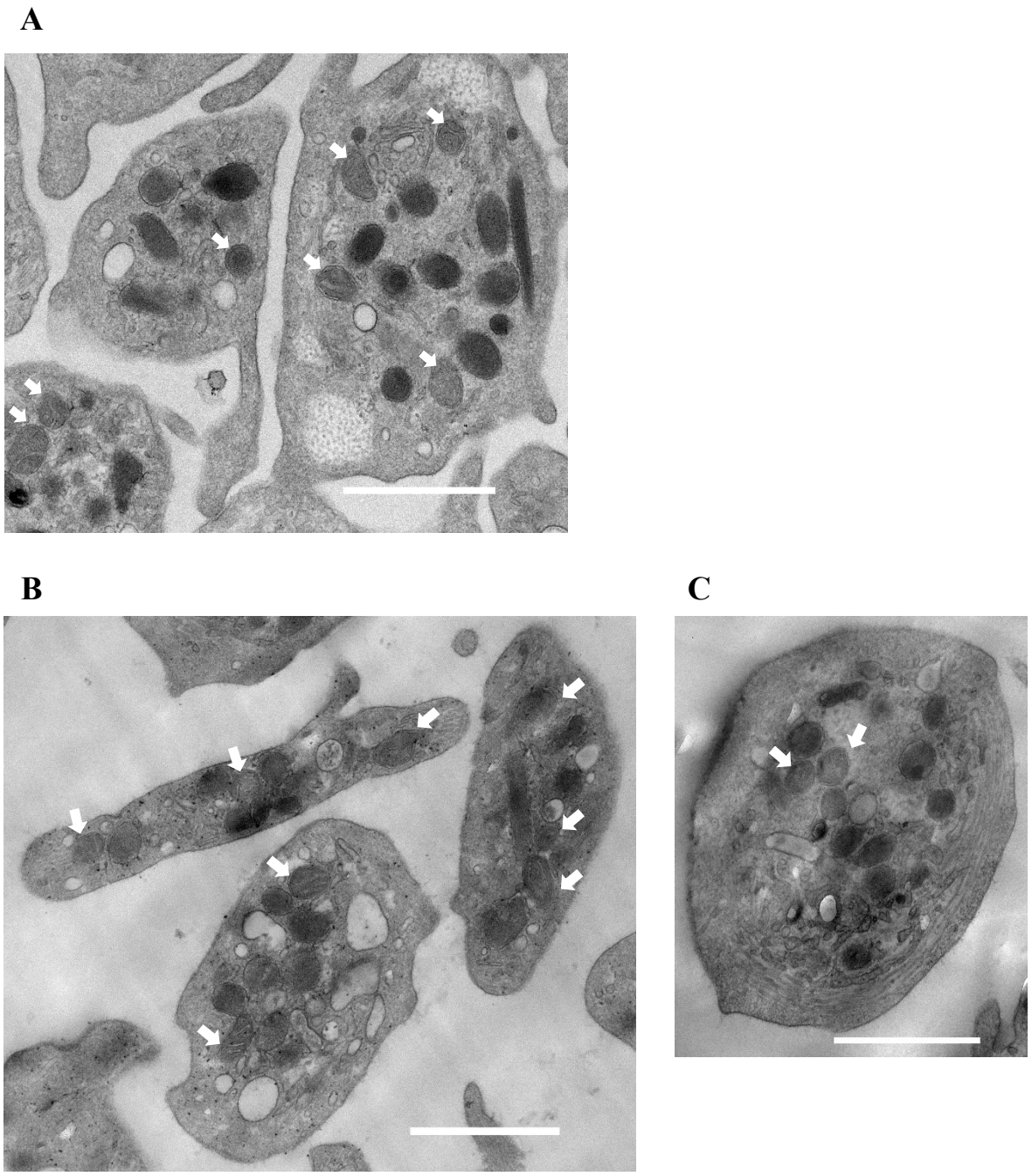

Fig. S5. Electron microscopy of platelets. (A) – healthy donor platelets, **(B-C) –** platelets from 2 WAS donors. Arrows show mitochondria. Bar 1 μM.

Figure S7

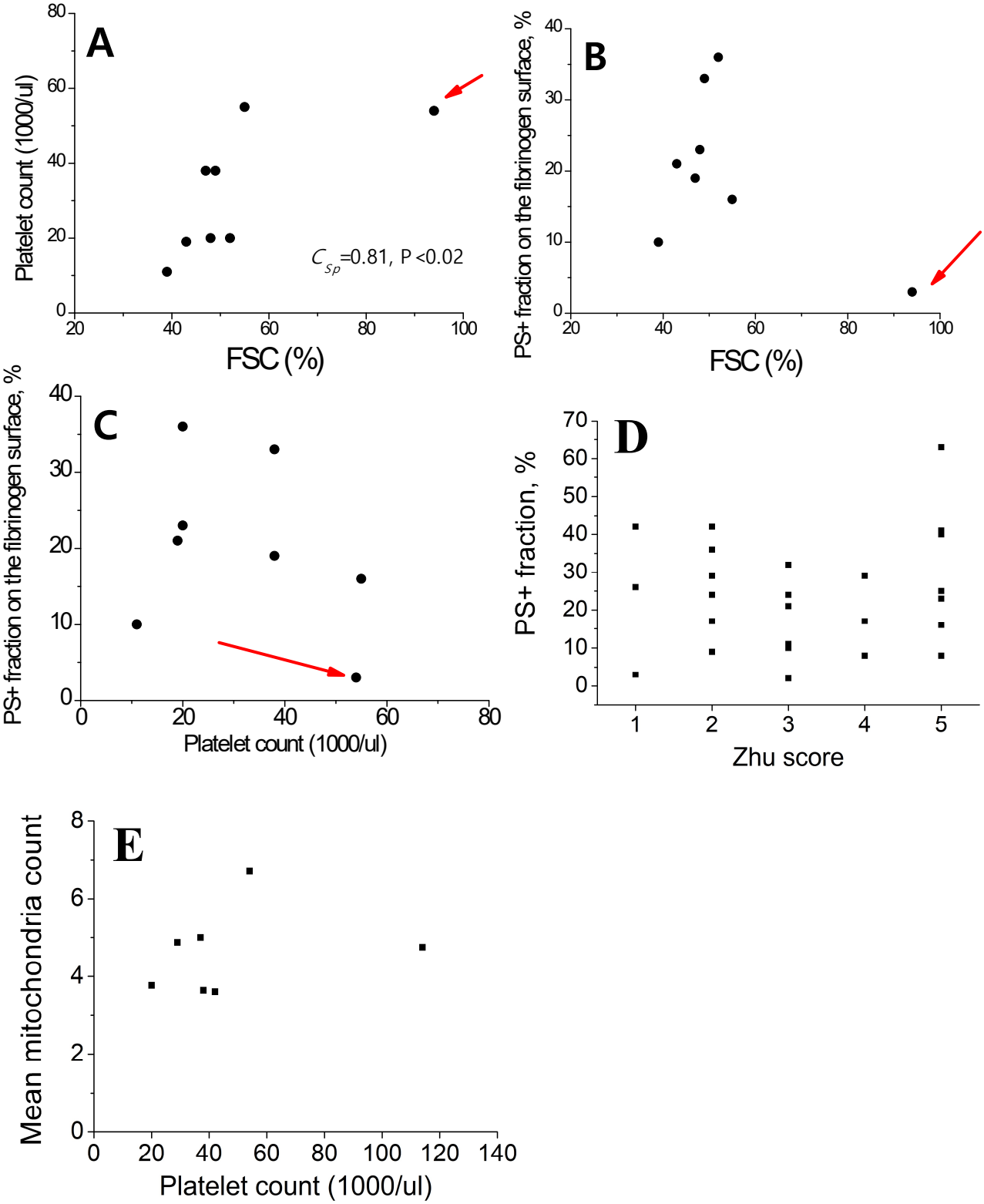

Fig. S7. Correlation of platelet size, platelet count and tendency to expose PS in the untreated WAS patients' platelets. The analysis is based on the PS exposure results in Fig. 1B, FSC results of Fig. 2A, and platelet counts in Table 1.

Supplemental methods

Patients and healthy donors. A total of 35 patients with WAS were included in the study (Table 1). The diagnosis was made according to European Society for Immunodeficiencies diagnostic criteria and genetically confirmed by WAS mutations identification. Samples from 12 patients were collected only when not on romiplostim, 17 patients were studied after at least 1 month of romiplostim treatment, and 5 patients were investigated both before treatment and on treatment. Romiplostim was administered off-label according to the institutional protocol at 9 μg/kg weekly. 12 of 35 patients had a Zhu score of 1 and 2. Control samples included blood from children and healthy adults as indicated in the experimental descriptions.

Reagents. The following materials were used: prostaglandin E1 (MP Biochemicals, Irvine, CA, USA); Fura Red AM, (Molecular Probes, Eugene, OR, USA); Alexa Fluor 647-conjugated annexin V, CD61- fluorescein isothiocyanate (FITC), CD61-phycoerythrin (PE) (Biolegend, San Diego, CA, USA); tetramethylrhodamine methyl ester (TMRM), nonyl acridine orange (NAO), 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) (Life Technologies, Grand Island, NY, USA); cyclosporine A, calcium ionophore A23187, calpeptin; Z-VAD-FMK, thapsigargin, CCCP, rotenone, oligomycin A, Xestospongin C (Tocris Biosciences, Bristol, UK); Phe-Pro-Arg chloromethyl ketone (PPACK) (Merck KGaA, Darmstadt, Germany), lactadherin-FITC and human thrombin (Haemotologic Technologies, VT, USA); osmium tetroxide (Ted Pella, CA, USA), luciferin-firefly luciferase reagent, dimetylsulphoxide, ATP-control (BCM ST, Russia), . All other reagents were from Sigma-Aldrich (San Diego, CA, USA). Integrin $\alpha_{IIb}\beta_3$ antagonist monafram[16] was a kind gift of Prof. A.V. Mazurov. Cysteine-containing version of cross-linked collagen-related peptide (CRP) was kindly provided by Prof. R.W. Farndale (University of Cambridge, Cambridge, UK).

Blood collection and platelet isolation. Investigations were performed in accordance with the Declaration of Helsinki under approval of the Children's for Hematology Ethical Committee, and written informed consent was obtained from all patients (or their parents) and donors. Washed platelets were prepared essentially as described[1]. Blood was collected into sodium citrate, and supplemented with prostaglandin E1 (1 μmol L^{-1}). Platelet-rich plasma was obtained by centrifugation at 100 × g for 8 min. Three parts of platelet-rich plasma were diluted with one part of 3.8% sodium citrate (pH 5.5), and centrifuged at 400 × g for 5 min (for WAS platelets, 700 g); platelets were then resuspended in buffer A (150 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 1 mmol L^{-1} $MgCl_2$, 0.4 mmol L^{-1} NaH_2PO_4, 20 mmol L^{-1} HEPES, 5 mmol L^{-1} glucose, 0.5% bovine serum albumin, pH 7.4).

Confocal microscopy experiments: general design. Glass coverslips (24 × 24 mm; Heinz Herenz, Hamburg, Germany) were cleaned by plasma cleaner (Harrick Plasma, Ithaca, NY, USA). They were coated with 1 mg mL^{-1} fibrinogen or monafram in PBS for 45 min at room temperature, rinsed, and used as the substrate for washed platelets. Washed platelets were attached to the protein-coated surface by incubating them at $1.3 \cdot 10^5$ μl^{-1} (or the maximal concentration attainable for WAS under conditions of thrombocytopenia) for 20 min and rinsing with buffer A with 1.5 mM $CaCl_2$. For the experiments on PS exposure, the fields were imaged once, and then again after 30 min of additional incubation. For experiments with intracellular dyes loading, continuous video-imaging was performed. Confocal images were acquired using an Axio Observer Z1 microscope (Carl Zeiss, Jena, Germany) equipped with a Yokogawa spinning disc confocal device (CSU-X1; Yokogawa Corporation of America, Sugar Land, TX) and a 1.3 numerical aperture × 100 objective. Optical filters (Semrock, Rochester, NY, USA) for fluorophores were used: Alexa Fluor 647–annexin V and Fura Red (647 nm long-pass filter); (FITC)–CD61 (520/35 nm); and TMRM (587/35 nm). The excitation wavelengths were 405 nm and 488 nm for Fura Red, 488 nm for FITC; 561 nm for TMRM; and 635 nm for Alexa Fluor 647. Analysis of the obtained images was carried out with ImageJ (http://imagej.nih.gov/ij/) software.

Cytosolic calcium signaling and mitochondrial membrane potential change. The methodology was essentially as described[2]. For experiments with calcium measurements washed platelets were incubated with 10 μmol L^{-1} Fura Red AM for 45 min at room temperature followed by centrifugation and resuspension in buffer A. Intracellular calcium levels of non-activated platelets were determined with confocal microscopy. Calibrations for ratiometric measurements were made using 10 mM calcium ionophore A23187 and 4 mM EGTA for minimal and maximal fluorescence ratios, separately for healthy and WAS platelets. Background signals were substracted from images and calcium concentrations were calculated using a formula for ratiometric indicators[3]. TMRM was used to detect mitochondrial potential dynamics. Washed platelets were spread on the fibrinogen-coated coverslip for 20 min in the presence of 200 nM TMRM at room temperature. Unattached cells were then removed with buffer A with 1.5 mm $CaCl_2$, and imaging was started as described above.

Characterization of platelet response to TRAP-6 in platelet-rich plasma (PRP). Samples of PRP were diluted with PPP to a final concentration of 20,000 μL^{-1} and buffered with HEPES at pH 7.4 (100 mM final concentration). The irreversible thrombin inhibitor Phe-Pro-Arg chloromethyl ketone (PPACK) was added to the final concentration of 100 μM in order to block spontaneous thrombin generation upon recalcification (Fig. S1). Platelets were recalcified by addition of calcium chloride (final concentration 20 mM, which corresponds to 2 mM of free calcium[4]) and activated by 25 μM TRAP-6 for 5 min at room temperature. They were stained with

CD61-FITC (antibody to membrane integrin $\alpha_{IIb}\beta_3$) and annexin V-Alexa Fluor 647 (marker of PS-positive platelets) for 5 min, diluted with buffer A containing 1.5 mM calcium chloride and immediately analyzed using flow cytometry.

Determination of ROS. Washed platelets at 20000 uL-1 were incubated with 10 μM CM H2-DCFDA for 30 min at 37°C in buffer A and activated for 10 min at RT with the indicated concentrations of agonists in the presence of 2,5 mM calcium chloride. Platelets were stained with CD61-PE and annexin V-Alexa Fluor 647 for 5 min at RT, diluted with buffer A containing 2,5 mM calcium chloride and immediately analyzed by flow cytometry. Flow cytometry analysis was performed using Accuri Flow Cytometer (BD Biosciences, San Jose, CA, USA).

Modulation of calcium signalling and mitochondrial function. Platelets in buffer A at 20,000 μL^{-1} were incubated with 1 μM TG in the presence of 1.5 mM calcium chloride for 10 min. At the same time platelets were stained with CD61-FITC and annexin V-Alexa Fluor 647. TG-treated stained platelets were diluted with buffer A containing 1.5 mM calcium chloride and immediately analyzed using flow cytometry. In the case of experiments with integrin $\alpha_{IIb}\beta_3$ antagonist, the platelets in buffer A at 20,000 μL^{-1} were preincubated with 200 μg/ml monafram for 20 min at RT before treatment with TG. For calcium-free experiments, platelets at 20,000 μL^{-1} were incubated with 1 μM TG in buffer A without addition of calcium chloride and stained with CD61-PE and lactadherin-FITC, then diluted with buffer A without addition of calcium chloride and immediately analyzed using flow cytometry. The mitochondrial inhibitors oligomycin (2,5 μM), rotenone (10 μM) and uncoupler CCCP (10 μM) were incubated with platelets at 20,000 μL^{-1} in buffer A for 15 min at RT before addition of TG.

ATP measurement in platelets. Platelets in buffer A at 100,000 μL^{-1} (for donors) and 30,000 μL^{-1} (for patients) were incubated with 1 or 5 μM TG in the presence of 1.5 mM calcium chloride for 5, 20 or 40 min or with 10 μM CCCP for 10 min. The samples were taken at indicated time points to determine ATP concentration and percentage of PS-positive platelets. In brief, part of the sample was lyzed (addition 90 μl DMSO to 10 μl platelet suspension) and analyzed by luciferase-luciferin assay as described[5, 6] while the other part was stained with CD61-FITC and annexin V-Alexa Fluor 647 and analyzed by flow cytometry.

Flow cytometry characterization of platelet functional activity. The experiments were performed essentially as described[7, 8] with minor modifications. Citrated blood samples were diluted 1:20 with buffer A. Platelets were either left intact or loaded with mepacrine (10 μM final concentration) for 30 min at 37ºC. Subsequently, they were either left unstimulated or stimulated with CRP at 20 μg/μl plus TRAP-6 at 12.5 μM for 10 min in the presence of 2.5 mM calcium chloride. Both resting and activated samples were incubated with antibodies against CD61, CD42b, CD62P, as well as PAC1 and annexin V for 10 min in the presence of 2.5 mM calcium

chloride. Subsequently, they were diluted 10-fold with buffer A containing 2.5 mM calcium, and analyzed using Novocyte (Acea Bioscience, San Diego, CA, USA) flow cytometer. Blood samples from healthy children claiming not to have used medication for a week prior to analysis were used for control.

Transmission electron microscopy. The protocol was essentially as described[9]. PRP was fixed for 1 hour with freshly prepared 2.5% glutaraldehyde in PBS (pH 7.4). After centrifugation and rinsing with PBS (pH 7.4), cells were postfixed in 1% osmium tetroxide for 1 hour. Then each sample was dehydrated in a graded acetone series and embedded in Epon 812. Ultrathin sections were produced using Ultracut E (Reichert, Vienna, Austria). The sections were stained with lead citrate followed by uranyl acetate and observed with the transmission electron microscope JEM-1400 (JEOL Tokyo, Japan).

Statistics. Data are presented as means ± standard deviations. The statistical significance of the differences between groups was determined with the non-parametric Mann–Whitney U-test (p). The effects in the paired samples were evaluated using Wilcoxon signed-rank test (p*). Differences were considered to be significant when the P-value was < 0.05.

Computational modeling of platelet calcium homeostasis. A systems biology model of platelet calcium signalling was described previously[18]. Briefly, it was a multicompartmental stochastic computational model of platelet calcium signaling resulting from stimulation of PAR1 receptor. In contrast to its predecessor pure calcium signalling/homeostasis models[10, 11], it had several mitochondrial compartments and included equations describing dependence of ATP production and calcium pumps activity on mitochondrial inner membrane potential. The model consisted of 35 ordinary differential equations. The set was solved using COPASI software (www.copasi.org) and stochastic adaptive SSA/tau-leap method.

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