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Wdr-1- is Essential for F-actin Interaction with Focal Adhesions in Platelets

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Abstract

OBJECTIVE—Wdr-1, an actin interacting protein, enhances cofilin's capacity to accelerate depolymerization of F-actin filaments. Wdr-1-deficient mice have impaired hemostasis due to defective inside-out integrin signaling in platelets. Here, we studied the role of Wdr-1 on outside-in signaling necessary for retraction of the clot and platelet spreading.

METHODS—Outside-in signaling was assessed by fibrin clot retraction assay and by adhesion and spreading of unstimulated platelets on fibrinogen substrate. The spatial distribution of actin, cofilin-1 and Wdr-1 were determined by immunofluorescence microscopy. Interaction of F-actin with focal adhesion kinase (FAK) was assessed in dual-color confocal images and by immunoblotting of F-actin filaments.

RESULTS—Clot retraction is markedly impaired in Wdr-1-deficient platelets. Wdr-1-deficient platelets adhere and spread poorly on fibrinogen substrate compared to wildtype controls. In resting platelets, Wdr-1 is colocalized with cofilin-1 in cortical actin. Following platelets spreading on fibrinogen substrate, Wdr-1 translocates to the cytoskeleton in association with cofilin-1. In Wdr-1-deficient platelets, cofilin-1 is aberrantly localized throughout the cytoplasm and there is no significant change following adhesion to fibrinogen substrate. The actin filaments formed upon spreading on fibrinogen are mostly in the periphery of the platelets and does not traverse the cytoplasm. Furthermore, there is diminished colocalization of actin filaments with focal adhesion kinase (FAK).

CONCLUSION—These studies show that Wdr-1 is essential for the localization of cofilin-1 to the platelet membrane skeleton. F-actin fails to attach to focal adhesions resulting in defective reorganization of actin filaments necessary for platelet spreading and clot retraction.

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Declaration of Interest statement
The authors declare no conflicts of interest

Compliance with Ethical Standards
A The investigation involving mice was conformed to the Guide for The Care and Use of Laboratory Animals as published by the US National Institutes of Health. All animals were treated in accordance with the protocol approved by the Animal Care and Use Committee (IACUC) of Baylor College of Medicine. Blood was obtained from human volunteers after an informed written consent under a protocol approved by the Institutional Review Board of Baylor College of Medicine.

Keywords

Actin reorganization; cofilin; Wdr-1; focal adhesions

Introduction

The actin cytoskeleton plays a central role in the hemostatic function of platelets (1). In resting unstimulated platelets, the actin filaments are located in the submembranous zone and provides a framework for discoid platelet shape and structure (2). During activation and spreading, there is extensive dismantling and reorganization of the actin filaments (3). The signals for the reorganization of actin network are derived from platelet integrin αIIbβ3, following binding to fibrinogen (outside-in signaling). Spreading is maintained by myosin-driven contractile force on newly formed actin network and the resistance generated by integrin binding to the matrix substrates. The contractile force also mediates the clot retraction.

The actin reorganization is orchestrated by a large number of actin-binding proteins (4). Wdr-1 is the mammalian homolog of actin interacting protein 1 (Aip1) in the yeast (5). In yeast and in mammalian cells, it enhances cofilin-1's capacity to accelerate depolymerization of existing actin filaments and promotes formation of barbed ends and reorganization. Wdr-1 deficiency in human causes an autoinflammatory disease characterized by periodic fevers, immunodeficiency, and intermittent thrombocytopenia (6). A hypomorphic allele of Wdr-1 has been described in mice which have spontaneous autoinflammatory disease and thrombocytopenia, similar to humans (7). We previously described that Wdr-1 deficiency leads to defective platelet aggregation due to impaired inside-out signaling (8). Here, we present evidence the Wdr-1 plays a significant role in outside-in signaling necessary for platelet spreading and clot retraction.

METHODS

Reagents

Goat antibody to Wdr-1 and rabbit antibody to focal adhesion kinase (FAK) were obtained from Santa Cruz Biotechnology, while rabbit anticofilin-1 antibody was from Cell Signaling Technology. Alexa 488-labeled sheep antirabbit antibody, Alexa 647-labeled donkey antigoat antibody and Alexa 488-labeled phalloidin were purchased from Invitrogen. Apyrase, polylysine and prostaglandin E_1 (PGE_1) were obtained from Sigma-Aldrich. Fibrinogen was isolated as described before (9). The hypomorphic allele of Wdr-1 mice has been described before (7). The mutant mouse ($Wdr\text{-}1^{rd/rd}$) has a T>A transversion in the second dinucleotide of the intron 9 splice donor and it produces a mutant transcript containing a 6-bp in-frame deletion that results in a incorrectly folded, nonfunctional protein (7). A small amount of normal splicing produces some wildtype protein, resulting in a hypomorphic allele (Figure 1, Panels A and B). All animals were treated in accordance with the protocol approved by the Animal Care and Use Committee (IACUC) of Baylor College of Medicine.

Isolation of murine platelets

To isolate mouse platelets, blood was obtained from the inferior vena cava into a one-tenth volume of citrate from ≥4-month-old mice under isoflurane anesthesia. Blood was diluted with an equal volume of modified Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 5 mM Hepes, 1 mM $MgCl_2$, 3 mM NaH_2PO_4, and 5.5 mM Dextrose, pH, 7.4) containing 0.5% bovine serum albumin (BSA). Platelet-rich plasma was obtained by centrifugation at 75 g for 15 minutes. PGE_1 (1 μM) and apyrase (0.5 unit) were added, and platelets were sedimented by centrifugation at 600 g for 10 minutes, washed once in Tyrode's buffer containing apyrase and suspended in Tyrode's buffer.

Clot retraction assay

For the fibrin clot retraction assays, PRP was isolated as described before. The platelet count was adjusted to 2×10^8 per mL with addition of platelet-poor plasma. To visualize the clot, 5 μl of washed red blood cells was added to 1 ml of PRP. An aliquot (400 μL) of this mix was placed into a glass cuvette. Thrombin (0.5 U/mL) was added and mixed with and clot retraction was allowed to proceed at 37°C for 1 hour. After 30 minutes, the clot was pulled out and the amount of liquid not incorporated into the clot was subtracted from the initial volume to determine the volume of clot. It is expressed as percentage of the initial volume.

Platelet adhesion and spreading

For adhesion assays, wells of 96-well plates were coated with fibrinogen (100 μL of 10 μg/mL) and blocked with 0.5 % denatured bovine serum albumin (BSA). Washed murine platelets were added and incubated for 30 minutes at 37°C. After washing, adhesion was quantified by assaying for acid phosphatase activity at 405 nM. Percent adhesion was calculated as number of fibrinogen adhered platelets divided by the total number of platelets added to the well and multiplied by 100 as previously reported (10). For spreading assays, coverslips were coated with fibrinogen (10 μg/mL) overnight at 4°C. Surfaces were washed and then blocked with denatured BSA (5 mg per mL) for 1 h at room temperature followed by subsequent washing with phosphate buffered saline (PBS) before use in spreading assays. Washed platelets (2×10^7 per mL) were allowed to spread on immobilized fibrinogen for 30 minutes at 37°C. Unbound platelets were removed by washing with PBS and differential interference contrast images of bound platelets were captured by Olympus UPlanSApo 100×/1.4NA oil objective on a GE Healthcare DVLive microscope. To compute the surface area, spread platelets were fixed, permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, washed thrice in PBS and then stained with Alexa 488-phalloidin (1 in 500) for 60 minutes and fluorescent images were obtained. ImageJ software was used to measure area of spread platelets.

Immunofluorescence microscopy

Washed platelets were immobilized on a polylysine- or fibrinogen coated cover slip fixed, permeabilized with 0.1%triton X-100 and incubated with anticofilin-1 and antiWdr-1 overnight at 4ºC temperature. Cells were washed and incubated with Alexa fluor conjugated appropriate secondary antibodies for one hour. For the analysis of F-actin-focal adhesion interaction, platelets adherent on fibrinogen-coated surface were fixed with 4%

paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained for FAK (red) and actin (green). Images are taken in a DeltaVision OMX microscope (GE Healthcare) at 1000 × magnification with oil immersion and deconvolved using SoftWoRX 6.5.2 that applies a 3D iterative constrained deconvolution algorithm.

Analysis of platelet cytoskeleton

Washed platelets (2×10^8/ml) from wildtype and Wdr-1-deficient mice were incubated on fibrinogen-coated surface on a petri dish. The adherent platelets were lysed with an equal volume 2 × lysis buffer (100 mM Tris-HCl (pH 7.4), 2% Triton X100 containing 'Halt protease and phosphatase inhibitor cocktail' (Thermo Scientific)) and kept on ice for 15 minutes. Lysates were centrifuged at 100,000 g for 1 hour to sediment Triton-X-100-insoluble F-actin. The F-actin pellets were washed twice in cold lysis buffer and solubilized in 8 M urea. The pellets were reconstituted to the same initial volume. Equal fractions were loaded onto SDS-polyacrylamide gels, electrophoresed, transferred onto PVDF membrane and probed with antibodies to focal adhesion kinase (FAK), or actin followed by peroxidase-labeled secondary antibody. The bands were analyzed and quantified in Odyssey Fc Dual-Mode Imaging System (LI-COR Biosciences) as described before (11). The ratio of the densitometric value of actin to FAK was determined in each separate experiment the values for wildtype platelets was arbitrarily normalized to 1 for comparison.

Statistical Analysis

All data were expressed as mean ± standard deviation of triplicate experiments. Comparisons between individual groups were performed using the Student T-test with paired and unpaired samples. A probability value (P) of 0.05 or below was considered statistically significant.

RESULTS

Impaired clot retraction in Wdr-1-deficient mouse platelets

Fibrin-bound activated platelets mediate clot retraction by transducing contractile forces onto the fibrin network, resulting in a more compact tensile clot. Clot retraction is markedly impaired in Wdr-1-deficient mice compared to their littermate controls (Figure 1, panels C and D). Impaired clot retraction implies a major defect in outside-in signaling.

Defective adhesion and spreading of Wdr-1-deficient mouse platelets

Outside-in signaling cascades generated by platelet αIIbβ3 binding to immobilized fibrin(ogen) support adhesion and spreading. We measured platelet adhesion and spreading of platelets to immobilized fibrinogen. Wdr-1-deficient mouse platelets show decreased adhesion on fibrinogen compared wildtype mouse platelets (Figure 2, Panels A). Wdr-1-deficient platelets exhibit a more rounded morphology compared to their wildtype littermate control platelets as shown by differential interference contrast images (Figure 2, Panels B and C). The surface area of spreading showed significant decrease in Wdr-1-deficient platelets compared to wildtype mouse platelets (Figure 2, Panel D).

Distribution of Wdr-1 and cofilin-1 in platelets

We examined the distribution of Wdr-1 by immunofluorescence microscopy in permeabilized mouse platelets under resting condition and following spreading on fibrinogen-coated surfaces. In resting platelets, Wdr-1 is seen in distinct patches in the plasma membrane (Figure 3, Panel A). Since Wdr-1 antibodies do not bind to intact non-permeabilized platelets (data not shown), these results show that Wdr-1 is localized to the submembranous cortical region. In addition, Wdr-1 colocalizes with cofilin-1 in the submembranous region in resting platelets (Figure 3, Panels B and C). Following spreading on fibrinogen–coated surfaces, Wdr-1 redistributes in a discrete granular pattern throughout the cytoplasm (Figure 3, Panel E) in association with cofilin-1 (Figure 3, Panels F and G). These findings are consistent with translocation of Wdr-1 in a complex with cofilin-1 from the cortical actin to the cytoskeleton following outside-in signaling. In resting Wdr-1-deficient mouse platelets, cofilin-1 is abnormally distributed throughout out the cytoplasm (Figure 3, Panel I and J) compared to platelets from wildtype littermate controls (Figure 3, Panel A) and there is no significant alteration in the distribution following adhesion to fibrinogen (Figure 3 Panel K and L). These findings show that Wdr-1-cofilin-1 interaction is essential for the cortical localization of cofilin-1 in resting platelets and WDR-1 is necessary for the cortical reorganization of actin that occurs during platelet spreading on fibrinogen.

Defective adhesion and abnormal actin filaments-focal adhesion interaction in Wdr-1-deficient mouse platelets

When platelets adhere to fibrinogen, integrin αIIbβ3 clusters and recruits a number of adapter proteins to the integrin cytoplasmic tails to form focal adhesions (12–16). In wildtype platelets, spreading on fibrinogen is associated with formation of long actin filaments that traverse through the cytoplasm and well-formed focal adhesions (Figure 4 Panel A). In Wdr-1-deficient platelets, the F-actin filaments are confined to the periphery of platelets (Figure 4, Panel B). There is impaired colocalization of actin with FAK in focal adhesions as indicated by the enhanced intensity of red fluorescence due to decreased colocalization of FAK and F-actin compared to wildtype platelets (Figure 4, Panel B). Consistent with these results, despite an increase in F-actin content in Wdr-1-deficient platelets, there is decreased incorporation of FAK to the actin filaments as measured by ratio of actin to FAK by densitometry of immunoblots of fibrinogen adherent platelets (Figure 4 Panels C and D). These results show that F-actins do not make the optimal contact with the focal adhesions, precluding the formation of an adequate mechanical and functional link between intraplatelet actin filaments and the extracellular matrix components through integrin αIIbβ3 in the absence of Wdr-1.

Discussion

When endothelial continuity is disrupted, platelets rapidly adhere to the subendothelial matrix to seal the breach in the vessel wall. The initial platelet activation occurs as a result of soluble agonists binding to platelet receptors, which initiates a cascade of signal transduction events (inside out signaling) that causes integrin activation (17). Integrin αIIbβ3, the major integrin in platelets, undergoes activation induced conformational changes and binds to several cell adhesion molecules to mediate platelet adhesion and aggregation and fibrinogen

is the most abundant ligand (18). Upon engagement with its ligands, integrins cluster and recruit various signaling and adaptor proteins to form focal adhesions, which function as transmembrane mechanical link between extracellular matrix and the cytoskeleton inside platelets. Integrins in focal adhesion plaques can sense the matrix environmental cues and transmit signals to the cytoskeleton (outside in signaling), resulting in platelet spreading (12, 13, 19). Platelet spreading is maintained by the tension is generated f by the actomyosin motors transmitted externally through integrins.

Clot retraction is a critical event in thrombus consolidation and is dependent the integrity of properly aligned platelet actin fiber assembly. It almost entirely depends on outside-in signaling by the integrin αIIbβ3, following binding to fibrin(ogen). During clot retraction, the platelet-rich thrombus contract to a more compact clot due to actin filaments pulling inward the attached fibrin through the integrins in focal adhesions.

Our finding shows Wdr-1-mediated cofilin-1 plays a crucial role in spreading on fibrinogen substrate and in clot retraction – the two functions where outside-in signaling plays a significant role. Cofilin-1 changes the twist of F-actin filament and leads to its fragmentation. Cofilin1 then depolymerizes filamentous F-actin without capping it. This depolymerization facilitates new actin fiber formation via the newly exposed barbed ends. Wdr-1,by binding to the cofilin–F-actin complex and strongly enhances the severing activity of cofilin1 (20). Aberrant spatial localization of cofilin-1 in Wdr-1 deficient platelets may not allow proper attachment to focal adhesions resulting in defective reorganization of actin filaments necessary for development mechanical strength necessary for spreading and clot retraction, resulting in an impaired hemostasis in Wdr-1 deficiency.

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References

1. Fox JE. Cytoskeletal proteins and platelet signaling. Thromb Haemost. 2001;86(1):198–213. [PubMed: 11487008]
2. Thon JN, Italiano JE. Platelets: production, morphology and ultrastructure. Handb Exp Pharmacol. 2012(210):3–22.
3. Hartwig JH. The platelet: form and function. Seminars in hematology. 2006;43(1 Suppl 1):S94–100. [PubMed: 16427392]
4. Lappalainen P Actin-binding proteins: the long road to understanding the dynamic landscape of cellular actin networks. Molecular biology of the cell. 2016;27(16):2519–22. [PubMed: 27528696]
5. Rodal AA, Tetreault JW, Lappalainen P, Drubin DG, Amberg DC. Aip1p interacts with cofilin to disassemble actin filaments. J Cell Biol. 1999;145(6):1251–64. [PubMed: 10366597]
6. Standing AS, Malinova D, Hong Y, Record J, Moulding D, Blundell MP, et al. Autoinflammatory periodic fever, immunodeficiency, and thrombocytopenia (PFIT) caused by mutation in actin-regulatory gene WDR1. J Exp Med. 2017;214(1):59–711. [PubMed: 27994071]

7. Kile BT, Panopoulos AD, Stirzaker RA, Hacking DF, Tahtamouni LH, Willson TA, et al. Mutations in the cofilin partner Aip1/Wdr1 cause autoinflammatory disease and macrothrombocytopenia. Blood. 2007;110(7):2371–80. [PubMed: 17515402]
8. Dasgupta SK, Le A, Da Q, Cruz M, Rumbaut RE, Thiagarajan P. Wdr1-Dependent Actin Reorganization in Platelet Activation. PLoS One. 2016;11(9):e0162897. [PubMed: 27627652]
9. Martinez J, Holburn RR, Shapiro SS, Erslev AJ. Fibrinogen Philadelphia. A hereditary hypodysfibrinogenemia characterized by fibrinogen hypercatabolism. J Clin Invest. 1974;53(2):600–11. [PubMed: 11344575]
10. Khatlani T, Pradhan S, Da Q, Gushiken FC, Bergeron AL, Langlois KW, et al. The beta isoform of the catalytic subunit of protein phosphatase 2B restrains platelet function by suppressing outside-in alphaII b beta3 integrin signaling. J Thromb Haemost. 2014;12(12):2089–101. [PubMed: 25330904]
11. Hartig SM. Basic image analysis and manipulation in ImageJ Current protocols in molecular biology / edited by Ausubel Frederick M [et al.]. 2013;Chapter 14:Unit14 5.
12. Sandmann R, Koster S. Topographic Cues Reveal Two Distinct Spreading Mechanisms in Blood Platelets. Scientific reports. 2016;6:22357 Epub 2016/03/05. [PubMed: 26934830]
13. Arias-Salgado EG, Lizano S, Sarkar S, Brugge JS, Ginsberg MH, Shattil SJ. Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. Proc Natl Acad Sci U S A. 2003;100(23):13298–302. [PubMed: 14593208]
14. Podolnikova NP, Yermolenko IS, Fuhrmann A, Lishko VK, Magonov S, Bowen B, et al. Control of integrin alphaIIb beta3 outside-in signaling and platelet adhesion by sensing the physical properties of fibrin(ogen) substrates. Biochemistry. 2010;49(1):68–77. [PubMed: 19929007]
15. Kasirer-Friede A, Kang J, Kahner B, Ye F, Ginsberg MH, Shattil SJ. ADAP interactions with talin and kindlin promote platelet integrin alphaIIbbeta3 activation and stable fibrinogen binding. Blood. 2014;123(20):3156–65. [PubMed: 24523237]
16. Gorbatyuk V, Nguyen K, Podolnikova NP, Deshmukh L, Lin X, Ugarova TP, et al. Skelemin association with alphaIIbbeta3 integrin: a structural model. Biochemistry. 2014;53(43):6766–75. Epub 2014/09/17. [PubMed: 25224262]
17. Ginsberg MH. Integrin activation. BMB reports. 2014;47(12):655–9. Epub 2014/11/13. [PubMed: 25388208]
18. Coller BS. alphaIIbbeta3: structure and function. J Thromb Haemost. 2015;13 Suppl 1:S17–25. [PubMed: 26149019]
19. Oakes PW, Banerjee S, Marchetti MC, Gardel ML. Geometry regulates traction stresses in adherent cells. Biophysical journal. 2014;107(4):825–33. [PubMed: 25140417]
20. Ono S Regulation of actin filament dynamics by actin depolymerizing factor/cofilin and actin-interacting protein 1: new blades for twisted filaments. Biochemistry. 2003;42(46):13363–70. [PubMed: 14621980]

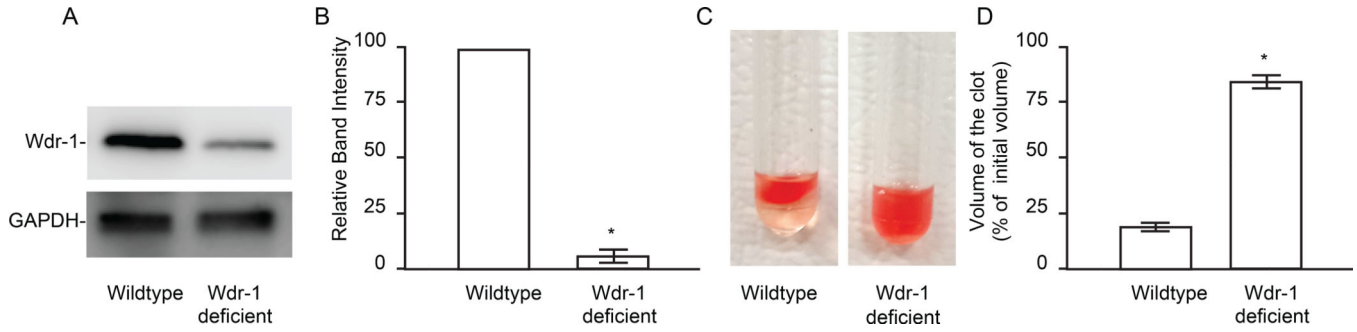

Figure 1. Clot retraction in Wdr-1-deficient mice.

Panels A. Platelets from wildtype and Wdr-1-deficient mice were isolated, solubilized in 1% SDS and subjected to PAGE followed by immunoblot with antibodies to Wdr-1 or GAPDH. **Panel B.** A representative ratios of relative band intensities of Wdr-1 (means and SD) of three independent experiments are shown where the relative intensity to the corresponding wild type was considered as 1 for comparison. **Panel C,** a representative image of clot retraction by platelets at 30 min after stimulation with 0.5 U/mL of thrombin. **Panel D**, the volume of clot expressed as percentage of the initial volume. * denotes a P value of less than 0.05 compared to wildtype.

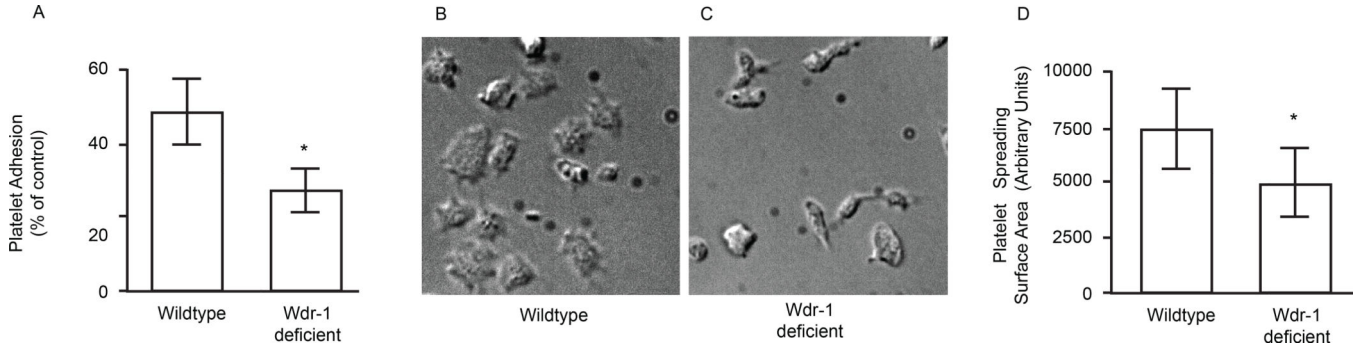

Figure 2: Adhesion and spreading of Wdr-1-deficient platelets on fibrinogen substrate.

Panel A. Washed platelets were incubated for 30 min at room temperature in microtiter wells coated with fibrinogen, and the adherent platelets solubilized. The supernatants were aspirated, the wells washed and platelets bound to each well was measured as described before (10). Washed platelets from wildtype littermate controls **(Panel B)** and Wdr-1-deficient **(Panel C)** mice were allowed to adhere and spread on fibrinogen-coated surface and examined by differential interference contrast microscopy. **In Panel D**, the platelet surface area was quantified using image J software and the mean and standard deviation of three independent experiments were plotted. * denotes a P value of less than 0.01 compared to wildtype.

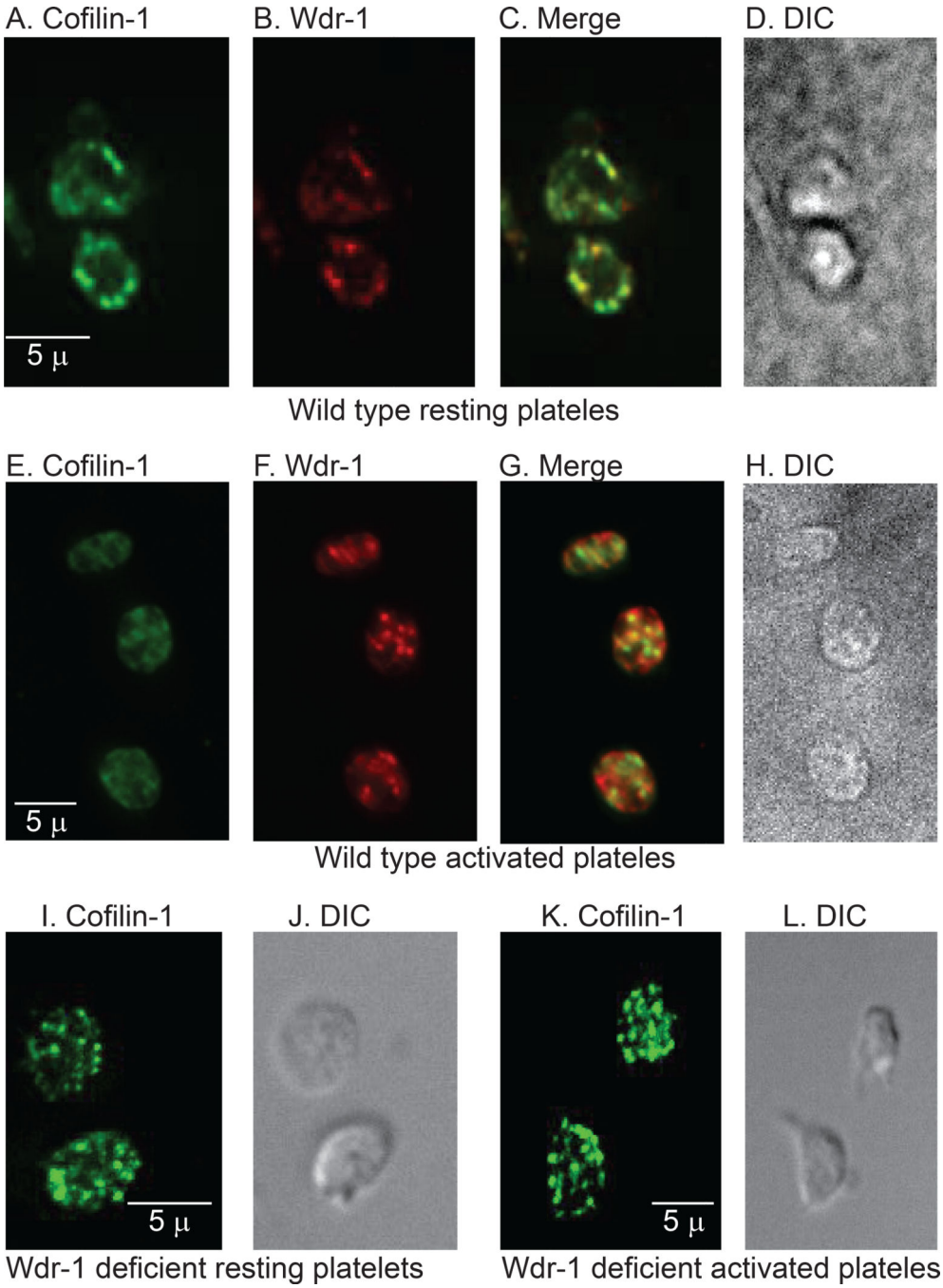

Figure 3. Cofilin-1 and Wdr-1 distribution and in wildtype platelets.

Panels A-H. Washed mouse platelets were immobilized on a polylysine-(Panels A-E) or fibrinogen-coated cover slips (Panels E-H), fixed, permeabilized and incubated with antiWdr-1 (Panels A and D) or anticofilin-1 (panels B and F) antibodies followed by appropriately-labeled secondary antibody. The merge of images is shown in Panels C and G. Images were taken on a deconvolution microscope with 1000 × magnification and the corresponding DIC images are shown in Panels D and H. **Cofilin-1 distribution in Wdr-1-deficient mouse platelets. Panels I-L.** Washed Platelets from Wdr-1-deficient mice were

immobilized on a polylysine (Panels I and J) or fibrinogen-coated cover slips (Panels K and L), fixed, permeabilized, cofilin-1 distribution was analyzed by deconvolution microscopy in Panels I and K and the corresponding DIC image are shown in Panels J and L

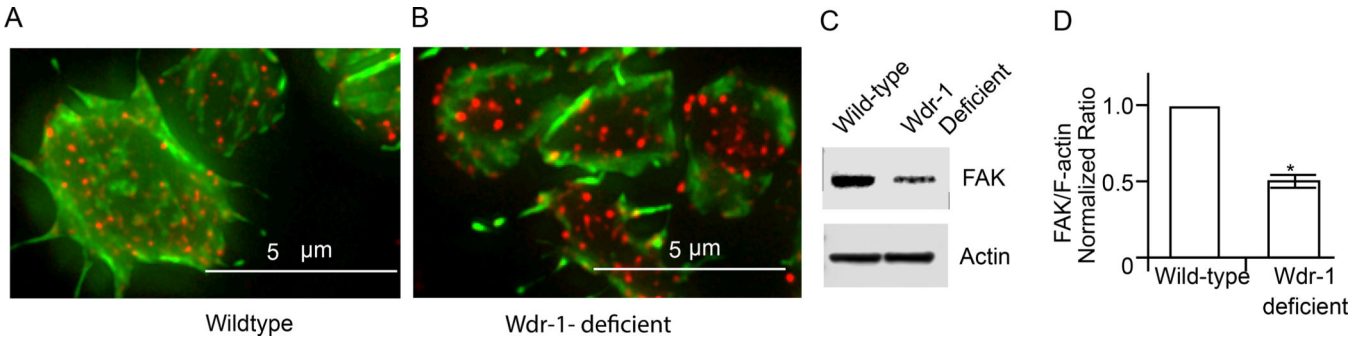

Figure 4. Defective interaction of F-actin with focal adhesion.

Wildtype **(Panel A)** and Wdr-1 deficient **(Panel B)** platelets were allowed to spread on fibrinogen, fixed and permeabilized and the F-actin fibers where visualized with Alexa 488 phalloidin (green fluorescence) and focal adhesions were visualized with antiFAK antibody (red fluorescence). Impaired colocalization was indicated by enhanced red fluorescence in Wdr-1-deficient mouse platelets. **Panel C**. F-actin filaments were isolated from wildtype and Wdr-1-deficient mice and the incorporation of FAK to actin filaments was determined by immunoblots. The ratio of the densitometry value of actin to FAK **(Panel D)** was determined in each separate experiment and the values for wildtype platelets were arbitrarily normalized to 1 for comparison. A representative blot of means and standard deviations of the ratios from three independent experiments are shown. * denotes a P value of less than 0.01 compared to wildtype.