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ORIGINAL ARTICLE

TC21/RRas2 regulates glycoprotein VI–FcRγ-mediated platelet activation and thrombus stability

S. JANAPATI,* J. WURTZEL,† C. DANGELMAIER,* B. K. MANNE,* D. BHAVANASI,* J. C. KOSTYAK,* S. KIM,*[1] M. HOLINSTAT,‡ S. P. KUNAPULI* and L. E. GOLDFINGER†§

**The Sol Sherry Thrombosis Research Center and Department of Pharmacology, Lewis Katz School of Medicine at Temple University; †The Sol Sherry Thrombosis Research Center and Department of Anatomy & Cell Biology, Lewis Katz School of Medicine at Temple University, Philadelphia, PA; ‡Department of Pharmacology, Department of Internal Medicine, Division of Cardiovascular Medicine, University of Michigan, Ann Arbor, MI; and §Cancer Biology Program, Fox Chase Cancer Center, Philadelphia, PA, USA*

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Essentials

- RAS proteins are expressed in platelets but their functions are largely uncharacterized.
- TC21/RRas2 is required for glycoprotein VI-induced platelet responses and for thrombus stability *in vivo*.
- TC21 regulates platelet aggregation by control of $\alpha_{IIb}\beta_3$ integrin activation, via crosstalk with Rap1b.
- This is the first indication of functional importance of a proto-oncogenic RAS protein in platelets.

Summary. *Background:* Many RAS family small GTPases are expressed in platelets, including RAC, RHOA, RAP, and HRAS/NRAS/RRAS1, but most of their signaling and cellular functions remain poorly understood. Like RRAS1, TC21/RRAS2 reverses HRAS-induced suppression of integrin activation in CHO cells. However, a role for TC21 in platelets has not been explored. *Objectives:* To determine TC21 expression in platelets, TC21 activation in response to platelet agonists, and roles of TC21 in platelet function in *in vitro* and *in vivo* thrombosis. *Results:* We demonstrate that TC21 is expressed in human and murine platelets, and is activated in response to agonists for the glycoprotein (GP) VI–FcRγ immunoreceptor tyrosine-based activation motif (ITAM)-containing collagen receptor, in an Src-dependent manner. GPVI-induced platelet aggregation, integrin $\alpha_{IIb}\beta_3$ activation, and α-granule and dense granule secretion, as well as phosphorylation of Syk, phospholipase Cγ2, AKT, and extracellular signal-regulated kinase, were inhibited in TC21-deficient platelets *ex vivo*. In contrast, these responses were normal in TC21-deficient platelets following stimulation with P2Y, protease-activated receptor 4 and C-type lectin receptor 2 receptor agonists, indicating that the function of TC21 in platelets is GPVI–FcRγ-ITAM-specific. TC21 was required for GPVI-induced activation of Rap1b. TC21-deficient mice did not show a significant delay in injury-induced thrombosis as compared with wild-type controls; however, thrombi were unstable. Hemostatic responses showed similar effects. *Conclusions:* TC21 is essential for GPVI–FcRγ-mediated platelet activation and for thrombus stability *in vivo* via control of Rap1b and integrins.

Keywords: blood platelets; collagen receptors; embolism and thrombosis; monomeric GTP-binding proteins; RAS proteins.

Correspondence: Lawrence Goldfinger, Department of Anatomy and Cell Biology and the Sol Sherry Thrombosis Research Center, Lewis Katz School of Medicine at Temple University, 3420 N. Broad Street, Philadelphia, PA, USA
Tel.: +1215 707 8157
E-mail: goldfinger@temple.edu

[1]Present address: College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk, Korea

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Introduction

Platelets rely on fast, highly coordinated cytosolic signal transduction to mediate rapid responses to prothrombotic stimuli. These 'inside-out' signaling responses lead to activation of $\alpha_{IIb}\beta_3$ integrin adhesion receptors, which bind fibrinogen, allowing platelets to maintain hemostasis in response to vascular injury on a time scale of seconds to minutes [1]. The RAS family small GTPase Rap1b is a proximal player in integrin activation by recruiting talin [2–9]. Many pathways of Rap1b activation in platelets have been studied; however, the molecular basis of platelet activation remains incompletely understood. Previous

studies have shown that other RAS small GTPases may have some functions in platelets, but the RAS isotypes involved in platelet functional responses were not identified [10,11]. Much of the knowledge regarding RAS small GTPases and integrins comes from overexpression studies. HRAS was found to suppress activation of chimeric, ectopically expressed integrins in CHO cells, whereas RRAS1 and RRAS2/TC21 can reverse suppression by HRAS [7,8,12,13]. Proteomic analysis of palmitoylated proteins demonstrated moderate expression of several RAS isotypes in platelets, including HRAS, NRAS, and RRAS1 [14].

RRAS2, also known as TC21 and encoded by the *RRAS2* gene, shares features with both oncogenic RAS and RRAS subgroups. RRAS2/TC21 can stimulate the RAL, phosphoinositide 3-kinase (PI3K) and RAF–MEK–extracellular signal-regulated kinase (ERK) pathways, and is strongly tumorigenic; also, somatic mutations that make it insensitive to inactivation by GAPs are prevalent in many human tumors. TC21 also regulates cell migration and survival; these functions resemble those of RRAS1 [15–19]. TC21, like RRAS1, can also reverse the suppression of activation of chimeric integrins by HRAS [13]. *Rras2*$^{-/-}$ mice are lymphopenic, owing to poor survival and homeostatic proliferation of peripheral T and B cells, reflecting a TC21-dependent T-cell receptor (TCR) recycling mechanism: TC21 constitutively associates with the TCR and B-cell receptor (BCR) immunoreceptor tyrosine-based activation motif (ITAM) and recruits PI3K to these sites, which is required for its activation [20,21]. Despite these defects, *Rras2*$^{-/-}$ knockout mice appear grossly normal (viable and fertile), but have a delay in mammary gland development, owing to a reduction in terminal end buds and ductal branching [20]. We have found that TC21 is highly expressed in murine and human platelets. In this study, we investigated roles for TC21 in inside-out integrin signaling, platelet aggregation, hemostasis, and thrombosis.

Materials and methods

Reagents

2MeSADP, sodium citrate, fucoidan, acetylsalicylic acid, apyrase (type VII) and bovine serum albumin (fraction V) were from Sigma (St. Louis, MO, USA). AYPGKF was custom-synthesized by Invitrogen (Carlsbad, CA, USA). Collagen-related peptide (CRP) was from R. Farndale at the University of Cambridge. Convulxin was purified as described previously [22]. Type I collagen was from Chrono-log (Havertown, PA, USA). Phycoerythrin-conjugated JON/A antibodies, fluorescein isothiocyanate (FITC)-conjugated anti-P-selectin antibodies and FITC-conjugated anti-glycoprotein (GP) VI antibodies were from Emfret Analytics (Wurzburg, Germany). Mouse anti-TC21 antibodies were from Abnova (Walnut, CA, USA). 2,3-Dihydro-3-[(1-methyl-1*H*-indol-3-yl) methylene]-2-oxo-1*H*-indole-5-sulfonamide (OXSI-2) was from Tocris Biosciences (Minneapolis, MN, USA). IV.3 (CD32) antibodies were from Stemcell Technologies (Cambridge, MA, USA), and goat anti-mouse IgG (Fab′2) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). FcRγ antibodies were from Sigma (St Louis, MO, USA). Anti-phospho-Syk (Tyr525/Tyr526), anti-phospho-phospholipase Cγ2 (PLCγ2) (Tyr759), anti-phospho-AKT (Ser473), anti-phospho-ERK (Thr202/Tyr204) and anti-β-actin antibodies were from Cell Signaling Technology (Beverly, MA, USA). PP2 and PP3 were from Enzo Life Sciences (Farmingdale, NY, USA). Human fibrinogen was from Enzyme Research Laboratories (Enzo Life Sciences, Farmingdale, NY, USA). PPACK (Enzo Life Sciences) was from Glycotech (Gaithersburg, MD, USA). RAF-1 and RALGDS glutathione-*S*-transferase (GST)–RBD proteins were prepared as described previously [23,24]. All other reagents were reagent-grade, and deionized water was used throughout.

Animals

Rras2$^{-/-}$ mice were a gift from B. Alarcón, Universidad Autónoma de Madrid, Madrid, Spain. These mice were backcrossed 10 times against a C57Bl/6 background. Wild-type (WT) C57Bl/6 mice were from Charles River Laboratories (Wilmington, MA, USA). All mice were housed in a pathogen-free facility, and all animal procedures were approved by the Temple University Institutional Animal Care and Use Committee. Age-matched and gender-matched WT mice were used as controls.

Preparation of human and murine platelets

Human and murine platelets were isolated from blood as described previously [25]. Approval was obtained from the Institutional Review Board of Temple University. Informed consent was provided prior to blood donation, in accordance with the Declaration of Helsinki.

Platelet aggregation and secretion

Platelet ATP release and aggregation were performed simultaneously with a lumi-aggregometer, as described previously [25].

Flow cytometry

Washed murine platelets were labeled with phycoerythrin-conjugated JON/A antibodies or FITC-conjugated anti-P-selectin antibodies, and detected as described previously [26].

Western blotting

Platelets were stimulated with agonists for the appropriate time, and protein levels and phosphorylation events were assessed as described previously [27].

GTP–TC21 and GTP–Rap1b pulldown assays

Washed platelets (0.5 mL, 2×10^9 mL^{-1}) were stimulated with different agonists in an aggregometer, and reactions were stopped by the addition of 250 μL of 3 × HEPES lysis buffer (55 mM HEPES, 174 mM NaCl, 30% glycerol, 6 mM EGTA, 6 mM $MgCl_2$, 3% NP40, pH 7.5) with 10 μM GTP and 20 μg GST–RBD of RAF-1 or RALGDS [23,24]. Platelet lysates were clarified by centrifugation at 13 000 × *g* for 5 min at 4 °C. Fifty-microliter supernatants were separated for whole cell lysate samples, and 40 μL of glutathione–agarose beads were added to the remaining supernatant and kept on a rocker for 2 h at 4 °C. Bead-bound complexes were washed three times with 1 × HEPES wash containing 10 μM GTP, eluted with SDS sample buffer, and analyzed by western blotting.

Immunofluorescence microscopy

These experiments were performed as described previously [28,29].

Platelet spreading and clot retraction

These experiments were performed as described previously [25].

$FeCl_3$-induced in vivo thrombosis and bleeding times

Adult mice (aged 10–12 weeks) were anesthetized by intraperitoneal injection of pentobarbital (40 mg kg^{-1}), and $FeCl_3$-induced thrombosis was assessed as described previously [30]. Bleeding times following tail clip were assessed in 6–8-week-old male and female mice, as described previously [30].

Blood flow over collagen

Blood was collected from 12–14-week-old mice via cardiac puncture into tubes containing 400 mM PPACK and 5 U mL^{-1} heparin as anticoagulant. Blood was passed over surfaces coated with 50 μg mL^{-1} type I collagen at 500 s^{-1} for 3 min by use of a parallel-plate flow chamber kit from Glycotech. The chamber was flushed with phosphate-buffered saline, and images were obtained with a Nikon Eclipse TE300 at × 400 magnification (Nikon, Melville, NY, USA). Thrombus area was quantified with IMAGEJ. At least five randomly chosen images were used for analysis per experiment.

Statistical analysis

Each experiment was performed at least three times unless otherwise indicated. All statistical tests were carried out using PRISM software (version 3.0). Data are presented as means + standard error of the mean. Statistical significance was determined with Student's *t*-test and ANOVA.

Results

Expression of the small GTPase TC21 in platelets and involvement in GPVI-induced platelet functional responses

Early studies showed that 'RAS' (isotype not specified) can be activated in platelets, and RAS proteins appear to be important for platelet functions [10,11,31]. RRAS1 is also expressed in human platelets [14], but its functions are unclear. Western blot analysis with TC21 mouse mAbs confirmed the findings of prior proteomic studies [32], and demonstrated that TC21 is expressed in both human and murine platelets, at levels similar to those in peripheral blood mononuclear cells, in which TC21 plays important functional roles [21,33,34] (Fig. 1A). There are currently no selective pharmacological inhibitors of TC21. To evaluate the functions of TC21 in platelets, we investigated agonist-induced platelet responses *ex vivo* in platelets isolated from TC21-null (*Rras2*$^{-/-}$) mice [21]. TC21 is expressed in WT murine platelets but not in TC21-null murine platelets (Fig. 1B). TC21-null mice had decreased circulating platelet counts relative to WT mice, indicating potential defects in either platelet production or platelet lifespan; however, mean platelet volumes were in the normal range (Fig. 1C). *Ex vivo* platelet aggregation and dense granule secretion induced by the GPVI-specific agonists CRP and convulxin were inhibited in TC21-null platelets as compared with WT platelets (Fig. 2A). These defects were reversed with higher concentrations of CRP, suggesting compensatory mechanisms in the absence of TC21 with higher doses of agonist. Similarly, collagen-induced aggregation and dense granule secretion were inhibited with low concentrations of agonist (0.5–0.75 μg mL^{-1}), but not at higher concentrations (Fig. 2B). However, platelet aggregation and secretion in response to the protease-activated receptor (PAR) 4-activating peptide AYPGKF or P2Y receptor agonist 2MeSADP were similar in TC21-null and WT platelets (Fig. 2C), indicating that G-protein-coupled receptor agonists induce normal platelet aggregation and secretion in the absence of TC21. Thus, TC21 is required for platelet aggregation and dense granule secretion downstream of GPVI receptor pathways.

TC21 regulates platelet GPVI-mediated integrin activation and α-granule secretion

Platelet aggregation results from the activation of integrin $\alpha_{IIb}\beta_3$, mediated by inside-out signaling downstream of

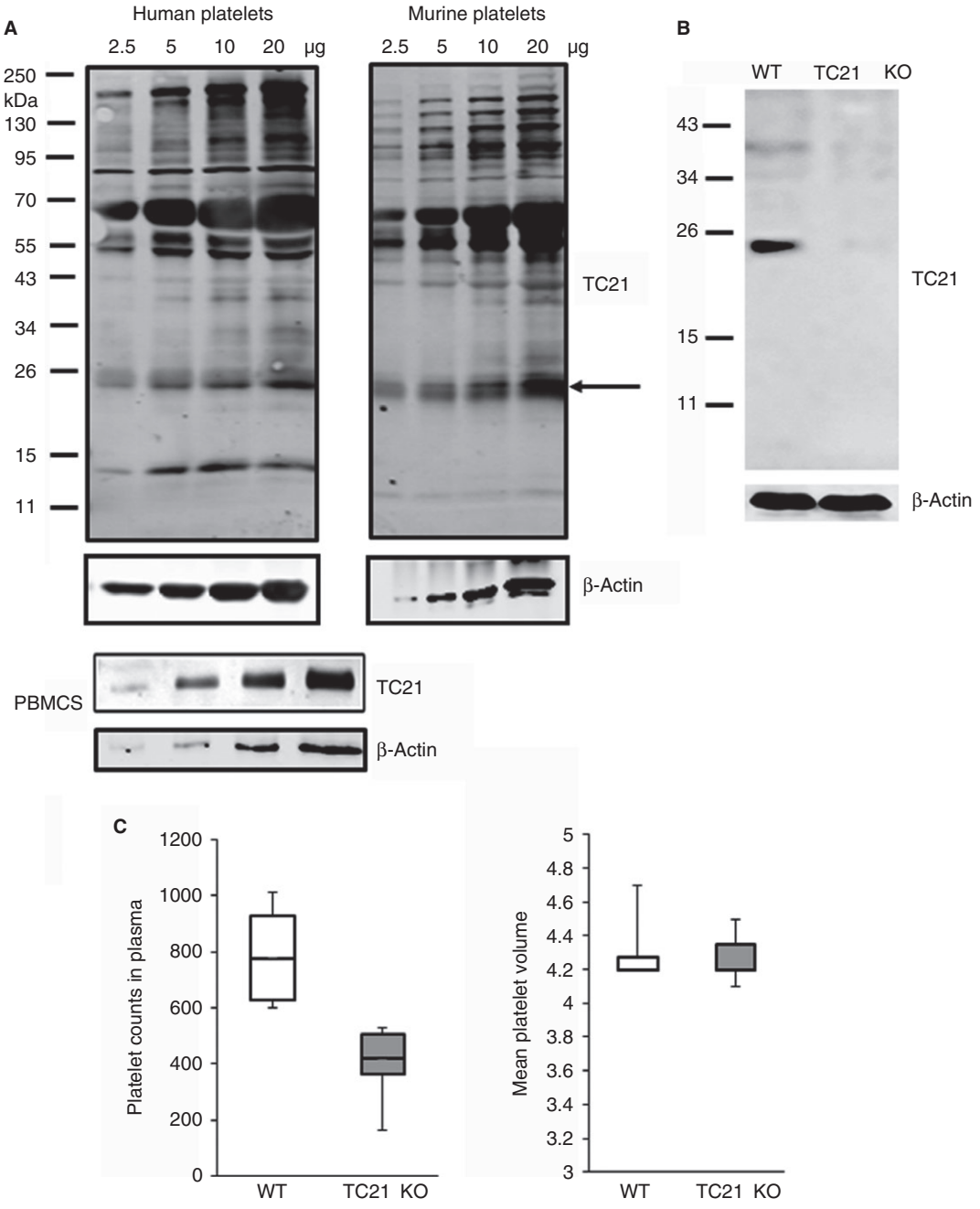

Fig. 1. Expression of TC21/RRas2 in human and murine platelets. (A) Whole cell lysates (WCLs) of washed human (2×10^8 mL^{-1}) and murine (1.5×10^8 mL^{-1}) platelets and peripheral blood mononuclear cells (PBMCs) were separated by SDS-PAGE and immunoblotted with TC21 antibodies. Hemavet analysis showed no detectable levels of leukocytes or erythrocytes. (B) Western blots of WCL samples from wild-type (WT) and TC21-null (TC21 knockout [KO]) C57Bl/6 mouse platelets, with TC21 antibodies. Hemavet analysis showed no detectable levels of leukocytes or erythrocytes. (C) Platelet counts in plasma (left) and mean platelet volume (right) were assessed by Hemavet analysis. $n = 6$.

ligation of signaling receptors [1]. We analyzed the agonist-induced activation of integrin $\alpha_{IIb}\beta_3$ by measuring binding of the conformation-dependent antibody JON/A to stimulated platelets by using flow cytometry [35]. Consistent with a defect in GPVI-mediated platelet aggregation, we found that JON/A binding induced by different concentrations of CRP and convulxin was significantly inhibited in TC21-null platelets as compared with WT platelets (Fig. 3A). Thus, TC21 is required for GPVI-mediated inside-out signaling leading to integrin activation and platelet aggregation. P-selectin surface expression induced by CRP and convulxin was also significantly blocked in TC21-null platelets (Fig. 3B), indicating defects in α-granule secretion [36]. However, at both low and high concentrations, AYPGKF-induced and 2MeSADP-induced JON/A binding and P-selectin

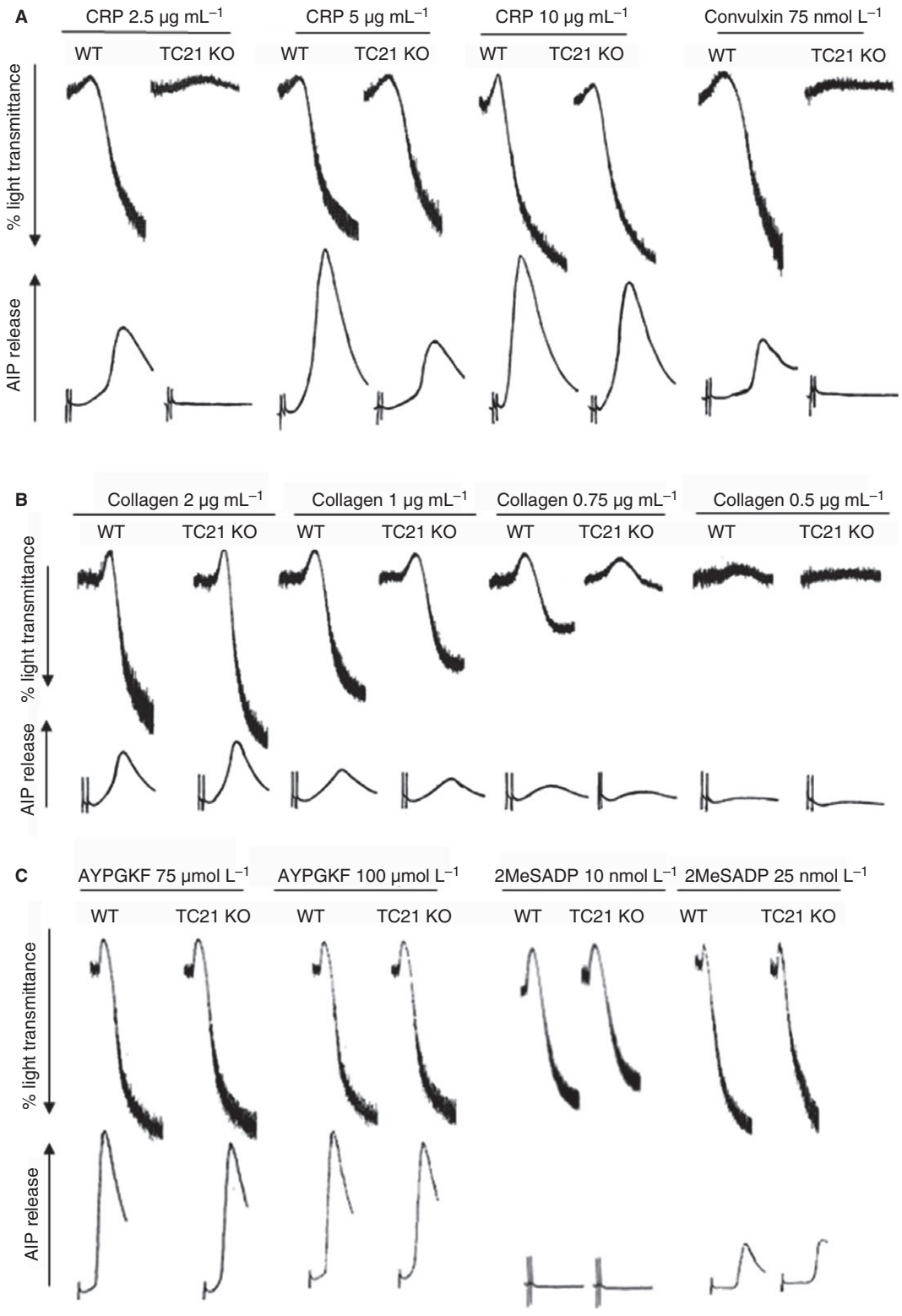

Fig. 2. TC21 is required for glycoprotein VI-induced platelet aggregation and dense granule secretion. TC21-null (TC21 knockout [KO]) and wild-type (WT) murine platelets isolated from whole blood were resuspended in phosphate buffer and apyrase at physiological pH, placed under stirring conditions in a lumi-aggregometer in the presence of luciferin–luciferase reagent, and then stimulated with collagen-related peptide (CRP) (2.5, 5 and 10 μg mL^{-1}) and convulxin (75 nM) (A), collagen (0.5, 0.75, 1 and 2 μg mL^{-1}) (B) or AYPGKF (75 and 100 μM) and 2MeSADP (25 nM) (C) for 3.5 min. Representative aggregation (top) and dense granule secretion (bottom) tracings from three independent experiments are shown.

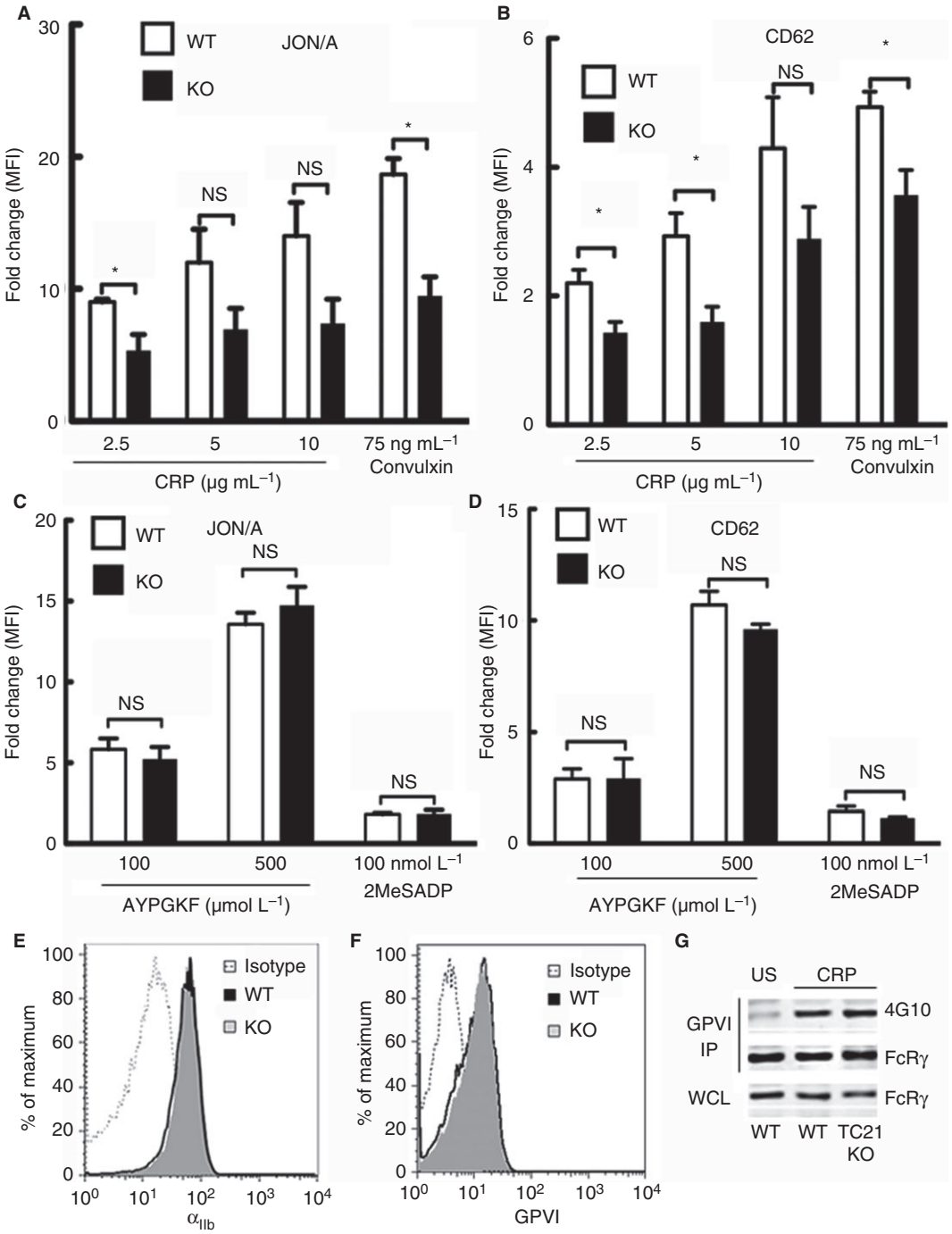

Fig. 3. TC21 is required for glycoprotein (GP) VI-induced platelet $\alpha_{IIb}\beta_3$ integrin activation and α-granule secretion. (A–D) Washed platelets from TC21-null (knockout [KO]) and wild-type (WT) murine platelets were stimulated with the indicated doses of collagen-related peptide (CRP) or convulxin (A, B), or AYPGKF or 2MeSADP (C, D) for 15 min in the presence of phycoerythrin-labeled JON/A antibodies (A, C) or fluorescein isothiocyanate-labeled anti-P-selectin antibodies (CD62) (B, D), and labeling was assessed by flow cytometry. Mean fluorescence intensities (MFIs) are shown as fold change from unstimulated (US) control, and are representative data from three independent experiments, + standard error of the mean. $*P < 0.05$. (E, F) Washed platelets were labeled with antibodies against GPVI (E) or α_{IIb} integrin (F), and fluorescence intensities were detected by fluorescence-activated cell sorting. (G) Washed platelets from TC21-null and WT mice were either kept US or treated with 2.5 μg mL^{-1} CRP for 60 s, lysed, and subjected to immunoprecipitation (IP) with anti-GPVI antibodies. IP fractions were separated by SDS-PAGE and western blotting with the indicated antibodies. The FcRγ band migrated at ~ 14 kDa. NS, not significant; WCL, whole cell lysate.

expression in TC21-null platelets were similar to those in WT platelets (Fig. 3C,D), indicating that G-protein-coupled receptor agonists cause integrin $\alpha_{IIb}\beta_3$ activation and α-granule secretion independently of TC21. Surface expression levels of integrin $\alpha_{IIb}\beta_3$ and GPVI in TC21-null platelets were comparable to those in WT platelets

(Fig. 3E, F). Expression of FcRγ was also similar to that in WT platelets, and FcRγ phosphorylation in response to platelet stimulation with CRP was also similar to that in WT platelets (Fig. 3G). Thus, TC21 is selectively required for GPVI-mediated activation of $\alpha_{IIb}\beta_3$ integrin, dense granule and α-granule secretion, and aggregation responses in platelets.

Inhibition of GPVI-mediated downstream signaling events in platelets in the absence of TC21

Many signaling players in the GPVI integrin activation pathway have been investigated in platelets [37,38]. To determine whether TC21 regulates these signaling events, we assessed GPVI-mediated signaling in lysates of TC21-null or WT platelets stimulated with GPVI agonists, by immunoblotting with phospho-specific antibodies: anti-Syk (Y525/526), anti-PLCγ2 (Y759), anti-AKT (S473), and anti-ERK1/2 (T202/Y204). CRP treatment of WT platelets at 2.5 μg mL^{-1} caused a rapid signaling response, including phosphorylation of each of these targets. In contrast, CRP-induced phosphorylation of Syk, PLCγ2, AKT and ERK1/2 was reduced in TC21-null platelets (Fig. 4A). Similar results were observed with CRP stimulation at 5 μg mL^{-1}, with repression of GPVI signaling responses in TC21-null platelets. However, with CRP stimulation at 10 μg mL^{-1}, GPVI signaling responses were similar in TC21-null and WT platelets (Fig. 4A). When platelets from TC21-null mice were stimulated with AYPGKF or 2MeSADP, phosphorylation levels of AKT and ERK were comparable to those in WT platelets (Fig. 4B). These results demonstrate that TC21 regulates GPVI downstream signaling responses, and suggest a receptor-proximal role, upstream of Syk, for TC21 in GPVI-mediated signaling phosphorylation events.

TC21 activation induced by GPVI receptor agonists requires Src family kinases (SFKs)

The above results demonstrate a requirement for TC21 expression in GPVI signaling in platelets, and suggest that the molecular function of TC21 may be an important factor in these responses. Therefore, we assessed TC21 activation in human platelets following GPVI agonist stimulation, by using a standard pulldown assay for GTP-bound RAS proteins, employing the RAS-binding domain of RAF-1 fused to GST (GST–RBD) as bait bound to glutathione–sepharose beads [23]. This GST fusion protein is predicted to bind to activated, GTP-bound TC21 [15,19], such that immunoblotting of the precipitated fractions indicates a ratio of activated TC21 and total TC21 from the whole platelet lysate. TC21 was precipitated by GST–RBD in lysates from human platelets stimulated with CRP and convulxin (GPVI agonists), but not in those from unstimulated platelets, confirming the utility of this approach for assessing TC21 cellular activation in platelets. CRP and convulxin – which are structurally distinct GPVI agonists – each caused rapid activation of TC21 in human platelets, as early as 30 s and persisting for up to 120 s (Fig. 5A). Thus, GPVI signaling activates TC21 in platelets. Interestingly, TC21 was also activated rapidly upon platelet stimulation with the PAR4 agonist AYPGKF, and the C-type lectin receptor 2 (CLEC-2) agonist fucoidan (Fig. 5B).

Although signaling through multiple receptors activated TC21 in platelets, the GPVI platelet response was selectively impaired by TC21 deletion (Figs 2–4). As GPVI-coupled Fc receptors harbor ITAM motifs that are subject to tyrosine phosphorylation by SFKs, leading to downstream signaling events [37,38], we investigated whether TC21 activation by GPVI requires SFK activation, by using the SFK inhibitor PP2 and the control analogue PP3. TC21 activation by CRP was completely blocked in the presence of PP2, but not in the presence of PP3, demonstrating that GPVI–FcRγ-mediated TC21 activation requires SFK activity (Fig. 5C). We evaluated CRP-induced TC21 activation in murine platelets, and found similar activation profiles as in human platelets (Fig. 5D, left panels; see also Fig. 5A). TC21 was upstream of Syk activation induced by GPVI stimulation (Fig. 4); however, TC21 could also be downstream of Syk in a putative feedback loop. Therefore, we tested CRP-induced TC21 activation under conditions of Syk inhibition in WT murine platelets, and found that TC21 activation was unaffected by the Syk inhibitor OXSI-2 (Fig. 5D). Thus, TC21 is upstream of Syk in the GPVI pathway and is not subject to negative feedback effects. However, negative feedback downstream of Syk at later time points could not be ruled out. On the basis of the ability of TC21 to interact with ITAM receptors in other cells, we considered whether TC21 may associate with the GPVI–FcRγ complex. GPVI was detected in TC21 immunoprecipitate fractions after CRP stimulation, indicating that TC21 physically associates with the GPVI complex upon receptor ligation (Fig. 5E). To investigate further a role for TC21 in ITAM signaling in platelets, we assessed TC21 activation following crosslinking of the FcγRIIa receptor, which is not expressed in murine platelets. This ITAM receptor drives platelet signaling through similar pathways as GPVI–FcRγ, to affect aggregation and thrombus formation [39,40]. Crosslinking of FcγRIIa in human platelets induced rapid activation of TC21 (Fig. 5F), suggesting a conserved ITAM-mediated mechanism for TC21 activation in platelets.

TC21 is required for GPVI-induced Rap1b activation in platelets

The RAS family small GTPase Rap1b plays an essential, proximal role in the activation of $\alpha_{IIb}\beta_3$ integrin in platelets, by stimulating the recruitment of talin and cofactors

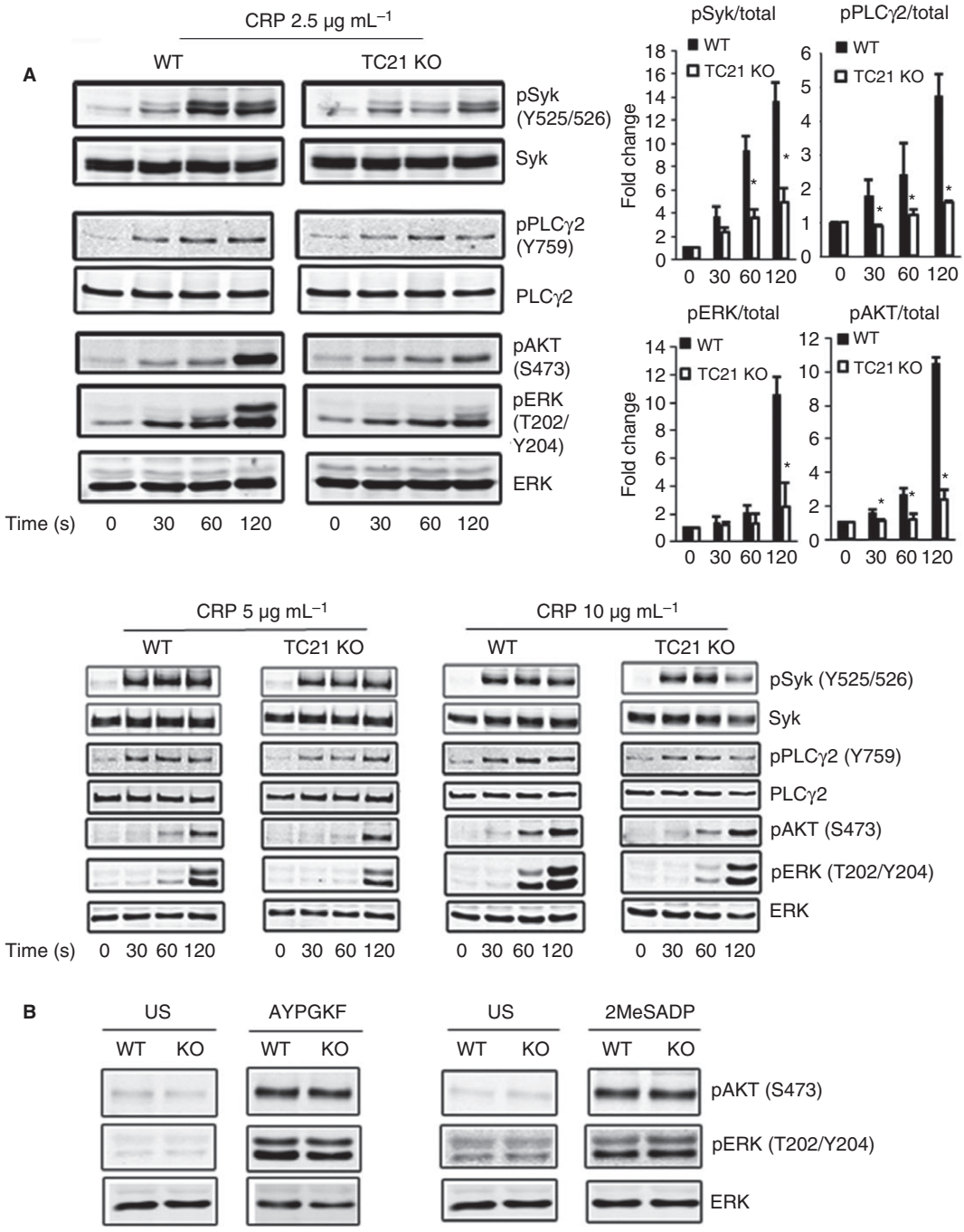

Fig. 4. Glycoprotein VI-mediated downstream signaling events are inhibited in TC21-null platelets. Washed platelets isolated from TC21-null (TC21 knockout [KO]) and wild-type (WT) mice were stimulated for the indicated times with collagen-related peptide (CRP) (A) at the indicated concentrations or (B) AYPGKF (100 μM) or 2MeSADP (25 nM) (60 s each), and lysed; lysates were immunoblotted with antibodies as shown. Phosphoprotein/total protein ratios from six independent experiments are shown on the right in (A) for 2.5 μg mL^{-1} CRP treatment. Representative blots are shown. $*P < 0.03$. ERK, extracellular signal-regulated kinase; PLCγ2, phospholipase Cγ2; US, unstimulated.

to the integrin tails [9]. As shown in Fig. 6, Rap1b activation induced by CRP was abolished in TC21-null platelets, in contrast to robust Rap1b activation in WT platelets. Thus, TC21 regulates GPVI-mediated integrin activation and platelet activation through crosstalk signaling required for activation of Rap1b.

CLEC-2-mediated platelet aggregation and downstream signaling events in TC21-deficient platelets

To confirm that the block in integrin-mediated platelet aggregation and secretion responses in TC21-null platelets was GPVI-specific, we assessed aggregation and

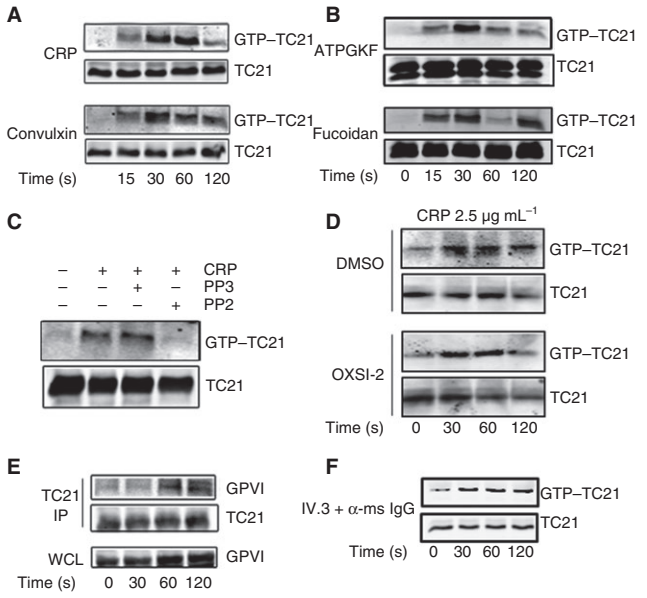

Fig. 5. TC21 activation by glycoprotein (GP) VI receptor agonists downstream of Src kinases. (A, B) Washed platelets were stimulated with 2.5 μg mL^{-1} collagen-related peptide (CRP) or 75 ng mL^{-1} convulxin (A), or 500 μM AYPGKF or 100 μg mL^{-1} fucoidan (B), and lysed; GTP-bound TC21 (upper panels) was then extracted from platelet lysates (lower panels, total TC21) by the use of glutathione-*S*-transferase–RAF-1-RBD coupled to gluthatione–sepharose beads. (C) TC21 activation induced by 5 μg mL^{-1} CRP for 60 s was evaluated in the presence of 10 μM PP2 or PP3 as indicated. (D) Washed platelets were incubated with 1 μM 2,3-dihydro-3-[(1-methyl-1*H*-indol-3-yl) methylene]-2-oxo-1*H*-indole-5-sulfonamide (OXSI-2) or dimethylsulfoxide (DMSO) vehicle for 5 min, stimulated with CRP as shown, and then evaluated for total and GTP-bound TC21 as in (A). (E) TC21 was immunoprecipitated from IgG–sepharose precleared lysates of GPVI-stimulated platelets, with 2 μg of anti-TC21 antibody. Washed immunoprecipitate fractions were subjected to western blotting with antibodies against TC21 and GPVI as indicated. (F) FcγRIIa was crosslinked with 2.5 μg mL^{-1} IV.3 antibodies followed by 50 μg mL^{-1} anti-mouse IgG (Fab′2) for the indicated times. GTP-bound, activated TC21 was precipitated from platelet lysates and detected by western blotting as above. All data are representative of three independent experiments each. α-ms, anti-mouse; IP, immunoprecipitation; WCL, whole cell lysate.

secretion in response to fucoidan, a sulfated polysaccharide that stimulates platelet activation via Syk and PLCγ2, but through the CLEC-2 receptor, which harbors a hemi-ITAM motif [41], independently from the GPVI–FcRγ receptor complex at low concentrations [42]. Fucoidan-induced platelet aggregation, $\alpha_{IIb}\beta_3$ integrin activation and α-granule secretion were normal in both WT and TC21-null platelets (Fig. S1A–C). In addition, fucoidan treatment induced Syk, PLCγ2, AKT and ERK1/2 phosphorylation in TC21-null platelets to similar levels as in WT platelets (Fig. S1D). These results indicate that TC21 is not important for platelet aggregation or Syk and AKT phosphorylation downstream of the CLEC-2 receptor, and confirm that TC21 selectively functions downstream of the GPVI–FcRγ receptor complex in platelet activation.

Regulation of hemostasis and thrombosis in vitro *and* in vivo *by TC21*

We next assessed the contributions of TC21 to thrombus formation and stability *in vitro* and *in vivo*. We first investigated thrombus formation *in vitro* in whole blood flowing over collagen-coated surfaces. As shown in Fig. 7A, thrombus formation over collagen-coated surfaces was significantly decreased in blood from TC21-null mice as compared with that from WT mice. On the basis of these results, we investigated putative roles for TC21 in hemostasis and injury-induced thrombus formation and stability *in vivo*. We first measured bleeding times following tail clip, and found significantly increased bleeding times in TC21 knockout mice (Fig. 7B). We measured time to occlusion in the carotid artery, following 7.5% $FeCl_3$-induced injury for 90 s, in TC21-null and WT mice. As shown in Fig. 8A, *in vivo* thrombosis at the site of injury appeared to be slightly delayed, as observed by increased time to occlusion, in TC21-null mice as compared with WT mice. The average time to occlusion after injury in TC21-deficient mice was 10.5 min, as compared with 8 min in WT mice (Fig. 8B). However, this delay in initial time to occlusion was not significant. In contrast, embolization of the thrombi was observed in 64% of TC21-null mice, as compared with 18% of WT mice ($n = 11$), indicating unstable thrombi after arterial injury in these mice (Fig. 8C). This result is consistent with prolonged bleeding in the tail clip thrombosis model in TC21-null mice. Thus, the small GTPase TC21 is required in platelets for rapid thrombus formation and maintenance of thrombus stability in response to arterial injury in mice.

TC21 effects on outside-in signaling in platelets

To obtain a broader picture of the contributions of TC21 to platelet responses, we analyzed platelet spreading on immobilized fibrinogen and thrombin-induced clot retraction *ex vivo*. TC21-null platelets spread to a similar degree as WT platelets (Fig. S2A). Similarly, thrombin-induced clot retraction with TC21-deficient platelets proceeded at the same rate as with WT platelets *ex vivo* (Fig. S2B). Thus, TC21 is not required for outside-in signaling in platelets. Fibrin has also been shown to activate GPVI by direct binding to the receptor [43,44]; however, we did not observe differences in spreading on fibrin between WT and TC21-null platelets, whereas TC21-null platelets did not spread efficiently on collagen or CRP as compared with WT platelets (Fig. S3), indicating that fibrin signaling in platelets does not require TC21.

Discussion

This study is the first to demonstrate a functional role for a proto-oncogenic RAS small GTPase – TC21/RRAS2 –

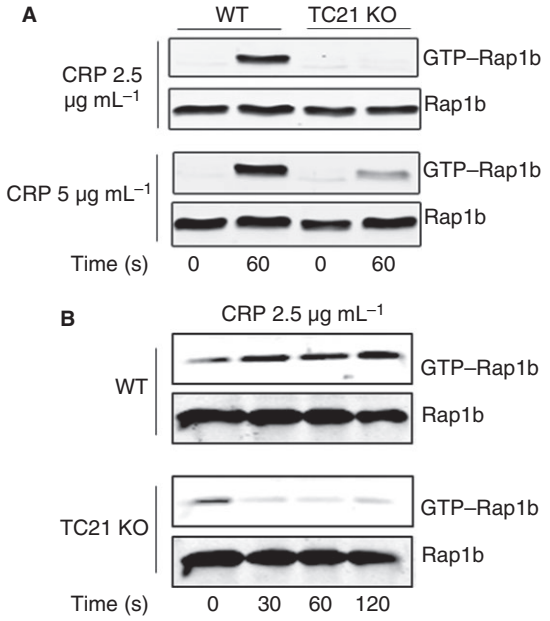

Fig. 6. TC21 is required for glycoprotein VI-mediated Rap1b activation. GTP–Rap1b pulldown (upper panels) with glutathione-*S*-transferase (GST)–RALGDS-RBD from lysates (total Rap1b, lower panels) of wild-type (WT) or TC21-null (knockout [KO]) platelets stimulated with collagen-related peptide (CRP) at the indicated concentrations and for the indicated times. (A) Sixty seconds of stimulation with 2.5 or 5 μg mL^{-1} CRP. (B) Time course with 2.5 μg mL^{-1} CRP.

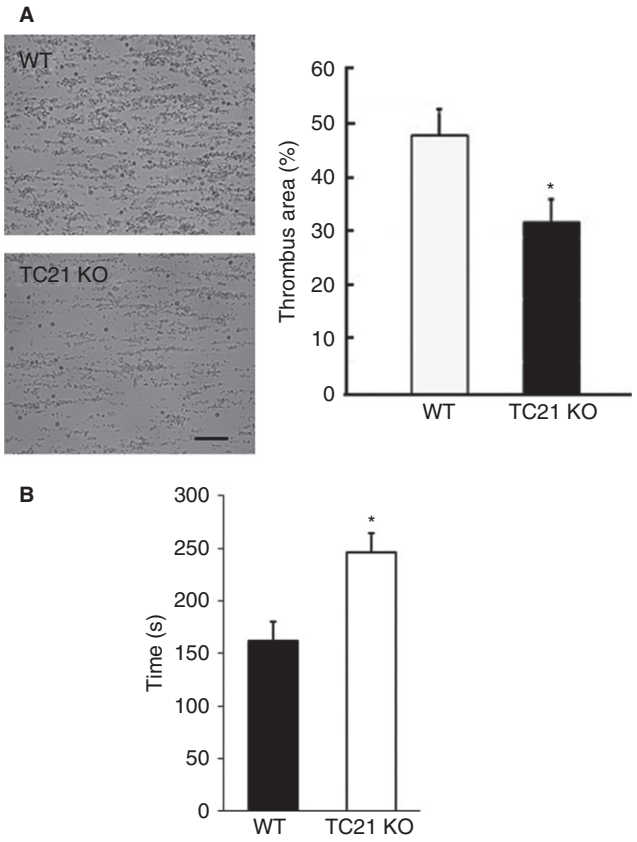

Fig. 7. TC21 regulates thrombus formation over collagen surfaces and *in vivo* hemostasis. (A) Representative images of thrombus formation on collagen surfaces in blood from wild-type (WT) and TC21-null (TC21 knockout [KO]) mice in a flow chamber. Percentages of the imaging area covered by platelets are shown to the right, + standard error of the mean. $*P < 0.05$. ($n = 5$). Scale bar: 50 μm. (B) Bleeding times following tail clip in WT and TC21-null mice. $*P < 0.002$. ($n = 20$).

in platelet functional responses, hemostasis, and thrombosis. TC21-deficient mice showed prolonged times to cessation of bleeding and a tendency to rebleed in a tail clip hemostasis model, reduced platelet deposition on collagen surfaces in whole blood under flow, and thrombus embolization in the carotid artery following injury, indicating a role for TC21 in both thrombus formation and thrombus stability. Thrombus instability was attributable to a defect in activation of $\alpha_{IIb}\beta_3$ integrins in TC21-deficient platelets downstream of impaired platelet signaling responses. TC21-dependent platelet responses were selective for the GPVI–FcRγ collagen receptor, and TC21 was activated by GPVI stimulation upstream of Syk, pointing to TC21 activation as a receptor-proximal event. TC21 was also required for downstream GPVI-mediated Rap1b activation, demonstrating RAS GTPase crosstalk and a RAS/Rap1b linkage in GPVI-mediated integrin activation in platelets.

Prolonged bleeding in TC21 knockout mice in a tail clip hemostasis model, and thrombus embolization in a vascular thrombosis model, together suggest that TC21 plays an important role in the strength of platelet adhesion and aggregation. This was supported by a defect in TC21-deficient platelet thrombus formation *in vitro* in blood flowing over collagen surfaces. In addition, decreased platelet counts in TC21-deleted mice may also contribute to impaired thrombosis and hemostasis. Many hereditary platelet functional disorders, such as Bernard–Soulier syndrome, von Willebrand disease, and Glanzmann's thrombasthenia, result from compromised platelet adhesive capacity, leading to complications of either spontaneous bleeding or thrombosis defects [45–48]. Thus, it is conceivable that somatic or inherited mutations in TC21 may correlate with bleeding tendencies in humans, but this merits further investigation.

The role of TC21 in platelet aggregation and thrombus stability correlated with a role in activation of the $\alpha_{IIb}\beta_3$ integrin fibrinogen receptor, indicating that TC21-mediated thrombus stability is attributable to a requirement for TC21 in platelet integrin activation. RAS family proteins, including HRAS, RRAS1, and TC21, have been associated with regulation of integrin activation in other contexts, and these studies indicated a positive role for TC21 in integrin activation [12,13,49]. However, these studies were focused on ectopic expression of both integrins and RAS proteins in heterologous cells. Thus, an integrin regulatory function for RAS proteins had not previously been recognized in platelets. Many studies have characterized upstream

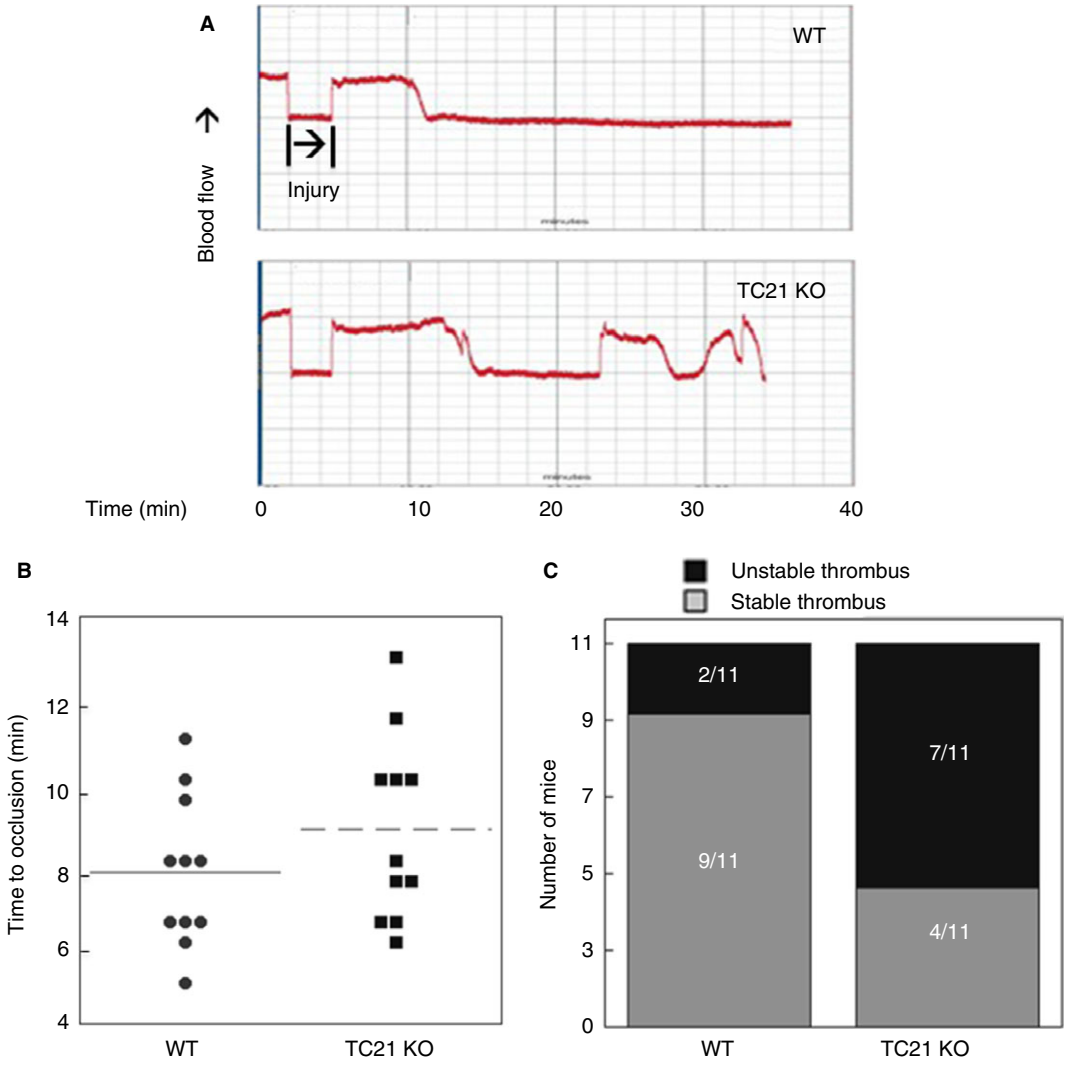

Fig. 8. Delay and instability of *in vivo* arterial thrombus formation in TC21-null mice. (A) Representative Doppler tracings of blood flow in the carotid artery following $FeCl_3$-induced injury in TC21-null (TC21 knockout [KO]) and wild-type (WT) mice. (B, C) Time to occlusion (B) and stability of thrombus formation (C) as determined by lack of restored blood flow in Doppler tracings, $n = 11$ each. [Color figure can be viewed at wileyonlinelibrary.com]

regulators of RAS as playing roles in platelet integrin activation and function, including CALDAG-GEFs, which are important for platelet adhesion and aggregation [50–54], and an early report identified the GRB2 RAS activation complex in these processes [55]. However, many of these GEF proteins also stimulate activation of Rap1, which is an integrin-proximal player essential for integrin activation [9,56]. Hence, a direct role for proto-oncogenic RAS in platelet integrin activation was not demonstrated. To our knowledge, this study is the first to demonstrate a functional role for a RAS protein (other than Rap1), i.e. TC21, in the regulation of native integrins in platelets, which is required for thrombus stability *in vitro* and *in vivo*.

TC21 signaling leading to integrin activation in platelets was selectively downstream of the GPVI collagen receptor, and TC21 appears to propagate signaling proximal to the receptor. TC21 was activated by GPVI agonists (CRP and convulxin), indicating receptor-mediated stimulation of a RasGEF promoting GTP loading of TC21. The notion of TC21 as a receptor-proximal signal transducer is consistent with our findings that GPVI-induced TC21 activation required Src family kinase activity, and was upstream of PLCγ2, AKT, and ERK [37,57–59]. TC21 was activated by GPVI stimulation under conditions of Syk inhibition, placing TC21 upstream of Syk with minimal negative feedback signaling from Syk to TC21. Moreover, TC21 associated with GPVI following CRP agonist stimulation, indicating that TC21 forms a molecular complex with GPVI upon ligation. Thus, a GPVI → Src → TC21 → Syk pathway regulates platelet activation. However, precisely how TC21 functions upstream of Syk in a Src-dependent manner remains to be fully elucidated. Fibrin can activate GPVI by direct binding[43,44]; however, the platelet signaling

responses of this interaction are poorly understood. We did not observe fibrin-dependent spreading or clotting defects in TC21-null platelets, suggesting that collagen-mediated GPVI signaling through TC21 represents a distinct pathway from fibrin–GPVI signaling. As TC21 was also activated by AYPGKF and fucoidan, it appears that TC21 can be activated by multiple upstream signaling pathways in platelets. Furthermore, TC21 was also activated in human platelets by crosslinking of the FcγRIIa receptor, which is similar to the GPVI/FcRγ receptor, signaling via ITAM motifs in the Fc chains. This pathway has parallels with roles of TC21 in lymphocytes, where TC21 associates with BCR (B cells) and TCR (T cells) via interaction with ITAM motifs in each receptor, both of which propagate signaling through Syk pathways [21,33,60,61]. The GPVI–FcRγ receptor complex also contains ITAM motifs (in the FcRγ chain) [38,62], suggesting a possible conserved mechanism of TC21–ITAM motif association in receptor signaling in hematopoietic cells. Many RAS family GEFs stimulate RAS activation as a result of recruitment to receptor phosphotyrosines, typically via adapter proteins – such a mechanism has been shown to connect the cell adhesion receptor CEACAM3 with activation of RAC via direct binding of the RAC-GEF VAV to phosphotyrosines in the receptor ITAM [56,63], suggesting the potential for a conserved mechanism of ITAM–GEF–RAS complex formation. However, Syk is generally considered to bind directly to ITAM motifs following phosphorylation by SFKs in many cells, including platelets [64,65]; thus, the role of TC21 in mediating this connection is unknown. Which GEFs mediate GPVI-induced TC21 activation in platelets, and their mechanism of action, also remain to be determined.

Our results demonstrate crosstalk between two RAS GTPases, i.e. TC21/RRAS2 and Rap1b, in platelet signaling and functional responses. TC21 was required for GPVI-mediated Rap1b activation, which is a requisite downstream signaling event leading to $\alpha_{IIb}\beta_3$ integrin activation in platelets, as well as for activation of other integrins through a common mechanism [9,51]. Thus, we predict that, in the absence of TC21, talin recruitment to integrin tails is compromised, preventing allosteric activation of the integrin ligand-binding domain [9,66,67]. The molecular mechanisms by which TC21 regulates Rap1b activation in platelets remain to be explored. However, TC21 may be involved in integrin regulation in other contexts with varied physiological effects, e.g. Schwann cell migration and tumor metastasis, which have been associated with increased TC21 activity [68–71].

Recent interest in hemostasis and thrombosis research has been focused on identifying pharmacological targets such as regulators of integrin activation, which strike a balance between minimal bleeding events and maintenance of hemostasis [72,73]. GPVI-deficient mice do not show impaired hemostasis [74]; however, the hemostasis defect observed in TC21-deficient mice may also be attributable to lower platelet counts. Deficiency of TC21 is compatible with life, suggesting that targeting a TC21 pathway in platelets may have few unwanted effects. Thus, the TC21 pathway may be a viable target for intervention in pathological conditions driven by thrombus instability, such as atherosclerosis and stroke.

Addendum

L. Goldfinger and S. Kunapuli contributed to the conception and design of the work. S. Janapati, C. Dangelmaier, J. Wurtzel, B. Manne, D. Bhavanasi, J. Kostyak, and S. Kim performed experiments and acquired data. L. Goldfinger, S. Kim, M. Holinstat, and S. Kunapuli were involved in the analysis and interpretation of data. M. Holinstat provided key reagents. S. Kunapuli and L. Goldfinger supervised the experiments and analysis. S. Janapati and L. Goldfinger wrote the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Fig. S1. CLEC-2-mediated aggregation and downstream signaling events in TC21-null platelets.
Fig. S2. TC21 is dispensable for outside-in platelet responses.
Fig. S3. TC21 is dispensable for platelet spreading on fibrin.

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