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Author(s)	Miyashita, Naohiro; Onozawa, Masahiro; Hayasaka, Koji; Yamada, Takahiro; Migita, Ohsuke; Hata, Kenichiro; Okada, Kohei; Goto, Hideki; Nakagawa, Masao; Hashimoto, Daigo; Kahata, Kaoru; Kondo, Takeshi; Kunishima, Shinji; Teshima, Takanori
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Title

A novel heterozygous *ITGB3* p.T720del inducing spontaneous activation of integrin α IIb β 3 in autosomal dominant macrothrombocytopenia with aggregation dysfunction

Authors

Naohiro Miyashita¹, Masahiro Onozawa¹, Koji Hayasaka², Takahiro Yamada³, Ohsuke Migita⁴,
Kenichiro Hata⁴, Kohei Okada¹, Hideki Goto¹, Masao Nakagawa¹, Daigo Hashimoto¹, Kaoru Kahata¹,
Takeshi Kondo¹, Shinji Kunishima⁵, Takanori Teshima¹

¹Departament of Hematology, Hokkaido University Faculty of Medicine, Graduate School of Medicine,
Sapporo, Japan

²Division of Laboratory and Transfusion Medicine, Hokkaido University Hospital, Sapporo, Japan

³Division of Clinical Genetics, Hokkaido University Hospital, Sapporo, Japan

⁴Department of Maternal-Fetal Biology, National Research Institute for Child Health and Development,
Tokyo, Japan

⁵Department of Advanced Diagnosis, Clinical Research Center, National Hospital Organization Nagoya
Medical Center, Nagoya, Japan

Corresponding author

Masahiro Onozawa, MD, PhD

Department of Hematology, Hokkaido University Faculty of Medicine, Graduate School of Medicine,

Sapporo, Japan

Kita 15, Nishi 7, Kita-ku, Sapporo, JAPAN 0608638

Phone: +81-11-706-7214

Fax: +81-11-706-7823

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

Abstract

We identified a novel heterozygous *ITGB3* p.T720del mutation in a pedigree with macrothrombocytopenia exhibiting aggregation dysfunction. Platelet aggregation induced by ADP and collagen was significantly reduced, while ristocetin aggregation was normal. Integrin α Ib β 3 was partially activated in a resting status, but platelet expression of α Ib β 3 was downregulated. Functional analysis using a cell line showed spontaneous phosphorylation of FAK in α Ib/ β 3 (p.T720del)-transfected 293T cells in suspension conditions. Abnormal cytoplasmic protrusions, membrane ruffling, and cytoplasmic localization of α Ib β 3 were observed in α Ib/ β 3 (p.T720del)-transfected CHO cells. Such morphological changes were reversed by treatment with an FAK inhibitor. These findings imply spontaneous, but partial, activation of α Ib β 3 followed by phosphorylation of FAK as the initial mechanism of abnormal thrombopoiesis. Internalization and decreased surface expression of α Ib β 3 would contribute to aggregation dysfunction. We reviewed the literature of congenital macrothrombocytopenia associated with heterozygous *ITGA2B* or *ITGB3* mutations. Reported mutations were highly clustered at the membrane proximal region of α Ib β 3, which affected the critical interaction between α Ib R995 and β 3 D723, resulting in a constitutionally active form of the α Ib β 3 complex. Macrothrombocytopenia caused by a heterozygous activating mutation of *ITGA2B* or *ITGB3* at the membrane proximal region forms a distinct entity of rare congenital thrombocytopenia.

Keywords

Congenital macrothrombocytopenia, FAK phosphorylation, *ITGB3*, integrin α Ib β 3, platelet

Introduction

Congenital macrothrombocytopenia is a group of rare platelet disorders characterized by a decreased platelet count with macrothrombocytes showing a varied bleeding tendency. Mutations causing congenital macrothrombocytopenia have been reported in more than 12 genes including *MYH9*, which is responsible for autosomal dominant *MYH9* disorders, and *GP1BA*, *GP1BB* and *GP9*, which are responsible for Bernard-Soulier syndrome [1-4]. Variants of integrin α Ib, coded by *ITGA2B*, and integrin β 3, coded by *ITGB3*, have also been identified in patients with congenital macrothrombocytopenia [5-12]. The integrin α Ib β 3 complex is expressed on the surface of platelets and megakaryocytes. α Ib β 3 plays essential roles in the processes of platelet aggregation, thrombus formation, and thrombopoiesis through actin remodeling [13-15]. These reported variants are associated with spontaneous, but partial, activation of α Ib β 3 and macrothrombocytopenia with aggregation dysfunction of platelets. The molecular consequences after spontaneous α Ib β 3 activation contributing to macrothrombocytopenia and aggregation dysfunction have not been fully elucidated. Here, we report a novel heterozygous *ITGB3* p.T720del variant in a pedigree of macrothrombocytopenia and its causative mechanisms. We reviewed

the literature and illustrated the distinct clinical entity of this rare congenital macrothrombocytopenia with platelet aggregation dysfunction.

Materials and Methods

Patients

The patients were a 56-year-old Japanese woman (i2 in Fig 1A) and her two sons (ii2 and ii3 in Fig 1A). They had no bleeding tendencies and near-normal bleeding time evaluated by Duke's method (Table 1). Hematological examination revealed mildly decreased platelet counts ($58-75 \times 10^9/L$) with an increase of mean platelet volume (13.4-14.5 fl, Table 1).

Written informed consent was obtained from all family members in accordance with the Declaration of Helsinki. This study was approved by the Institutional Review Board of Hokkaido University Faculty of Medicine.

Genetic analysis

Genomic DNA was obtained from the three affected family members (i2, ii2, and ii3 in Fig 1A) and a non-affected member (ii1 in Fig 1A) in the pedigree. Whole exome sequencing (WES) was performed by HiSeq1500/2500 (Illumina, San Diego, CA) using SureSelect - Human ALL ExomeV6 (Agilent Technologies, Santa Clara, CA). The *ITGB3* gene was amplified using the primer set 5'-

CTCCTGCTTCTTCACAACC-3' and 5'-GGTCTGAGACTCTTAAGTGAAG-3'. The identified

ITGB3 p.T720del variant was confirmed by direct sequencing using the primer 5'-

CTCCTGCTTCTTCACAACC-3'.

Structural analysis

Interaction between α IIb and β 3 molecules was evaluated by in silico model construction using SWISS-MODEL and Cn3D 4.3.1. [16,17].

Platelet glycoprotein analysis

Surface expression of α IIb, β 3, α IIb β 3, and GPIb was evaluated by mean fluorescent intensity (MFI) of CD41 (5B12; Dako, Glostrup, Denmark), CD61 (SZ21; Beckman-Coulter, Fullerton, CA), CD41 (P2; Beckman-Coulter, Fullerton, CA), and CD42b (AN51; Dako, Glostrup, Denmark), respectively.

The activation status of α IIb β 3 was evaluated by binding of the ligand-mimetic antibody PAC-1 (BD Biosciences, Franklin Lakes, NJ) with or without 1 mM of an Arg-Gly-Asp-Ser (RGDS) mimetic antagonist (Sigma-Aldrich, St. Louis, MO) [18]. Platelets were discriminated from other blood cells by GPIb staining. MFI ratio was calculated by dividing MFI of PAC-1 binding on platelets by that of PAC-1 binding in the presence of the RGDS mimetic antagonist. MFI ratio was also calculated by dividing MFI of PAC-1 binding on platelets in the presence of 1 μ M adenosine diphosphate (ADP) by MFI of PAC-1

binding in the absence of ADP. Activation index is defined as $(F_x - F_{\min}) / (F_{\max} - F_{\min})$. F_x is the MFI of PAC-1 binding to the platelets, F_{\min} is the MFI of PAC-1 binding in the presence of the RGDS mimetic antagonist, and F_{\max} is the MFI of PAC-1 binding in the presence of 200 mM phorbol-12-myristate-13-acetate (PMA; Wako, Osaka, Japan).

The activation state of platelets was evaluated by expression of P-selectin, which was measured by the binding of anti-human CD62P antibody (AK-4; BD Pharmingen, Franklin Lakes, NJ) in the presence or absence of 1 μ M ADP. Platelets were discriminated from other blood cells by β 3 staining.

To examine total expression levels of α Ib β 3 in platelets including the cytoplasmic pool, Western blotting was performed using platelet-rich plasma (PRP) lysed with SDS lysis buffer containing 1.7% SDS, 60 mM Tris-HCl, pH 6.8, 0.85% 2-mercaptoethanol, and proteinase inhibitor cocktail (Roche, Basel, Switzerland). α Ib β 3 was detected with anti-integrin β 3 antibody (AB2984, Millipore, Bedford, MA), and protein loading of each well was controlled by anti-GAPDH antibody (V-18, Santa Cruz Biotechnology, Santa Cruz, CA). Platelet specific protein fraction was determined by anti- β 1 tubulin antibody (PD033, Medical & Biological Laboratories, Nagoya, Japan) [19].

Cloning, mutagenesis, and transfection assay

ITGA2B and *ITGB3* cDNAs were amplified from a normal individual's platelet cDNA and cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). *ITGB3* p.D723H and *ITGB3* p.T720del were introduced to

pcDNA3.1/*ITGB3* using a site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). *ITGB3* p.D723H was a functionally proven variant previously reported as a cause for macrothrombocytopenia resembling our cases [6].

The pcDNA3.1/*ITGA2B* and pcDNA3.1/*ITGB3* or its mutant were transiently transfected into 293T cells or Chinese hamster ovary (CHO) cells by Lipofectamine[®] 3000 Reagent (Thermo Fisher Scientific, Waltham, MA). Transfected 293T cells were washed with phosphate-buffered saline and lysed with 1% Triton X-100, 1 mM sodium vanadate, and proteinase inhibitor cocktail to examine tyrosine phosphorylation of focal adhesion kinase (FAK) by Western blotting. Integrin α IIb and integrin β 3 were detected with anti-integrin α IIb antibody (B-9, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-integrin β 3 antibody, respectively. FAK and tyrosine-phosphorylated FAK were detected with anti-FAK antibody (EP695Y; Abcam, Cambridge, UK) and anti-phospho-FAK (Tyr397) antibody (3283, Cell Signaling, Danvers, MA), respectively. Protein loading of each well was assessed by anti-GAPDH antibody. CHO cells cotransfected with pcDNA3.1/*ITGA2B* and pcDNA3.1/*ITGB3* or its mutant were subjected to an adhesion assay to examine morphological changes. Twenty-four hours after transfection, the cells were seeded onto a 100 μ g/ml fibrinogen-coated glass-based dish (IWAKI, Chiba, Japan). After 2-hour incubation at 37°C, the cells were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton-X-100. CHO cells were stained with AB2984 and Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan) followed by Alexa 594-conjugated secondary antibody (Thermo Fisher Scientific,

Waltham, MA) staining. Images were obtained using a Keyence BZ-X700 all-in-one fluorescence microscope (Keyence, Osaka, Japan). Morphological changes were defined as either rhomboidal changes, abnormal cytoplasmic protrusions, or membrane ruffling. One hundred cells were counted to calculate the percentage of cells with morphological changes.

For the FAK inhibitor assay, transfected 293T and CHO cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin/streptomycin and in Roswell Park Memorial Institute 1640 medium with 10% fetal bovine serum and 1% penicillin/streptomycin, respectively, with or without FAK Inhibitor 14 (1,2,4,5-Benzenetetraamine tetrahydrochloride (Y15), Sigma-Aldrich, St. Louis, MO). After 24-hour incubation at 37°C with 5% CO₂, transfected cells were reseeded and tyrosine phosphorylation of FAK and morphological changes were evaluated by Western blotting and immunofluorescence staining, respectively.

Statistical analysis

Total expression levels of $\beta 3$ in the patient's (i2) platelets and those in the control's platelets, levels of tyrosine phosphorylation of FAK in 293T cells transfected with wild-type or mutated $\alpha \text{IIb}/\beta 3$ vectors, and rate of morphological changes in CHO cells transfected with wild-type or mutated $\alpha \text{IIb}/\beta 3$ vectors were compared by the t-test. *P*-values < 0.05 were considered as a statistical significance.

Results

Pedigree of a family with congenital macrothrombocytopenia

In all affected family members, giant platelets were observed in a peripheral blood smear (Fig 1B). Platelet aggregation induced by ADP (10 μ mol/l) and collagen (2 μ g/ml) was obviously reduced, though platelet aggregation induced by ristocetin (1.5 mg/ml) was within the normal limit (Table 1, Fig 1C). The pedigree of the family indicates that the inheritance pattern is autosomal dominant (Fig 1A). Congenital diseases exhibiting macrothrombocytopenia, including MYH9 disorders, Bernard-Soulier syndrome, and type 2B von Willebrand disease, were excluded by aggregation dysfunction of platelets in response to ADP and collagen, normal expression levels of GPIb α (Table 1) and the inheritance pattern, and normal activity of von Willebrand factor (data not shown), respectively.

*Identification of *ITGB3* p.T720del variant*

A total of 1526 gene alterations were detected by WES in the three affected members but not in a non-affected member. Then, gene alterations without exonic and splice site variants and synonymous variants were excluded. As a result, 128 non-synonymous or non-frameshift gene alterations remained. Among them, a novel in-frame heterozygous 3-bp deletion (*ITGB3*, c.2236_2238delACC) resulting in the loss of Thr720 (p.T720del) (Human Genome Variation Society nomenclature for the mature protein) was identified. Direct sequence analysis revealed that all of the three affected family members had *ITGB3*

p.T720del variant as a heterozygous variant (Fig 1D). *ITGB3* p.T720del variant resulted in one threonine loss at the submembrane domain of integrin $\beta 3$.

In silico analysis of the interaction between α IIb and $\beta 3$ molecules revealed that *ITGB3* p.T720del induces conformational alteration of the α IIb $\beta 3$ complex (Fig 1E). *ITGB3* p.T720del variant causes a one amino acid shift downstream, thereafter, resulting in a change from $\beta 3$ -D723 to $\beta 3$ -R723. The positively charged α IIb-R995 faces the negatively charged $\beta 3$ -D723 in wild-type α IIb $\beta 3$, while the positively charged α IIb-R995 faces the positively charged $\beta 3$ -R723 in α IIb $\beta 3$ with *ITGB3* p.T720del. This subtle change expands the distance between the α IIb-R995 and $\beta 3$ -723 positions, like previously reported *ITGB3* p.D723H [6], in which the positively charged α IIb-R995 faces the positively charged $\beta 3$ -H723 in α IIb $\beta 3$ with *ITGB3* p.D723H.

Decreased surface expression and spontaneous partial activation of α IIb $\beta 3$

Flow cytometry was performed to evaluate the expression levels of integrin molecules on the surface of platelets. Surface expression levels of α IIb, $\beta 3$, and α IIb $\beta 3$ on platelets of patient i2 were decreased in comparison with the levels in the control, not only in resting conditions but also in response to ADP and PMA (Table 2). However, Western blotting using the lysate from PRP revealed that there were no significant differences in ~~total relative~~ expression levels of integrin $\beta 3$ compared to $\beta 1$ -tubulin between the affected member and the control (Fig 2A), suggesting that the marked reduction in surface expression

of α IIB β 3 was due to cytoplasmic retention of α IIB β 3. [Relative expression of integrin \$\beta\$ 3 compared to GAPDH was decreased in an affected member reflecting thrombocytopenia \(Fig 2A\).](#) PAC-1 is an antibody that recognizes an epitope exposed on the activated form of the α IIB β 3 complex. Spontaneous PAC-1 binding to the resting patient's (i2) platelets was detected, and α IIB β 3 was more activated in the patient's (i2) platelets than in the control's platelets (Fig 2B). Activation status of α IIB β 3 was evaluated by the activation index, which was higher in the patient's platelets (Fig 2C). On the other hand, α IIB β 3 was less activated in response to ADP in the patient (i2) (Fig 2D). MFI of anti-CD62P binding to resting platelets in the patient (i2) was equal to that in the control, but that in response to ADP was lower in the patient (i2) than in the control (Fig 2E). These findings suggested that α IIB β 3 of the affected family member was spontaneously and partially activated.

p.T720del causes spontaneous phosphorylation of FAK.

FAK is a tyrosine kinase that physically interacts with α IIB β 3, and phosphorylation of the molecule transduces α IIB β 3 outside-in signaling to downstream [20,21]. Phosphorylated FAK promotes cell proliferation and affects cell mobility via actin remodeling [22]. Physiologically, FAK is phosphorylated in adhesion cells, but phosphorylation is cancelled in suspension cells. To evaluate FAK phosphorylation, expression vectors of *ITGA2B* and wild-type or mutated *ITGB3* were transfected to a cell line. In 293T cells cotransfected with *ITGA2B* and *ITGB3* expression vectors, expression of transfected α IIB and β 3

was verified (Fig 3A). One day after transfection, the cells were re-seeded and incubated for one hour. Cells from the suspension fraction and adhered fraction were separately collected for Western blotting. FAK in α IIb/ β 3 (p.D723H)-expressing cells and that in α IIb/ β 3 (p.T720del)-expressing cells were highly phosphorylated with significant differences even under suspension conditions (t-test, $P=0.025$ vs. WT and $P=0.013$ vs. WT, respectively), although there was no significant difference between them in the adhered conditions (Fig 3B).

Mutated β 3 causes morphological changes that can be reversed by an FAK inhibitor.

CHO cells expressing α IIb/ β 3 (p.D723H) or α IIb/ β 3 (p.T720del) showed rhomboidal changes, abnormal cytoplasmic protrusions, and membrane ruffling (Fig 3C). Immunofluorescence staining using anti- β 3 antibody showed membrane localization of α IIb/ β 3 in wild-type α IIb/ β 3-expressing cells and cytoplasmic localization in α IIb/ β 3 (p.T720del)-expressing cells and α IIb/ β 3 (p.D723H)-expressing cells (Fig 3C, right). Few cells with morphological changes were observed in wild-type α IIb/ β 3-expressing CHO cells, but significantly increased numbers of cells with morphological changes were observed in α IIb/ β 3 (p.T720del) or (p.D723H)-expressing cells (Fig 3D). We treated the cells with an FAK inhibitor to determine whether these morphological changes observed in mutated β 3-transfected cells are caused by FAK phosphorylation. FAK Inhibitor 14 is a selective FAK inhibitor that displays no significant activity for a range of other kinases including PDGFR, EGFR, and IGF-IR. FAK Inhibitor 14 has been shown to

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3 prevent FAK autophosphorylation at the putative activation site, tyrosine 397 [23]. The effect of the FAK
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6 inhibitor was verified using 293T cells. FAK Inhibitor 14 hindered tyrosine phosphorylation of FAK in a
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9 dose-dependent manner (Fig 3E). The FAK inhibitor reversed the morphological changes observed in
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12 mutated $\beta 3$ -transfected cells in a dose-dependent manner in CHO cells (Fig 3F).
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19 Discussion

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22 Dysfunction pattern of platelet aggregation in our patients resembles that of Glanzmann
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25 thrombasthenia (GT). GT is caused by a homozygous mutation on *ITGA2B* or *ITGB3*, resulting in a loss
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28 or reduced level of α Ib β 3. These mutations causing GT mostly occur in the extracellular domain of
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31 integrin [24] and are considered to be loss-of-function mutations. Previously, autosomal dominant
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34 macrothrombocytopenia was reported in cases with membrane proximal region variants of either *ITGA2B*
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37 or *ITGB3* (Table 3). So far, 4 variants of *ITGA2B* (p.G991C, p.F993del, p.R995W, p.R995Q) and 3
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40 variants of *ITGB3* (p.L718P, p.D723H, p.L718del) have been reported [12,6,8,10,9,11]. Variants of each
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43 gene are highly clustered in a small region, amino acids 991-995 for *ITGA2B* and 718-723 for *ITGB3*
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46 (Table 3). All reported cases showed giant platelets, mild thrombocytopenia and decreased surface
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49 expression of α Ib β 3 on platelets with only mild or no clinical bleeding tendency. All of the cases had a
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52 heterozygous integrin variant same as our cases. These cases are considered to have the same mechanisms
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55 by which their clinical characteristics developed. Three variants of *ITGA2B* (p.F993del, p.G991C,
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p.R995W) were shown to cause constitutive phosphorylation of FAK [9,10]. We verified the *ITGB3* membrane proximal cytoplasmic tail variant confers constitutive activation of α IIb β 3 and phosphorylation of FAK, as do *ITGA2B* variants.

We confirmed spontaneous but partial α IIb β 3 activation using the patient's (i2) platelets. In a physiological state, inside-out signaling induces a change in α IIb β 3 from an inactive bent form to an extended and activated form by stimulation of agonists such as ADP, collagen, and thromboxane A2 [25-28]. A salt bridge between α IIb R995 and β 3 D723 is an essential interaction to maintain an activation-constraining clasp [29]. However, as shown in Fig 1E, the deletion of threonine (T720) unclasps the highly conserved membrane proximal complex of α IIb and β 3 cytoplasmic tails and constitutes the extended form even in resting conditions (Fig 4). Spontaneous α IIb β 3 activation leads to tyrosine phosphorylation of FAK via constitutive outside-in signaling, which results in abnormal actin remodeling in megakaryocytes, leading to abnormal regulation of proplatelet formation [30,31]. The abnormal actin remodeling is associated with a decreased number and increased size of proplatelet tips, leading to macrothrombocytopenia [6,10]. Thus, the variants around the juxtamembrane region of either α IIb or β 3 work as gain-of-function variants. Morphological changes in α IIb/ β 3 (p.T720del)-expressing CHO cells support these possible mechanisms (Fig 3C). For the first time, we showed that an FAK inhibitor hindered the morphological changes induced by *ITGB3* p.T720del and p.D723H variant. This finding strongly suggests that the FAK signaling directly contributes to the morphological change of transfected

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3 cells. Megakaryocytes extend long cytoplasmic projections called proplatelets, and the tips of the
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6 proplatelets are released as platelets [32]. The RhoA-ROCK-myosin-IIA pathway suppressed proplatelet
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9 formation in premature megakaryocytes [33,34]. Partially activated α IIB β 3 decreased RhoA activity and
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12 induced proplatelet-like protrusion in CHO cells [35]. Phosphorylated FAK would cause
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15 macrothrombocytopenia via RhoA suppression (Fig 4)
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22 Surface expression levels of α IIB β 3 were decreased on the patient's platelets, though the total
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25 expression level of β 3 in patient i2 was the same as that in the control. This observation suggested that
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28 *ITGB3* p.T720del induces the localization change of α IIB β 3. Immunofluorescence staining of transfected
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31 CHO cells also demonstrated cytoplasmic localization of β 3 in α IIB/ β 3 (p.T720del)-expressing CHO cells
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34 (Fig 3C). In platelets, the majority of β 3 exists as α IIB β 3, and the total expression level of β 3 is
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37 considered to represent the total expression level of α IIB β 3 [27,36]. Physiologically, α IIB β 3 is retained
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40 not only on the platelet surface membrane but also in the cytoplasmic pool on the actin cytoskeleton or on
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43 the membrane of α -granules [11,37]. It is also known that activated α IIB β 3 preferentially internalizes in
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46 normal platelets as a mechanism for downregulating adhesiveness of activated platelets in the circulation
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49 [38,39]. Considering this mechanism, the localization change in our patients would be due to the
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52 internalization of spontaneously activated α IIB β 3 (Fig 4).
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We found a novel heterozygous *ITGB3* p.T720del variant that is responsible for autosomal dominant congenital macrothrombocytopenia with abnormal α IIb β 3 localization and aggregation dysfunction of platelets. Although *ITGA2B* and *ITGB3* are mutated molecules in GT, submembranous variant forms a distinct clinical entity that is characterized by autosomal dominant macrothrombocytopenia with aggregation dysfunction. Clinically, this disease is even rarer than GT, but it is important for it to be known widely by physicians in the field. Each congenital thrombocytopenia should be correctly diagnosed to avoid unnecessary administration of corticosteroids under diagnosis as immune thrombocytopenia.

Compliance with ethical standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments. Informed consent was obtained from all patients for being included in the study.

Figure legends

Fig 1.

(A) The family pedigree shows macrothrombocytopenic family members (filled) and non-affected family members (open). Circles and squares mean female and male, respectively.

(B) Peripheral blood smear of the patient (i2) stained with May-Giemsa. The arrows indicate giant platelets.

(C) Results of platelet aggregation tests in response to ADP (Black, 1.0 μ M, Red, 10.0 μ M), collagen (Green, 2.0 μ g/ml, Blue, 5.0 μ g/ml), and ristocetin (1.5 mg/ml) stimuli in platelet-rich plasma from a patient (ii3).

(D) Genomic DNA was amplified by polymerase chain reaction and sequenced. A 3-base-pair deletion (c.2236_2238delACC) was found as a heterozygous variant. One threonine (T720) highlighted in red was expected to be lost in the affected family members.

(E) In silico modeling of α IIb and β 3 interaction. The extracellular domain of α IIb and β 3 was omitted. Wild-type α IIb β 3 complex has a close interaction between α IIb R995 and β 3 D723 at the submembrane region. β 3 p.D723H variant and β 3 p.T720del variant unclasp the membrane proximal interaction of α IIb and β 3 cytoplasmic tails in resting conditions. The upper panel shows a horizontal view and the lower panel shows a view from cytoplasmic tails. Amino acid 723 position is aspartic acid (D) in the wild type but is substituted to histidine (H) in *ITGB3* p.D723H and to arginine (R) in *ITGB3* p.T720del.

Fig 2. Functional analyses of *ITGB3* p.T720del variant using patient's platelets

(A) Western blotting was performed using platelet-rich plasma lysed with SDS lysis buffer. Protein loading was controlled by GAPDH. ~~Although relative expression total amount of β 3 compared to~~

~~GAPDH was decreased in an affected member reflecting thrombocytopenia, relative expression of $\beta 3$ compared to $\beta 1$ -tubulin was not decreased.~~ The upper bar graph~~s~~ shows~~s~~ relative expression of integrin $\beta 3$ compared to $\beta 1$ -tubulin which is platelet specific protein. ~~The lower bar graph shows~~ relative expression of integrin $\beta 3$ compared to GAPDH which reflects both platelet and contaminated mononuclear cells. Values of each samples were calibrated to that of control. Although relative expression of integrin $\beta 3$ compared to GAPDH was decreased in an affected member reflecting thrombocytopenia, relative expression of integrin $\beta 3$ compared to $\beta 1$ -tubulin was not decreased.

Results of immunoblotting are representative of 3 independent transfection experiments. $*P < 0.05$.

N.S., not significant.

- (B) Spontaneous binding of PAC-1 to α IIb β 3 in platelets of an affected family member (i2). Immediately after blood collection, platelets were incubated with PAC-1 in the presence or absence of 1 mM Arg-Gly-Asp-Ser (RGDS) mimetic antagonist and analyzed by flow cytometry. MFI ratio was estimated by dividing MFI of resting platelets by that of resting platelets incubated with RGDS.
- (C) Activation index of α IIb β 3. Relative PAC-1 binding in resting platelets compared with maximal PAC-1 binding in platelets stimulated with PMA is defined as activation index. Activation index in an affected family member (i2) was compared to that in the healthy volunteer.
- (D) Activation of platelet α IIb β 3 in response to ADP in an affected family member (i2). Immediately after blood collection, platelets were incubated with PAC-1 in the presence or absence of 1 μ M ADP and

analyzed by flow cytometry. MFI ratio was estimated by dividing MFI of platelets stimulated with ADP by that of resting platelets.

(E) Lower expression levels of CD62P in an affected family member (i2) in response to ADP.

Immediately after blood collection, platelets were incubated with CD62P in the presence or absence of 1 μ M ADP and analyzed by flow cytometry. MFI ratio was estimated by dividing MFI of platelets stimulated with ADP by that of resting platelets.

Fig 3. Results of transfection assay using cell lines

(A) Expressions levels of integrin α IIb and β 3 in transiently transfected 293T cells were shown by Western blotting. Whole cell lysates were immunoblotted.

(B) Tyrosine phosphorylation of FAK. Whole cell lysate was immunoblotted by anti-FAK (EP695Y) and anti-phospho-FAK (3283). Quantification of the phosphorylation state of FAK is also shown. The phosphorylation state of FAK was quantified as a density ratio of protein bands, pFAK/FAK. Values of each samples were calibrated to that of WT in an adhered condition. Results of immunoblotting are representative of 3 independent transfection experiments. * $P < 0.05$ vs. WT in a suspension condition. FAK in α IIb/ β 3 (p.D723H)-expressing cells and that in α IIb/ β 3 (p.T720del)-expressing cells were highly phosphorylated with significant differences even under the suspension conditions.

(C) CHO cells transfected with α IIb/ β 3 (p.D723H) and α IIb/ β 3 (p.T720del) were seeded onto

fibrinogen-coated coverslips. After 2-hour incubation at 37°C, the cells were fixed and stained with AB2984 and Hoechst 33342 followed by staining with Alexa 594-conjugated secondary antibody. Rhomboid-like change, abnormal cytoplasmic protrusions, and membrane ruffling were observed by α IIB β 3 mutants. Arrowheads indicate abnormal cytoplasmic protrusions. Scale bar represents 15 μ m. The panel on the right shows the intensity of the β 3 fluorescence signal along with x axis obtained by ImageJ (NIH, Bethesda, MD).

(D) One hundred cells were counted to calculate the percentage of cells with morphological change.

Results are means plus or minus SD from 3 independent counts. * $P < 0.01$.

(E) Whole cell lysate was collected 24 hours after transfection of *ITGA2B* and each *ITGB3* expression vector with or without the FAK inhibitor Y15 (10 μ M, 50 μ M). Phosphorylation status of FAK was analyzed by Western blotting. The bar graph shows relative phosphorylation of FAK analyzed by pFAK/FAK. In each *ITGB3* transfection, the value of FAK inhibitor-treated samples was calibrated to an FAK inhibitor untreated sample. Results are means plus or minus SD from 3 independent experiments. * $P < 0.05$. N.S., not significant.

(F) Morphological changes of *ITGA2B* and *ITGB3*-transfected CHO cells were analyzed. One hundred cells were counted to calculate the percentage of cells with morphological change. Results are means plus or minus SD from 3 independent counts. * $P < 0.05$.

Fig 4. Molecular mechanisms of gain of function induced by *ITGB3* p.T720del.

ITGB3 p.T720del unclasps the highly conserved membrane proximal complex of α Ib and β 3 cytoplasmic tails and constitutes the extended form of the α Ib β 3 complex, leading to constitutive phosphorylation of FAK. In megakaryocytes, tyrosine-phosphorylated FAK downregulates RhoA, which induces dissociation of the actin-myosin IIA bridge and promotes abnormal actin remodeling. Formation of proplatelets formation in premature megakaryocytes resulted in production of giant platelets and thrombocytopenia. In platelets, activated α Ib β 3 is internalized and the surface expression of α Ib β 3 decreases, leading to aggregation dysfunction.

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Fig 1

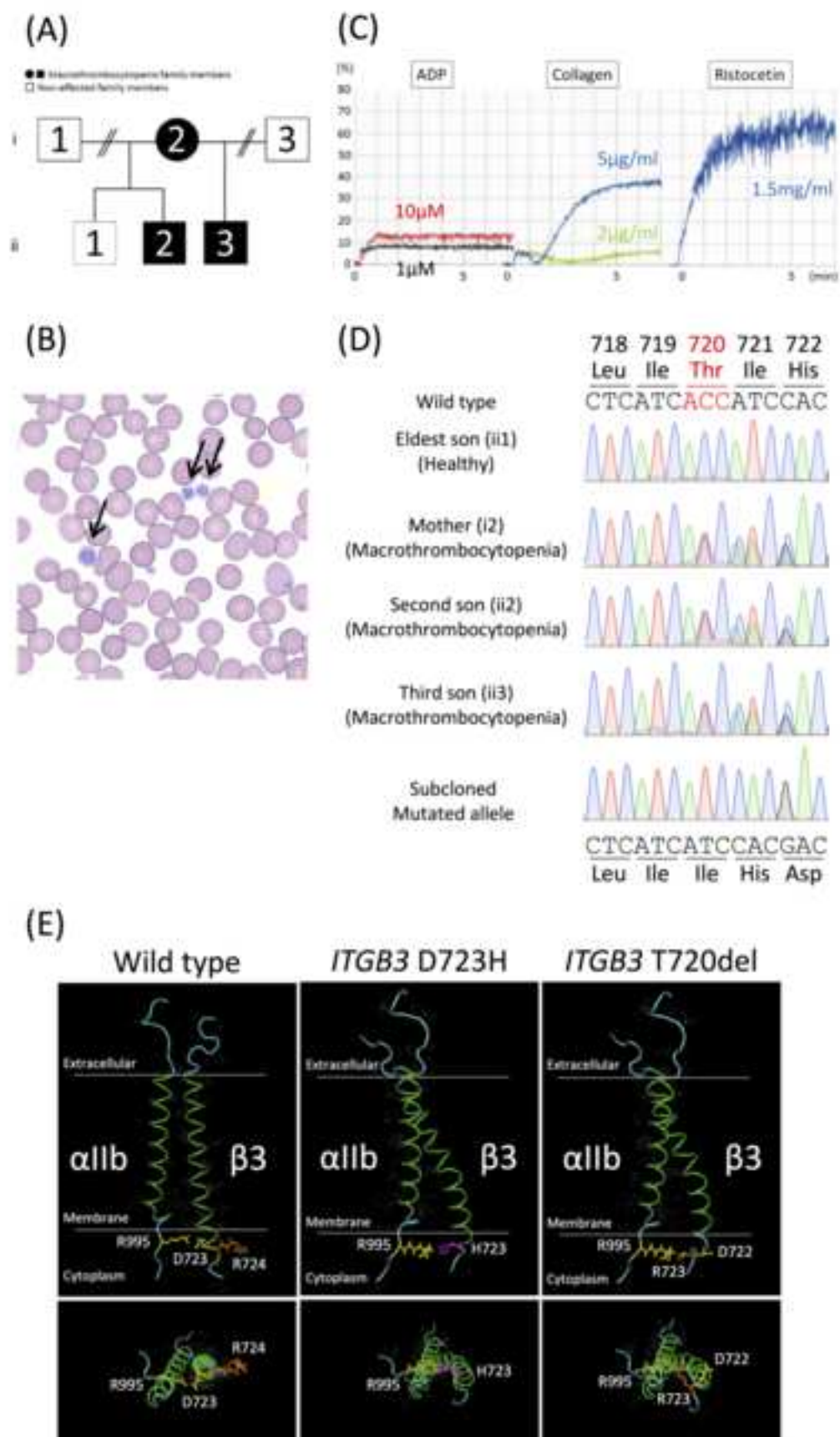


Fig 2

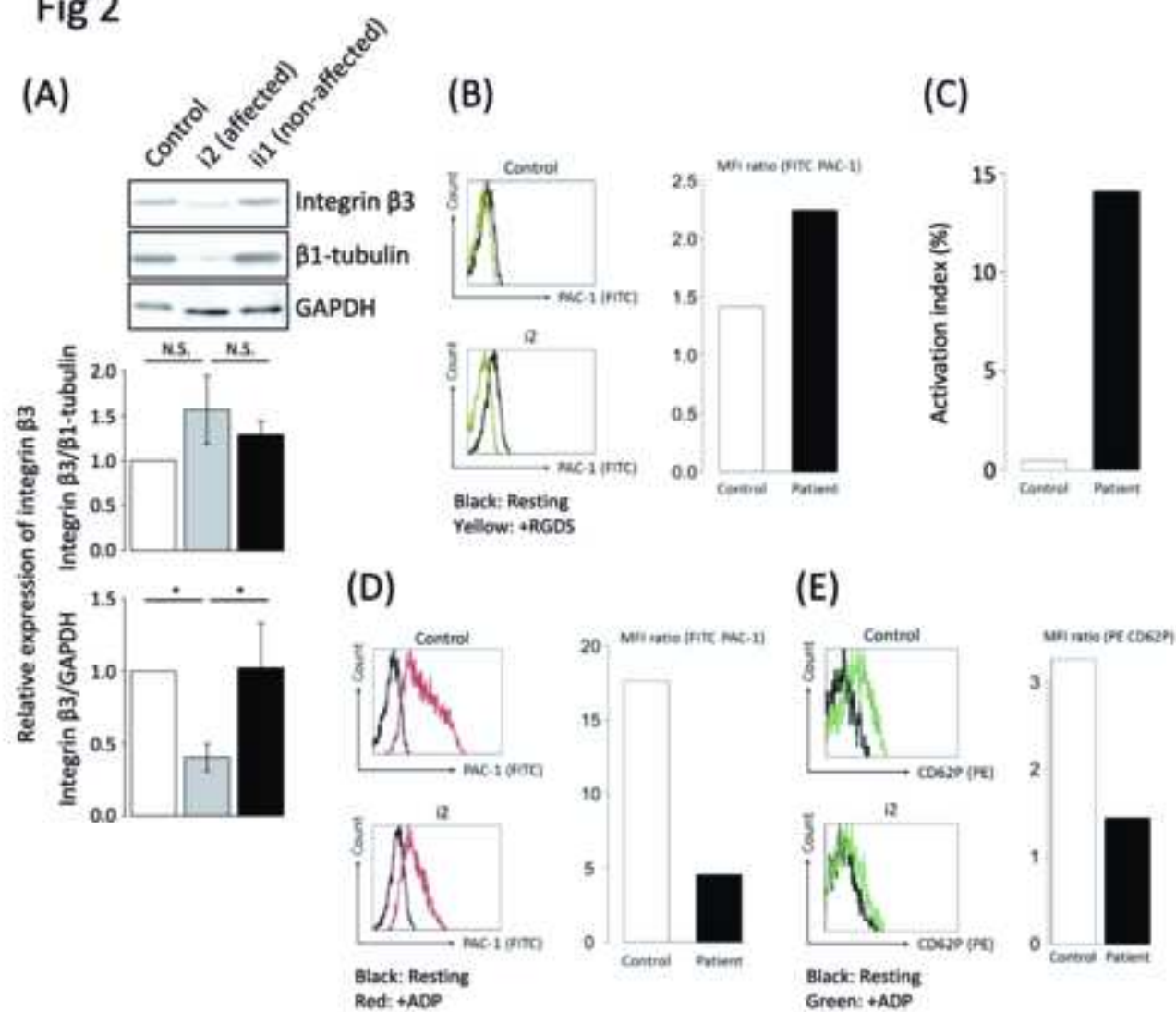


Fig 3

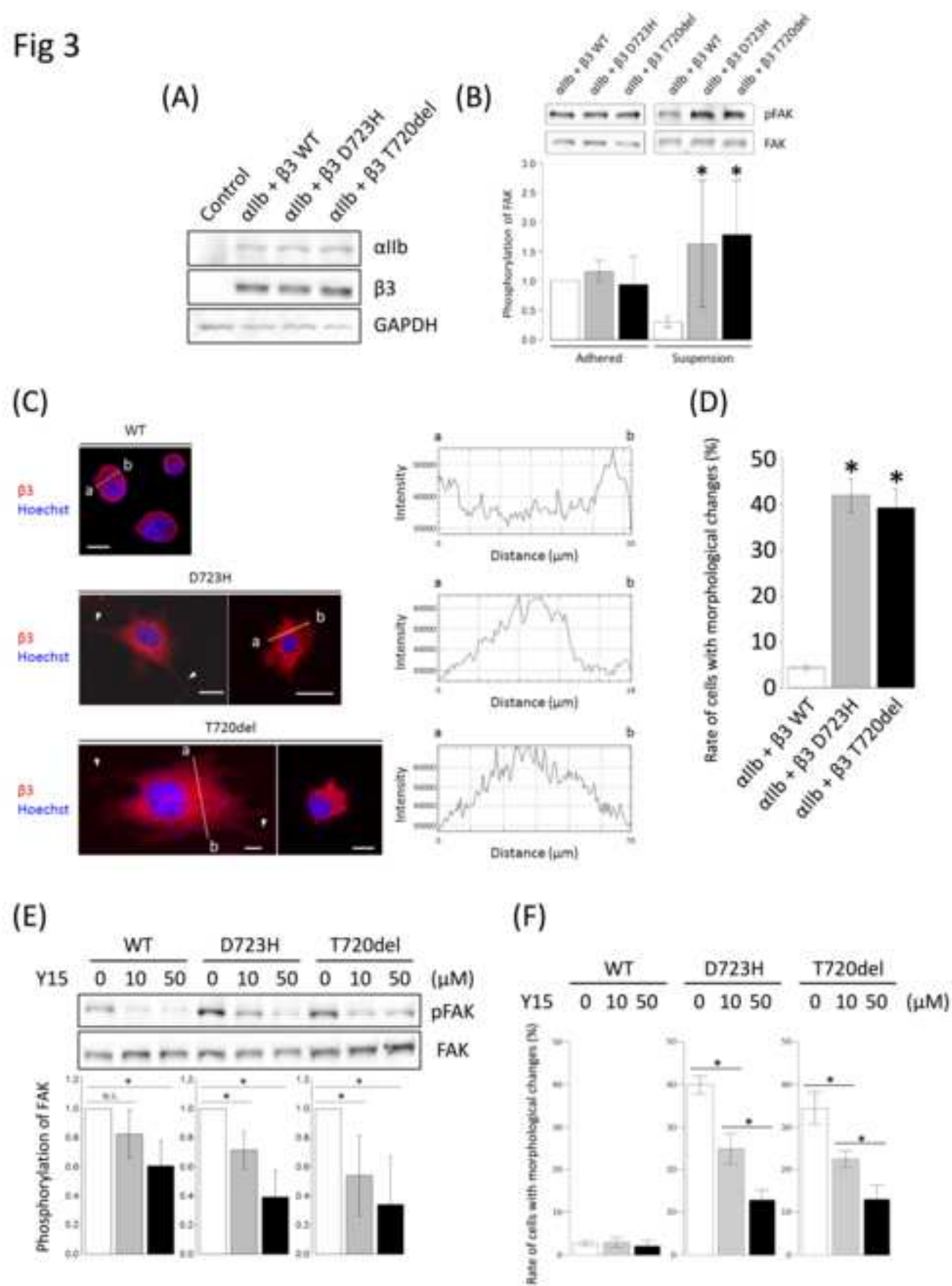


Fig 4

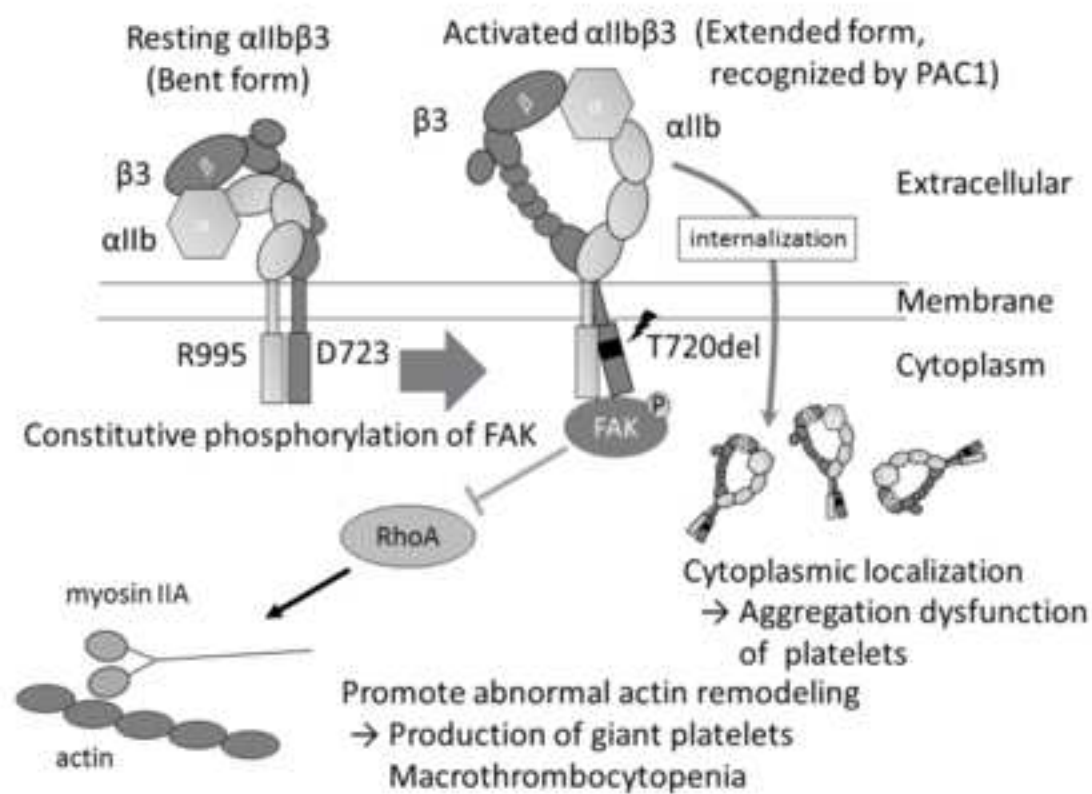


Table 1. Characteristics of 3 affected members (i2, ii2, and ii3) and a non-affected member (ii1)

Family member	Sex	Age (years)	Platelet count (x10 ⁹ /l)	MPV (normal range 7.0–11.0 fl)	Duke bleeding time (normal range 3.0–5.0 min.)	Platelet aggregation (normal range)		
						ADP (70-90%) (10μM)	Collagen (70-90%) (2μg/ml)	Ristocetin (60-90%) (1.5mg/ml)
i2	F	56	74	13.4 fl	5.3 min.	34 %	39 %	73 %
ii2	M	19	75	14.5 fl	4.0 min.	14 %	7 %	73 %
ii3	M	25	58	ND	5.0 min.	30 %	22 %	62 %
ii1	M	28	227	10.0 fl	NA	84 %	90 %	106 %

Notes: Significant results are shown in bold.

Abbreviations: MPV, Mean platelet volume; min, minutes; ND, not determined; NA, not available; ADP, adenosine diphosphate.

Table 2. Surface expression relative to control platelets

Family member	Surface expression relative to control platelets (%)											
	Resting				ADP				PMA			
	αIIb	β3	αIIbβ3	GPIIbα	αIIb	β3	αIIbβ3	GPIIbα	αIIb	β3	αIIbβ3	GPIIbα
i2	40.8	43.1	41.1	137.3	21.6	38.0	35.8	149.0	40.8	37.9	43.0	148.5
ii1	111.9	119.5	107.3	88.7	111.9	129.7	156.3	93.1	85.3	81.2	87.7	100.9

Abbreviations: ADP, adenosine diphosphate; PMA, phorbol-12-myristate-13-acetate.

Table 3. Reported gain of function variants of $\alpha\text{IIb}\beta 3$ resulting in macrothrombocytopenia

Mutation	Platelets characteristics		bleeding tendency	Aggregation assay			Flow cytometry			Functional assay		Reference
	Platelet count ($\times 10^9/\text{L}$)	MPV		ADP	Collagen	Ristocetin	$\alpha\text{IIb}\beta 3$ expression on platelets	$\alpha\text{IIb}\beta 3$ activation index (platelets)	$\alpha\text{IIb}\beta 3$ activation index (transfected cells)	FAK phosphorylation	Morphological changes in transfected cell (CHO)	
<i>ITGB3</i> T720del	58–86	12.8–14.5 fl	no	10–34%	17–39%	62–73%	decreased (40%)	0.14	NA	+	long protrusions, rhomboid-like, membrane ruffling	Our cases
<i>ITGB3</i> D723H	80	17.0 fl	no	normal	–	normal	decreased	NA	NA	NA	long protrusions	Ghevaert <i>et al</i> , 2008 [6]
<i>ITGB3</i> L718P	127	NA	depends on individual	10–20%	10–20%	normal	decreased (47%)	NA	NA	–	long protrusions	Jayo <i>et al</i> , 2010 [8]
<i>ITGB3</i> L718P	49–72	9.8–10.9 fl	mild	15%	10%	normal	decreased (43–75%)	NA	0.23 (CHO)	NA	long protrusions, rhomboid-like	Kobayashi <i>et al</i> , 2013 [31]
<i>ITGB3</i> L718del	100–120	11.5–12.5 fl	submucosal bleeding	decreased	decreased	normal	decreased	NA	NA	NA	NA	Nurden <i>et al</i> , 2017 [11]
<i>ITGA2B</i> F993del	59–111	11.9–12.8 fl	no	NA	NA	NA	decreased (74–82%)	NA	0.77 (293T)	+	NA	Kashiwagi <i>et al</i> , 2013 [9]
<i>ITGA2B</i> G991C	22–109	10.0–14.8 fl	purpura	NA	NA	NA	decreased (3–76%)	0.29	0.65 (293T)	+	NA	Kashiwagi <i>et al</i> , 2013 [9]
<i>ITGA2B</i> R995Q	100–160	10.3 fl	gingival bleeding	decreased	decreased	NA	decreased (15%)	NA	0.23 (CHO)	NA	NA	Peyruchaud <i>et al</i> , 1998 [12]
<i>ITGA2B</i> R995W	65–122	NA	absent – mild	11–43%	9–44%	72–77%	decreased (50–70%)	NA	0.19 (293T)	+	membrane ruffling, cytoplasmic protrusion with bulbous tips	Kunishima <i>et al</i> , 2011 [10]

MPV, Mean platelet volume; NA, not available; ADP, adenosine diphosphate; FAK, Focal adhesion kinase; CHO, Chinese hamster ovary.