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Regular Article

PLATELETS AND THROMBOPOIESIS

Novel variants in *GALE* cause syndromic macrothrombocytopenia by disrupting glycosylation and thrombopoiesis

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KEY POINTS

- ***GALE*** **is expressed during late-stage megakaryopoiesis, regulating the glycosylation and surface exposure of GPIbα and β1 integrin.**
- **Novel *GALE* variants cause syndromic macrothrombocytopenia associated with impaired platelet formation and function.**

Glycosylation is recognized as a key process for proper megakaryopoiesis and platelet formation. The enzyme uridine diphosphate (UDP)-galactose-4-epimerase, encoded by *GALE*, is involved in galactose metabolism and protein glycosylation. Here, we studied 3 patients from 2 unrelated families who showed lifelong severe thrombocytopenia, bleeding diathesis, mental retardation, mitral valve prolapse, and jaundice. Whole-exome sequencing revealed 4 variants that affect *GALE*, 3 of those previously unreported (Pedigree A, p.Lys78ValfsX32 and p.Thr150Met; Pedigree B, p.Val128Met; and p.Leu223Pro). Platelet phenotype analysis showed giant and/or grey platelets, impaired platelet aggregation, and severely reduced alpha and dense granule secretion. Enzymatic activity of the UDP-galactose-4-epimerase enzyme was severely decreased in all patients. Immunoblotting of platelet lysates revealed reduced GALE protein levels, a significant decrease in N-acetyl-lactosamine (LacNAc), showing a hypoglycosylation pattern, reduced surface expression of gylcoprotein Ibα-IX-V (GPIbα-IX-V) complex and mature β1 integrin, and increased apoptosis. In vitro studies performed with patients-derived megakaryocytes showed normal ploidy and maturation but decreased proplatelet formation because of the impaired glycosylation of the GPIbα and β1 integrin, and reduced externalization to megakaryocyte and platelet membranes. Altered distribution of filamin A and actin and delocalization of the von Willebrand factor were also shown. Overall, this study expands our knowledge of *GALE*-related thrombocytopenia and emphasizes the critical role of *GALE* in the physiological glycosylation of key proteins involved in platelet production and function.

Introduction

Inherited thrombocytopenias (ITs) are a group of rare and heterogeneous platelet disorders characterized by low platelet count, which lead to variable increased risk of bleeding depending on the degree of thrombocytopenia and the concomitant association of significant platelet dysfunction.[1] It is currently well-established that many ITs evolve with the development of other serious congenital defects that affect different organs or additional diseases including hematological malignancies, bone marrow failure, and/or nonhematological defects.[2,3] ITs are caused by genetic alterations in megakaryopoiesis-related genes. Early stages of megakaryopoiesis that lead to megakaryocyte (MK) differentiation can be disrupted by alterations in genes that have major roles in the fine-tuning of hematopoietic stem cell homeostasis or regulating the thrombopoietin (TPO) signaling axis. The late stages of the megakaryopoiesis, which include MK maturation

and migration, proplatelet formation, and platelet release into the bloodstream, can be impaired by not only rare molecular changes in genes encoding for critical transcriptional factors, vesicle trafficking, or biochemical signals, but also cytoskeletal proteins, platelet surface glycoproteins (GP), ion channels, metabolic genes, and genes affecting platelet survival.[4-10] Protein glycosylation and linked sialylation are common and complex post-translational modifications, with a critical role in different biological processes such as protein clearance and degradation, immunity, thrombosis and, hemostasis.[11] These modifications are also important in megakaryopoiesis[12] because mice with genetic deficiencies in glycosylation/sialylation present thrombocytopenia.[11,13,14] The use of whole-exome sequencing (WES) unveiled rare cases of syndromic ITs caused by recessive variants in crucial genes for regulating sialylation, such as *GNE*, involved in the sialic acid synthesis,[15,16] the sialic transporter gene *SLC35A1,*[17,18] or genes implicated in glycosylation, such as *GALE*, which encodes the uridine diphosphate (UDP)-galactose-4-epimerase.[19-21] Here, we report 3 patients from 2 unrelated families who showed lifelong syndromic thrombocytopenia and platelet dysfunction caused by compound heterozygosity of 4 rare *GALE* variants, 3 of those previously unreported. Our data further support the key role of *GALE* in protein glycosylation. We unveiled *GALE* variants driving novel mechanisms of pathological thrombopoiesis that consists of impaired glycosylation and externalization of GPIbα and β1 integrin during the late stages of megakaryopoiesis, which lead to altered proplatelet formation by disrupting the filamin A and actin cytoskeleton and to the production of platelets with impaired morphology, function, and viability.

Patients, materials, and methods

Additional methodological details can be found in the supplemental Material, available on the *Blood* website.

Patients, platelet phenotyping, whole-exome sequencing, and variant interpretation

Three patients from 2 unrelated and nonconsanguineous families presenting with syndromic thrombocytopenia of unknown origin were investigated (Figure 1A). Patients' general features and characteristics are summarized in Table 1. Venous blood samples were drawn into EDTA or sodium citrate according to the different studies. Assessment of platelet phenotype, WES, and variant classification were performed in 4 members of pedigree A and 3 members of pedigree B (Figure 1A), as previously described.[22-24]

UDP-galactose-4-epimerase activity and immunoblotting

The UDP-galactose-4-epimerase activity was measured in dried blood spots by high-performance liquid chromatography with tandem mass spectrometry, adapted from previous publications.[25] Protein levels were assessed in platelet lysates by standard immunoblotting and probed with selected antibodies. Protein levels in MK were evaluated by automated capillary-based Western blotting.

Human megakaryocyte differentiation

$CD45^+$ or $CD34^+$ cells were separated by immunomagnetic bead selection (Miltenyi Biotec, Bologna, Italy) and cultured in the presence of TPO, as previously described.[26,27] At the end of the culture (14th day), polyploidization, maturation, apoptosis, and total and surface (permeabilized and non-permeabilized cells, respectively) protein expression were analyzed by flow cytometry.[26,27] Mature MKs were characterized by microscopy using a Leica TCS-SP5 or TCS-SP8 confocal microscope. Images were analyzed using ImageJ software.

Adhesion to extracellular matrices

Control and patient platelets were set in coverslips (1×10^6 total platelets per coverslip) coated with laminin, fibronectin, collagen, or Haemate-P, as described,[28] and stained with selective antibodies.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 Software (GraphPad Software). Differences among groups were tested with t-student, 1- and 2-way analyses of variance, and Tukey's multiple comparisons test. Statistically significant difference was achieved for *P*-values <.05.

Ethics statement

The study was abided by the Declaration of Helsinki and approved by the Ethics Committees of the Instituto de Investigación Biomédica de Salamanca (IBSAL) (reference PI5505/2017). All patients, family members, and healthy controls gave written informed consent. Umbilical cord blood units were collected after obtaining the parents' written informed consent at enrollment in accordance with the ethical committee of the I.R.C.C.S. Policlinico San Matteo foundation of Pavia and the principles of the Declaration of Helsinki.

Results

Clinical presentation of 2 unrelated families

The 3 probands (siblings II.1 & II.4 in pedigree A; II.2 in pedigree B) presented lifelong severe macrothrombocytopenia associated with moderate to severe bleeding tendency and other clinical features such as mental retardation, mitral valve prolapse, and increased bilirubin levels (Figure 1A; Table 1). Proband A.II.1 and B.II.2 displayed 76% and 43% of reticulated platelets, respectively (Table 1). Notably, siblings II.1 & II.4 in pedigree A had been previously misdiagnosed with immune thrombocytopenia, were treated with corticosteroids and immunoglobulins, and lastly underwent splenectomy (A.II.1). In contrast, an IT was suspected in proband II.2 from pedigree B and he was only treated with platelet transfusions before invasive procedures (Table 1).

Assessment of patients' platelet phenotype

Blood film revealed a significant proportion of enlarged, giant, and/or grey platelets in all probands (Figure 1B; Table 1). Moreover, typical morphological findings of patients undergone splenectomy, such as erythroblast, were observed in the blood film from proband A.II.1. No other relevant morphological abnormalities appeared.

All probands displayed significantly reduced platelet aggregation upon stimulation with several agonists (supplemental Figure 1A; available on *Blood* website). Flow cytometry

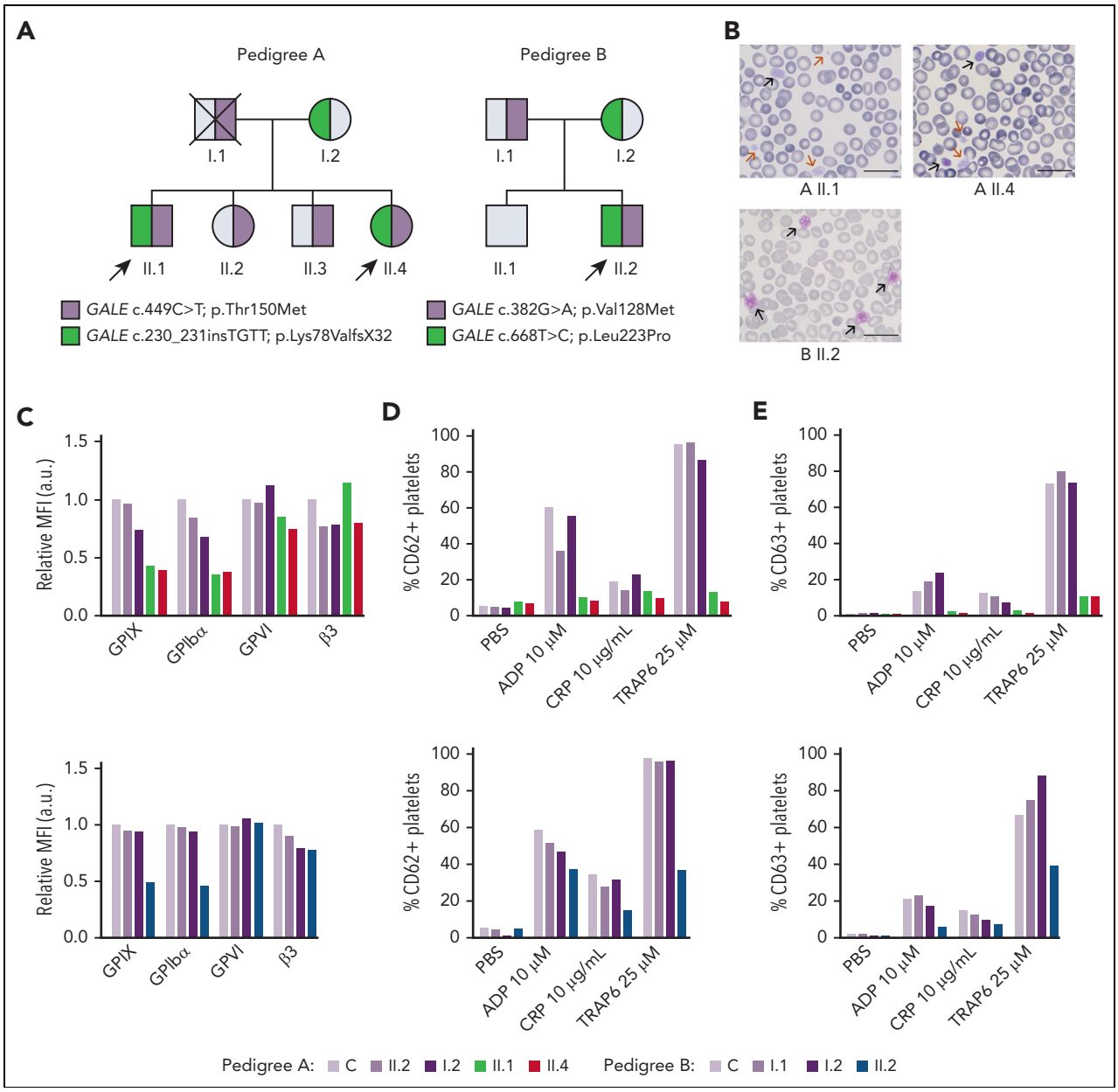

Figure 1. Patients carrying *GALE* variants showed giant and/or grey platelets in blood films and impaired granules secretion. (A) Family segregation of the variants affecting *GALE* identified by whole-exome sequencing. Probands are indicated with black arrows. Partially filled symbols in purple or green represent heterozygous status for the indicated *GALE* variant. (B) Representative peripheral blood films from probands of the 2 pedigrees after May-Grunwald Giemsa staining (×100). Red and black arrows indicate grey and giant platelets, respectively. (C) GPs expression in control and patients, assessed by flow cytometry with fluorescent-labeled antibodies anti-CD42a, CD42b, GPVI, and CD61. The MFI of each antibody, standardized by size (FSC) and relative to the control sample, is represented. (D-E) Platelets from healthy control and family members were stimulated with agonists in the presence of appropriate monoclonal antibodies to assess (D) alpha granules secretion (anti-CD62P), (E) dense granules secretion (anti-CD63) evaluated by flow cytometry. Plots show the percentage of positive platelets. a.u., arbitrary units; ADP, Adenosine diphosphate; CRP; collagen-related peptide; MFI, mean fluorescence intensity; PBS; phosphate-buffered saline; TRAP; thrombin receptor activator peptide.

analysis showed a comparable expression of GPVI and $\alpha_{IIb}\beta_3$ integrin in probands vs nonaffected relatives and healthy control but a significant reduction in the GPIbα and GPIX levels (GPIb-IX-V complex) (50%-70%) (Figure 1C). Furthermore, agonist-induced P–selectin and CD63 exposure were severely impaired (Figures 1D-E). Lastly, patients' platelets also showed a slightly reduced fibrinogen binding and normal thrombin receptor activating peptide 6 (TRAP-6)–induced platelet spreading on fibrinogen, suggesting substantial preservation of the $\alpha_{IIb}\beta_3$ function (supplemental Figures 1B-D).

WES analysis in patients and relatives identifies *GALE* candidate variants

WES unveiled the presence of compound heterozygous variants in *GALE* (NM_001127621.2) in all the probands (Figure 1A), according to the recessive inheritance of their disease. Patients from pedigree A (II.1 and II.4) carried the previously reported missense variant c.449C>T [p.Thr150Met],[21] which affects exon 5, and the novel insertion c.230_231insTGTT [p.Lys78ValfsX32], which affects exon 3. The proband from pedigree B (II.2) carried the 2 novel missense variants c.382G>A [p.Val128Met] and

Table 1. Clinical and laboratory characteristics of 3 patients from the 2 unrelated families

Patient	Pedigree A. II.1	Pedigree A. II.4	Pedigree B. II.2
Age, (y)	45	37	38
Sex	Male	Female	Male
Bleeding score, ISTH-BAT	8	10	6
Bleeding symptoms	Mucocutaneous (1), epistaxis (2), minor wounds (2) conjunctival and vitrectomy (3)	Mucocutaneous (1), epistaxis (2), dental extraction (2), menorrhagia (2), surgical hemostasis after ovarian cyst (3)	Mucocutaneous (2) and gastrointestinal (4)
Hemoglobin level, g/dL (N, 12-16 g/dL)	12.1	11.5	13
White blood cells (N, 3.8 × 10^9/L to 11 × 10^9/L)	9.9 × 10^9/L	3.5 × 10^9/L	8.8 × 10^9/L
Neutrophils (N, 1.4 × 10^9/L to 6.5 × 10^9/L);	5.1 × 10^9/L	1.6 × 10^9/L	5.6 × 10^9/L
Platelet count (N, 150 × 10^9/L to 450 × 10^9/L)	12.7 × 10^9/L	5.3 × 10^9/L	17 × 10^9/L
MPV (fL) (N, 7.2-11.1 fL)	17.4	19.2	20
IPF, % (N, 1.1%-6.1%)	76	NA	43
Blood film (MPD)	Large: 14% Giant: 30% Grey: 54%	Large: 24% Giant: 48% Grey: 24%	Large: 16% Giant: 72% Grey: 8%
Bone marrow aspirate	Erythroid hyperplasia and increased MKs	Erythroid hyperplasia and increased MKs	Not done
Previous diagnosis	Immune thrombocytopenia	Immune thrombocytopenia	DiGeorge syndrome Grey platelet syndrome
Treatments	Corticosteroids Immunoglobulins Splenectomy	Corticosteroids Immunoglobulins	No
Red blood cells transfusions	Yes	Yes	Yes
Platelet transfusions	Yes	Yes	Yes
Additional symptoms:	Yes	Yes	Yes
Neurological	Mental retardation	Mental retardation	Mental retardation
Cardiovascular	Mitral valve prolapse and mitral insufficiency	Mitral valve prolapse and mitral insufficiency	Mitral valve prolapse and mitral insufficiency
Ophthalmological	Retinal disease Cataracts	Retinal disease Cataracts	No
Orthopedic	No	Hip dysplasia	Equine foot
Hepatic	Jaundice Hepatomegaly	Jaundice Hepatomegaly	Jaundice Hepatomegaly
The parents and other relatives in both families were asymptomatic and had normal platelet count and volume.			

Bleeding symptoms were scored (n) by the ISTH-BAT questionnaire.

IPF, immature platelet fraction; ISTH-BAT, International Society on Thrombosis and Haemostasis bleeding assessment tool; MPD, mean platelet diameter; MPV, mean platelet volume; N, normal; NA. not available.

c.668T>C [p.Leu223Pro] at exons 5 and 7, respectively. Of note, all these mutations are located nearby NAD^+ binding site or the substrate-binding site of the *GALE*-encoded protein, UDP-galactose-4-epimerase (supplemental Figure 2). Considering these data, variants were reclassified as pathogenic variants (supplemental Table 1).

GALE variants lead to reduced levels and impaired enzymatic activity of UDP-galactose-4-epimerase, and increase in platelet apoptosis

Immunoblotting assays revealed reduced levels of UDP-galactose-4-epimerase (GALE protein) in platelet of patients compared with that of unaffected family members and healthy controls. As shown in Figure 2A, platelets from patients A.II.1 and A.II.4 had a 50% overall expression of GALE in comparison with control platelets, because of the p.Lys78ValfsX32 variant. In addition, samples from patients A.II.1 and A.II.4 presented a tiny protein band with different electrophoretic mobility corresponding to the mutant form of the enzyme (p.Thr150Met). The total expression of the enzyme in the unaffected A.II.2 was similar to that in the healthy control, but 2 bands corresponding to native and mutant (p.Thr150Met) proteins were detectable (Figure 2A). In contrast, A.I.2, the patient's mother carrying p.Lys78ValfsX32, displayed a 50% reduced expression of the protein, which is consistent with haploinsufficiency (Figure 2A). In pedigree B, platelet from proband II.2 displayed 35% of UDP-galactose-4-epimerase levels vs control platelets, whereas platelets from the unaffected relatives B.I.1 and B.I.2 showed a milder decrease (51% and 65% expression, respectively), suggesting the existence of a protein instability phenomenon, in agreement with the negative effect reported for other mutations.[29,30]

In consequence, all patients exhibited a sharp decrease in the enzymatic activity of the UDP-galactose-4-epimerase, in comparison with healthy controls (A.II.1, 15%; A.II.4, 15%; and B.II.2, 7.5%) (Figure 2B).

Because UDP-galactose-4-epimerase catalyzes the interconversion of UDP-glucose to UDP-galactose and UDP-N-acetyl-glucosamine to UDP-N-acetyl-galactosamine, thus acting on 4 molecules involved in the N-linked and O-linked glycosylation (supplemental Figure 3), we decided to assess the glycosylation pattern in platelet lysates. Immunoblotting assays with lectin ECL, which specifically binds to LacNAc (a dimer of N-acetyl-glucosamine and galactose), showed decreased LacNAc levels in platelets from the patients (A.II.1 & II.4; B.II.2) compared with control platelets, thus reflecting the deleterious effect of these GALE variants on the enzymatic activity (Figure 2C, supplemental Figure 3). It is known that proteins involved in apoptotic pathways carry many glycosylation sites, and *GALE*-deficient human embryonic kidney-293 (HEK-293) cells are hypersensitive to FasL-induced apoptosis.[31] To assess the potential effect of hypoglycosylation on platelet lifespan, we evaluated apoptosis-linked caspase activity in platelet lysates. Probands from pedigree A displayed increased levels of both procaspase 8 and cleaved caspase 8, in comparison with control and healthy relatives. In contrast, proband B.II.2 displayed a normal level of procaspase 8 but an increased amount of active caspase 8 (Figure 2D).

Platelets from patients carrying *GALE* variants show a sharp reduction of GPIbα and glycosylated β1 integrin

Given the hypoglycosylated profile found in patients' platelets, we assessed the level of several relevant platelet proteins known to undergo glycosylation. Interestingly, we found a sharp reduction in GPIbα and mature/glycosylated β1 integrin in all probands, whereas the levels of the hypoglycosylated β1 isoform were similar compared with that in control and healthy members (Figure 3). In contrast, normal levels of β3 integrin and TPO receptor (TPO-R) were detected in probands, suggesting that physiological glycosylation and/or maturation of these receptors is preserved in these *GALE* patients. Assessment of VWF in the platelet lysates showed reduced levels in the probands from pedigree A (Figure 3), in accordance with the high percentage of grey platelets observed in A.II.1 and A.II.4 blood films (54% and 24%, respectively) (Table 1). In contrast, B.II.2 proband, who presented <10% grey platelets in blood film, displayed normal VWF storage (Figure 3; Table 1).

GALE protein expression increases with MK maturation and is localized in the endoplasmic reticulum (ER)

To get insight of the role of GALE in megakaryopoiesis, we used cord blood–derived $CD34^+$ cells, a well-characterized source of in vitro differentiated human MKs.[32] We observed that GALE is poorly expressed in $CD34^+$ hematopoietic stem and progenitor cells (supplemental Figure 4A). During the TPO-induced differentiation, the expression of GALE was increased, being higher in mature MKs ($CD61^+CD42b^{high}$) than in immature MKs ($CD61^+CD42b^{low}$) (supplemental Figures 4A-B). In released platelet-like particles, the expression of GALE was reduced (supplemental Figure 4A). These results suggest that GALE is mainly expressed during the late stage of megakaryopoiesis.

To assess the cellular distribution of GALE in MKs, we performed a colocalization assay of GALE with GPIbα (plasma membrane) and the ER protein, calreticulin (supplemental Figure 4C). GALE showed a higher level of colocalization with calreticulin (Pearson's value, 0.86) than with GPIbα (Pearson's value, 0.48).

Normal MK maturation but impaired proplatelet formation in patients carrying GALE p.Lys78ValfsX32, p.Thr150Met, p.Val128Met, and p.Leu223Pro variants

To further investigate the mechanisms of thrombocytopenia, we differentiated MKs in vitro from peripheral blood progenitors from probands A.II.4 and B.II.2 and from 5 healthy controls, according to our standard protocols.[26,27] We observed normal MK differentiation, evaluated as $CD61^+$ MKs at the end of the culture, between the patients and the healthy controls (Figure 4A). However, we noticed a 60% to 80% reduced expression of GALE in mature MKs from probands A.II.4 and B.II.2 vs healthy controls (Figures 4B-C).

No differences in MK viability was found between controls and the probands A.II.4 and B.II.2 (Figure 4D). Indeed, both

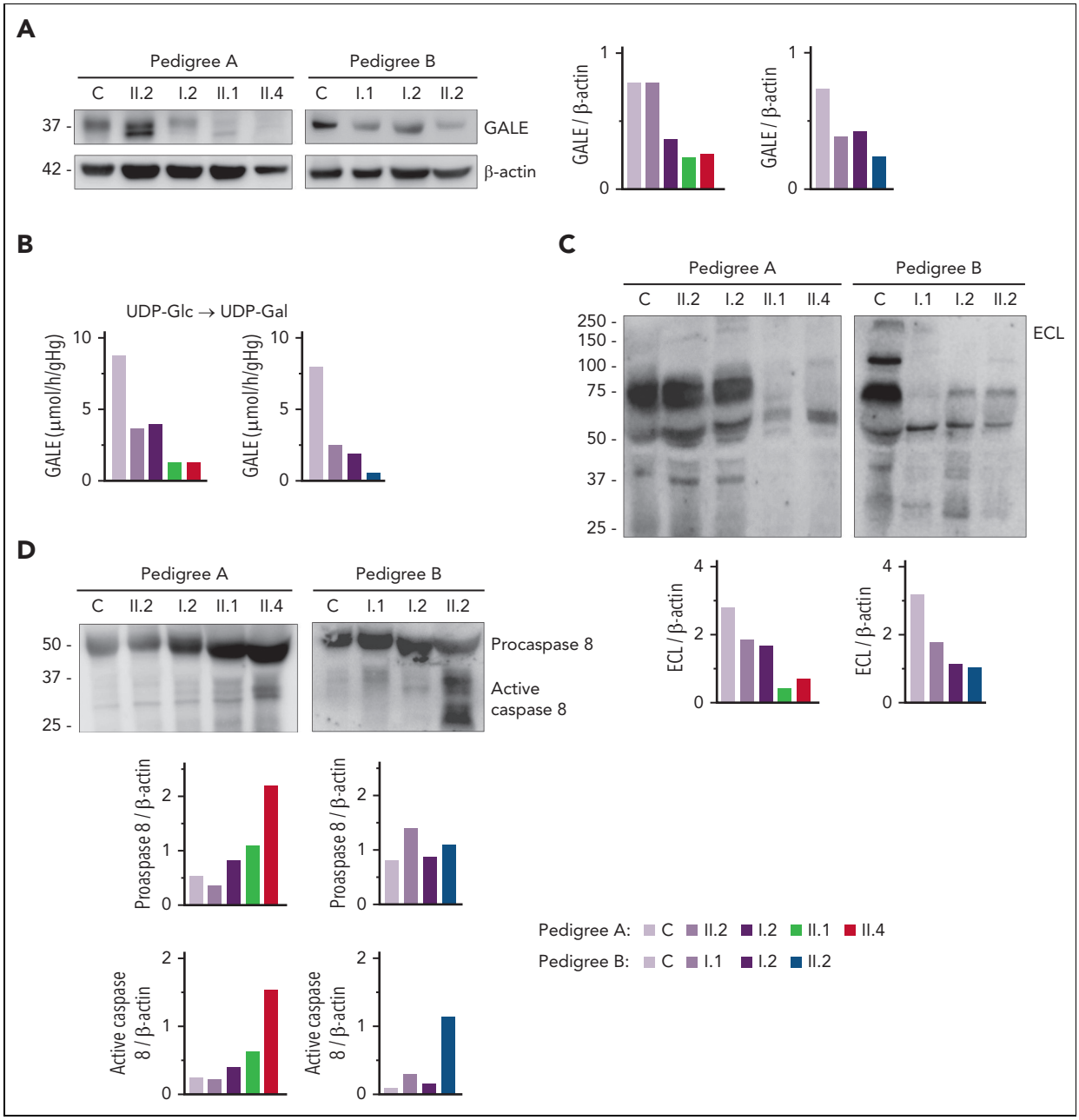

Figure 2. *GALE* variants associated with reduced GALE protein levels, impaired enzymatic activity, platelet hypoglycosylation, and increased apoptosis. (A) Western blotting from platelet lysates of patients from both pedigrees, unaffected relatives, and controls. Membranes were blotted with anti-GALE and anti-β-actin as an internal control. A.II.2 presented total levels of GALE protein similar to controls, but 2 GALE bands with different electrophoretic mobility were observed (top, GALE-WT; bottom, GALE p.Thr150Met), whereas A.I.2 displayed a 50% reduction of total GALE protein. Probands from pedigree A (II.1 and II.4) showed reduced protein levels (50%) and a tiny band corresponding to GALE p.Thr150Met. Individuals from pedigree B: I.1, I.2, and II.2 showed reduced total GALE protein levels (51%, 65%, and 35%, respectively) vs control. (B) Enzymatic activity of UDP-galactose-4-epimerase assessed in members of pedigrees A and B. The activity of a healthy control was evaluated in parallel. Probands of both families showed a sharp reduction in the GALE enzymatic activity (A.II.1, 15%; A.II.4, 15%; and B.II.2, 7.5%), whereas heterozygous carriers in both families displayed a moderate reduction (A.II.2, 42%; A.I.1, 45%; B.I.1, 31%; and B.I.2, 24%). (C) To assess the glycosylation profile, platelet lysates were probed with lectin ECL, which specifically binds to LacNAc, a dimer of N-acetyl-glucosamine and galactose molecules. β-actin staining, shown in panel A, was used as an internal control. A sharp decrease in ECL binding was found in all probands, less pronounced in heterozygous carriers of a single GALE variant. (D) Platelet lysates were probed with antibodies against procaspase 8 (56 kDa) (top band) or the active-cleaved form of caspase 8 (bottom bands). β-actin (shown in panel A) was the internal protein control. Proband from pedigree A displayed increased procaspase 8 and active caspase 8 level, whereas in the proband from the pedigree B only the active caspase 8 was increased. Band intensities were quantified by densitometric analysis using the 'Image J' software. ECL, *Erythrina crista-galli* lectin; LacNAc, N-acetyl-lactosamine; UDP-Glc, UDP-glucose; UDP-Gal, UDP-galactose.

patients' MKs displayed a similar percentage of polyploid cells with respect to the healthy controls (Figure 4E) and normal MKs size and maturation (Figures 4F-G). However, both patients A.II.4 and B.II.2 had a significantly reduced proportion of MKs extending proplatelets vs controls, both in suspension (data not shown) and in adhesion on fibrinogen (A.II.4, 7.3%; and B.II.2, 6.2% vs Controls, 12.3%) (Figures 4H-I). The patient's proplatelets exhibited an impaired morphology that was characterized

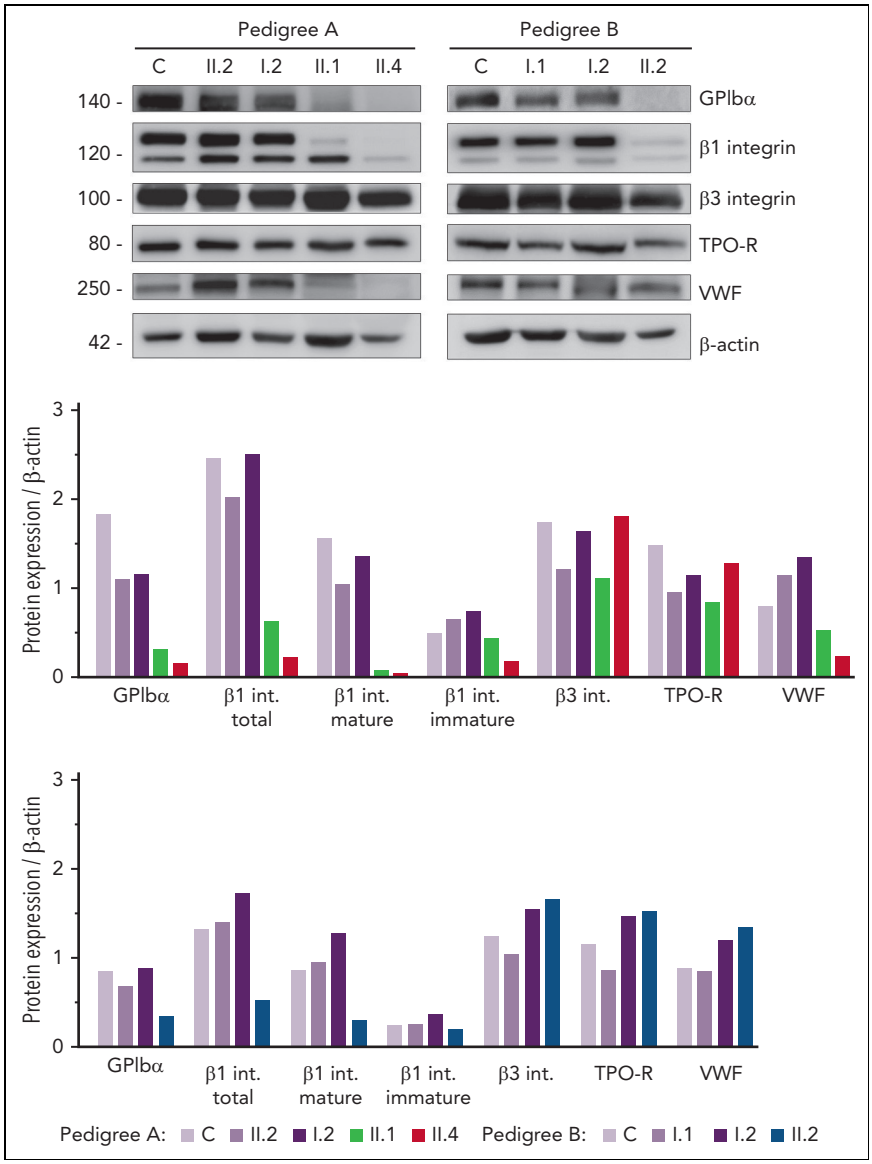

Figure 3. Immunoblotting of platelet lysates revealed severely reduced levels of GPIbα and glycosylated β1 integrin. Platelet lysates of controls, healthy relatives, and patients from both pedigrees were immunoblotted with anti-GPIbα, anti-β1 integrin, anti-β3 integrin, anti-TPO receptor, anti-VWF, and anti-β-actin as an internal control. Band intensities in blots were quantified by densitometric analysis using the 'Image J' software. Bar graphs represent each protein expression relative to β-actin. Impaired GPIbα and β1 integrin levels were observed in the proband from both pedigrees. VWF level was also reduced in pedigree A patients. VWF, von Willebrand factor.

by reduced length and bifurcation of the shafts, resulting in a smaller number of proplatelet-free ends and tips with increased size (Figures 4H-I).

GALE p.Lys78ValfsX32, p.Thr150Met, p.Val128Met, and p.Leu223Pro affect GPIbα and β1 integrin glycosylation, externalization, and delivery to platelets

Probands and the healthy controls had a comparable surface expression of the β3 integrin but reduced GPIbα (A.II.4, 22%; B.II.2, 38% vs the healthy control, 89%) and β1 integrin (A.II.4, 58%; B.II.2, 33% vs the healthy control, 114%) (Figures 5A). The total levels of GPIbα and β1 integrin in both patients were similar to that in control MKs (Figure 5A), thus suggesting that the observed reduction at the MK and platelet surfaces could be because of their impaired externalization (Figures 3 and 5A). Immunoblotting analysis of MK lysates revealed comparable levels of GPIbα and β1 integrin between probands and healthy controls but the presence of bands of a slightly lower molecular weight, probably because of the hypoglycosylation profile of these proteins (Figure 5B). To further confirm their reduced exposure to the membrane and a preferential accumulation of the hypoglycosylated forms within the ER, we assessed their colocalization with the ER protein, calreticulin. We found that GPIbα and β1 integrin were increasingly associated with calreticulin in probands A.II.A and B.II.2 vs the healthy control (GPIbα, 61% and 66% vs 28%, respectively; β1 integrin, 83% and 89% vs 51%, respectively) (Figure 5C). Moreover, GPIbα and β1 integrin fluorescence was distributed at the plasma membrane of control Mks, whereas the signal was mainly localized within the cytoplasm of patients' MKs (Figure 5D).

B.II.2 patient platelets displayed severely reduced adhesion onto VWF, moderate decrease onto laminin and fibronectin, and slight impaired adhesion onto collagen, compared with control platelets (supplemental Figure 5). No results are available for patient A.II.4. However, the lack of GPIbα and β1

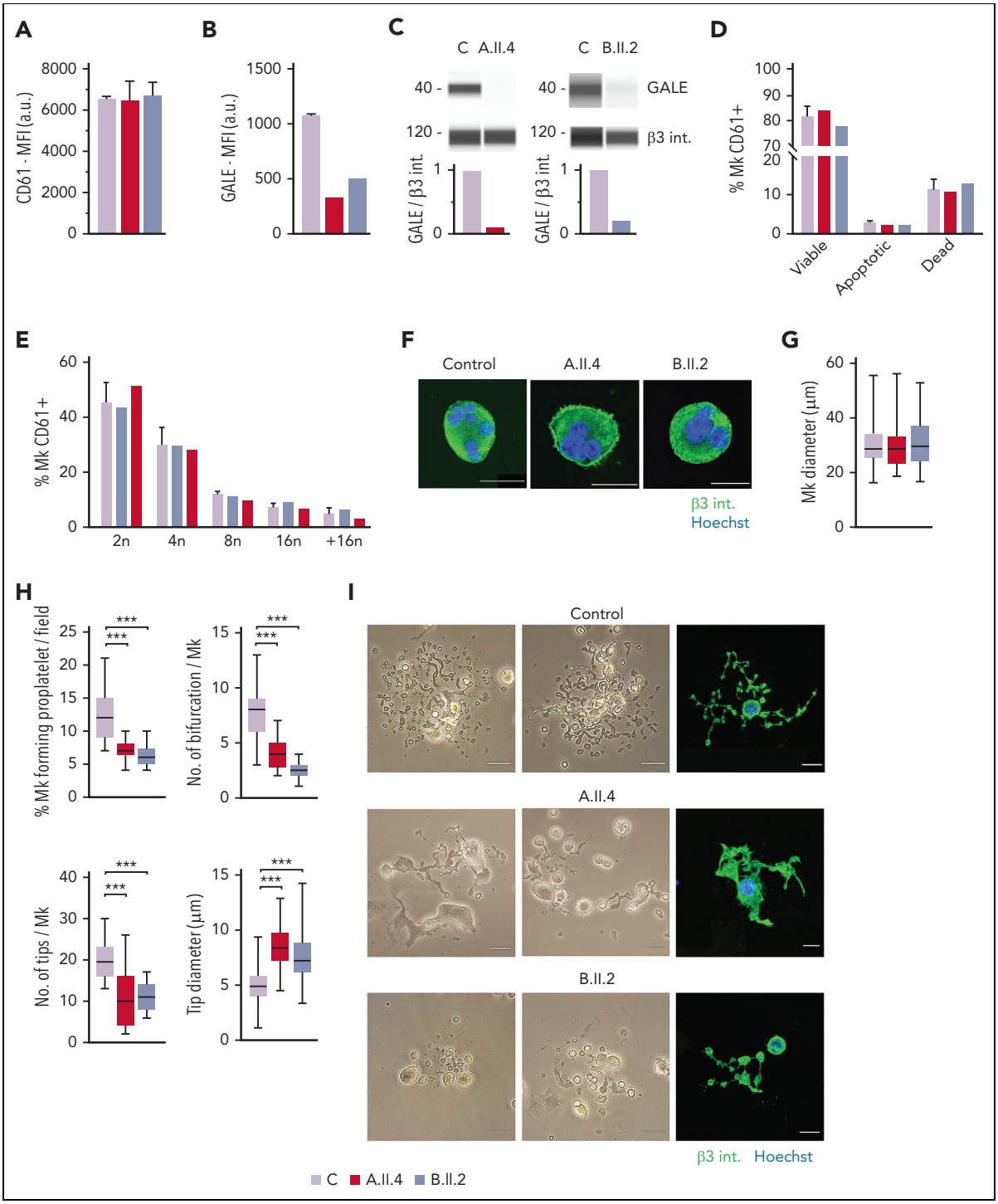

Figure 4. MK culture from probands carrying GALE p.Lys78ValfsX32, p.Thr150Met, p.Val128Met, and p.Leu223Pro variants showed normal polyploidization and maturation but impaired proplatelet formation. MKs were differentiated from peripheral blood progenitor cells from probands A.II.4 and B.II.2 through a 14-days culture, in parallel with healthy controls. (A) MK differentiation assessed as $CD61^+$ cells were measured by MFI by flow cytometry. (B) MFI of GALE, evaluated by flow cytometry, in permeabilized MKs from controls, and probands A.II.4 and B.II.2. Data was relativized with 1 control value. (C) Immunoblotting of MK lysates. Control, A.II.4, and B.II.2 samples were probed with anti-GALE and anti-β3 integrin (internal control). (D) Assessment of MK viability and apoptosis rates at the end of the culture using Annexin V–propidium iodide(PI) labeling. The percentage of $CD61^+$ MKs that are negative for both markers (viable cells), Annexin V^+ PI^- (apoptotic cells), and double-positive (dead cells) is represented. (E) Percentage of polyploid MKs after PI labeling. (F) Representative images of mature and polynuclear MKs labeled with an anti–β3 integrin antibody (green fluorescence). Hoechst (blue) was used for counterstaining nuclei. Scale bars, 20 μm. (G) Diameter measurement of MK from A.II.A, B.II.2, and healthy controls, in fibrinogen-coated coverslips (n = 100). (H) Characterization of impaired proplatelet formation in fibrinogen-coated coverslips in probands A.II4 and B.II.2 vs control, (1) Rate of proplatelet formation measured as the proportion of MKs displaying ≥1 proplatelet with respect to the total number of MKs; (2) Number of bifurcations of proplatelet shafts per MK; (3) Number of proplatelet-free ends (tips); (4) Diameter measurement of proplatelet-free ends (tips) from A.II.4, B.II.2, and healthy control (n = 100). Box and whiskers graphs are shown. The central line represents the median value, whereas percentiles 25 to75 is included in the box. Whiskers represent the minimum and maximum values. (I) Representative image of proplatelet formation in optical and immunofluorescence microscope. MKs was plated on fibrinogen-coated coverslips and incubated for 16 hours at 37°C and 5% CO_2. Cells were stained with an anti-CD61 antibody (green). Hoechst (blue) was used for counterstaining nuclei. Scale bars, 20 μm. ***$P < .001$; 2-tailed Student t test.

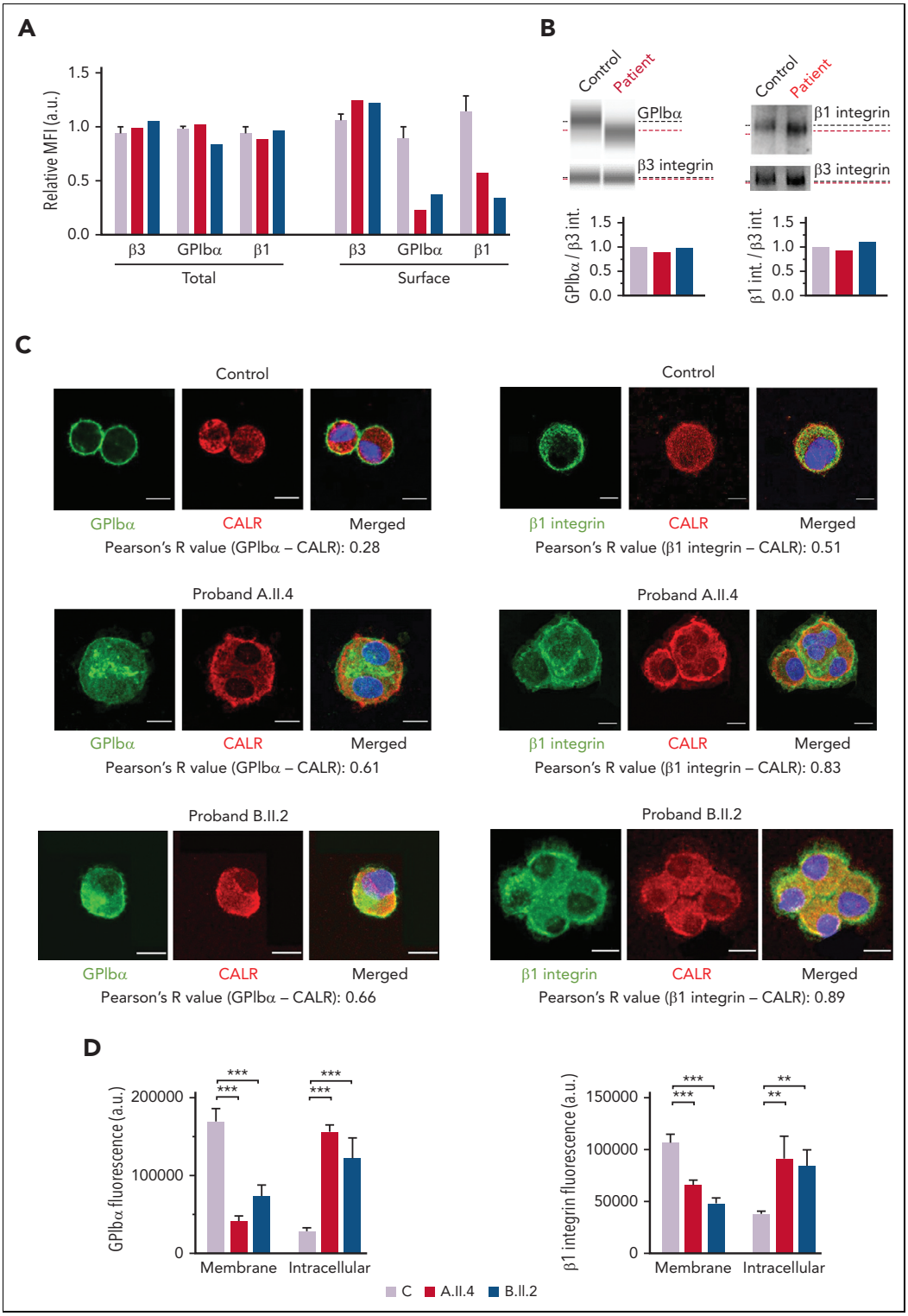

Figure 5. MKs from probands carrying *GALE* variants displayed impaired GPIbα and β1 glycosylation and externalization. MKs were differentiated from peripheral blood progenitor cells (probands A.II.4 and B.II.2) through a 14-days culture, in parallel with healthy controls. (A) MFI of β3 integrin (anti-CD61), GPIbα (anti-CD42b), and β1 integrin (anti-CD29) in permeabilized MKs (total levels) and nonpermeabilized MKs (surface levels) from controls, A.II.4, and B.II.2 are represented. Data were normalized against 1 control value. (B) Representative analysis of the automized immunoblotting of GPIbα and Western blotting of β1 integrin from a healthy control and proband B.II.2. Red dotted lines highlight bands of lower molecular weight in MKs from patients. Densitometry analysis demonstrated comparable levels of total proteins between patients and controls. β3 integrin was used as an internal control. (C) Representative image of control, A.II.4, and B.II.2 MKs. Cells were cytospinned and stained with anti-GPIbα or anti-β1–integrin antibodies (green). The ER was stained with anti-calreticulin (red fluorescence). Hoechst (blue) was used for counterstaining nuclei. Pearson's R values indicate the colocalization rate between GPIbα or β1 integrin with calreticulin in control and probands A.II.4 and B.II.2 MKs. (D) Fluorescence intensity distribution of GPIbα and β1 integrin in control vs patients. Control MKs presented a major distribution of GPIbα and β1 integrin in the membrane, with reduced intracellular levels, whereas patients' fluorescence distribution was mainly intracellular. Scale bars, 10 μm. ***$P < .001$; **$P < .01$; 2-tailed Student *t* test. CALR, calreticulin.

integrin in the MK surface, makes it conceivable that these receptors cannot be delivered to mature platelets, thus affecting their adhesion to physiologically relevant extracellular matrices.

GALE variants altered the distribution of filamin A and actin in MKs from patients

Because filamin A promotes cellular adhesion by linking membrane glycoproteins such as GPIbα to the actin cytoskeleton,[33] we investigated several structural proteins in MKs from both patients with *GALE* variants. As shown in Figure 6A, control MKs displayed a uniform actin distribution, colocalizing with filamin A, in mature MKs, in the proplatelets, and in the proplatelet tips. However, in MKs from probands A.II.4 and B.II.2, we observed a heterogeneously disorganized and nonuniform actin cytoskeleton, actin filaments distributed in patches, and a heterogeneous delocalization between filamin A and actin, forming actin clusters with decreased levels of filamin A (Figure 6A). In contrast, no differences were found in microtubule assembly (Figure 6B).

Finally, we observed similar levels of β-actin in both probands' MKs compared with controls', suggesting a qualitative but not quantitative defect in the MK cytoskeletal proteins in patients with *GALE* variants (Figure 6C).

Patients with *GALE* variants displayed VWF delocalization within the MK membrane

We lastly examined the VWF distribution, and we observed that mature and proplatelet-forming MKs from controls had the signal from granules both within the cytoplasm and near the cell membrane, which is an essential step to ensure physiological proplatelet formation (Figure 7A-B).[28] Nevertheless, A.II.4 and B.II.2 MKs displayed a preferential location of VWF within the cell cytoplasm, with a significant reduction in its localization at the plasma membrane (Figure 7A-B). This alteration was more pronounced in proband A.II.4 (Figure 7B) and could justify the presence of grey platelets in peripheral blood (Figures 1B and 3).

Discussion

In the last decade, high-throughput sequencing technology and particularly WES, has revolutionized the genetic landscape of ITs with the identification of novel causative genes.[34-36] One of these is the *GALE* gene,[37] encoding an enzyme, the UDP-galactose-4-epimerase, which is involved in a wide range of biological processes like N-linked and O-linked glycosylation (supplemental Figure 3).[31] Genetic variantions in *GALE* cause an autosomal recessive disorder (Online Mendelian Inheritance in Man, OMIM database 230350). Compound heterozygous variants of *GALE* usually provoke partial impairment of GALE activity, being associated with nongeneralized forms or even asymptomatic presentations, whereas homozygous variants are related to severe forms of this condition, which usually present generalized galactosemia.[38-41] In addition, long-term complications may include learning difficulties, developmental delay, and poor growth,[42] although cardiac failure and dysmorphic features are less common manifestations.[43] Until recently, there was no evidence linking *GALE* defects and hematological alterations. In 2019, Seo et al reported for the first time 6 patients from 1 pedigree affected by severe thrombocytopenia, febrile neutropenia, and mild anemia, who were homozygous for the GALE p.Arg51Trp variant.[19] Febres-Aldana et al have recently described a child with bone marrow dysfunction and complex congenital heart disease associated with compound heterozygosity in GALE (p.Arg51Trp and p.Gly237Asp).[20] In 2021, Markovitz et al reported a patient with pancytopenia and immune dysregulation because of a previously reported homozygous GALE variant (p.Thr150Met).[21] In these previous reports, the authors agreed that the identification of new patients and/or further in vitro studies were necessary to consolidate the correlation between *GALE* mutations and abnormal organ morphogenesis or hematologic disorders. In contrast with previously reported cases, our patients with compound heterozygosity of 4 variants affecting *GALE* displayed a syndromic type of IT, characterized by mental retardation, cardiovascular abnormalities, jaundice, giant and/or grey platelets, and remarkable reduction in granule secretion.

From a mechanistic point of view, these variants caused a severe reduction in the protein levels and the enzymatic activity of UDP-galactose-4-epimerase, which is reflected in a strongly impaired N-linked and O-linked protein glycosylation in platelets[11,14,44] In addition, the fact that all the phases of the heart valve morphogenesis are mediated by N-glycosylated proteins may explain that our 3 probands had mitral valve prolapse that had to be corrected at birth.[20,42,43] Taken together, these data pointed out the essential role of *GALE* in the glycosylation process. Proper performance of glycosylation is critical for physiological hematopoiesis because it would favor proliferation and clearance of the hematopoietic precursors,[11,44] which are especially relevant in platelet development.[44,45] The hypoglycosylation profile found in platelets from our patients was associated with reduction in surface expression of the GPIb-IX-V complex and almost absence of glycosylated β1 integrin. Also, hypoglycosylated platelets were prone to increased apoptosis, in accordance with previous studies.[46,47]

Recently, thrombocytopenia has been described in other congenital disorders of glycosylation/sialylation, including the *GNE*-related disorder, but the mechanisms remain unclear.[15,16,48,49] To the best of our knowledge, there is scarce information about the performance of megakaryopoiesis in patients with thrombocytopenia associated with *GALE* variants. Here, we showed that *GALE* variants disrupt thrombopoiesis because of the impaired glycosylation of the GPIbα and the β1 integrin and altered distribution of filamin A and F-actin cytoskeleton, leading to a reduction in proplatelet formation (supplemental Figure 6).

It is well established that polyploidization is dispensable for terminal MK maturation.[50] Normal ploidy was observed in MKs cultured from probands A.II.4 and B.II.2. Mature MK develop the demarcation membrane system (DMS), suffering a remodeling into pseudopodia by a process based on microtubules, especially the β1 tubulin, which guides proplatelet shaft initiation and elongation[23,51,52] and the actomyosin cytoskeleton that is critical for the DMS formation,[53,54] and to drive the formation of proplatelets.[55]

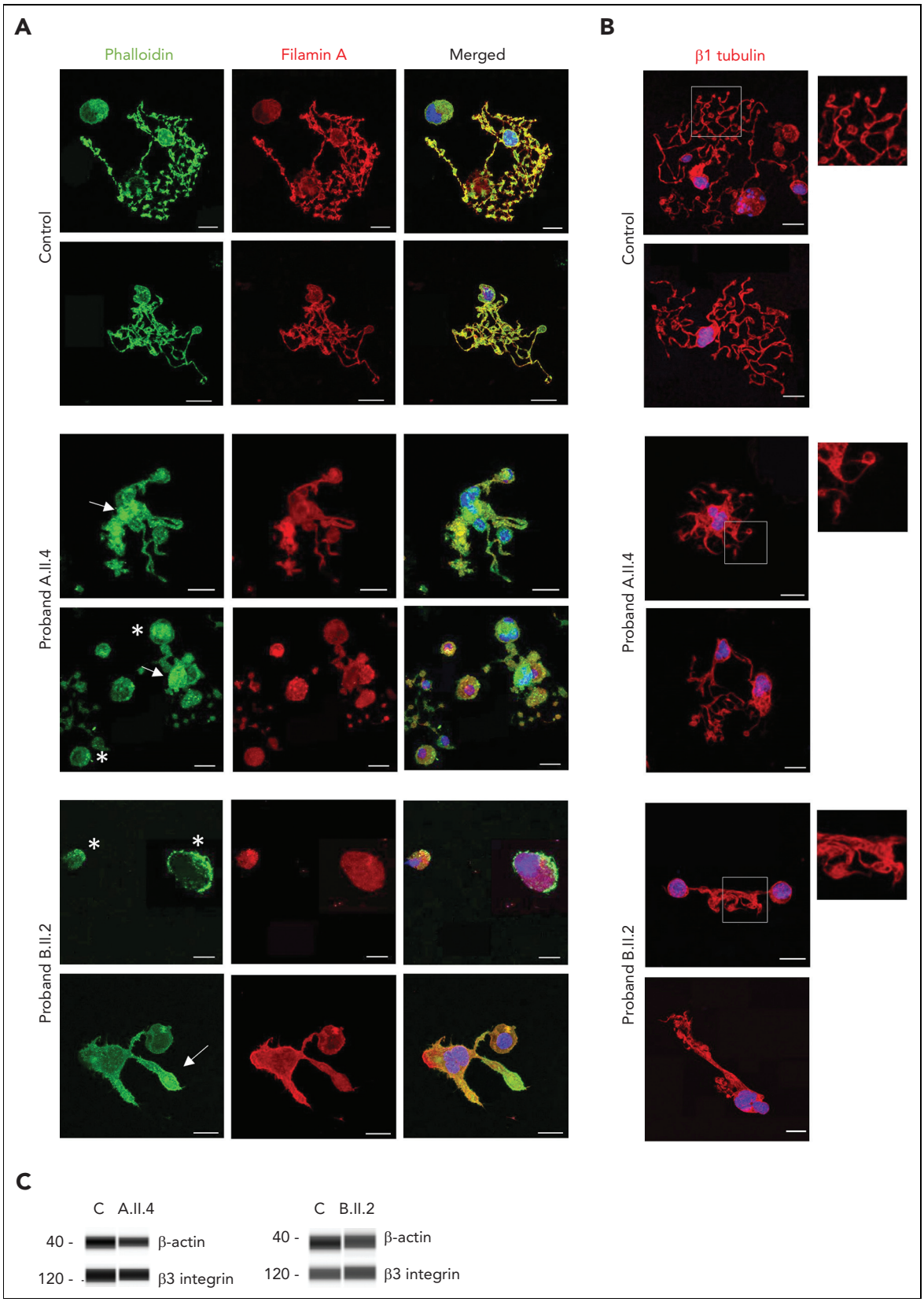

Figure 6. GALE p.Lys78ValfsX32, p.Thr150Met, p.Val128Met, and p.Leu223Pro variants impaired actin and filamin A distribution among the MK cytoplasm. MKs were differentiated from peripheral blood progenitor cells (probands A.II.4 and B.II.2) through a 14-days culture, in parallel with healthy controls. (A) Representative image of MKs stained with phalloidin (green) and anti-filamin A (red) antibodies in control, A.II.4, and B.II.2 samples. We observed a heterogeneously disorganized and nonuniform actin cytoskeleton, based on the presence of actin filaments distributed in patches (white asterisk), a heterogeneous delocalization of filamin A with actin, and the presence of actin in clusters with reduced levels of filamin A (white arrow). (B) Representative image of MKs stained with an anti-β1–tubulin to investigate microtubule assembly in MKs forming proplatelets. Hoechst (blue) was used for counterstaining nuclei. Scale bars, 20 μm. (C) Immunoblotting of Mk lysates. Control, A.II.4, and B.II.2 samples were probed with anti-β actin and anti-β3 integrin (internal control).

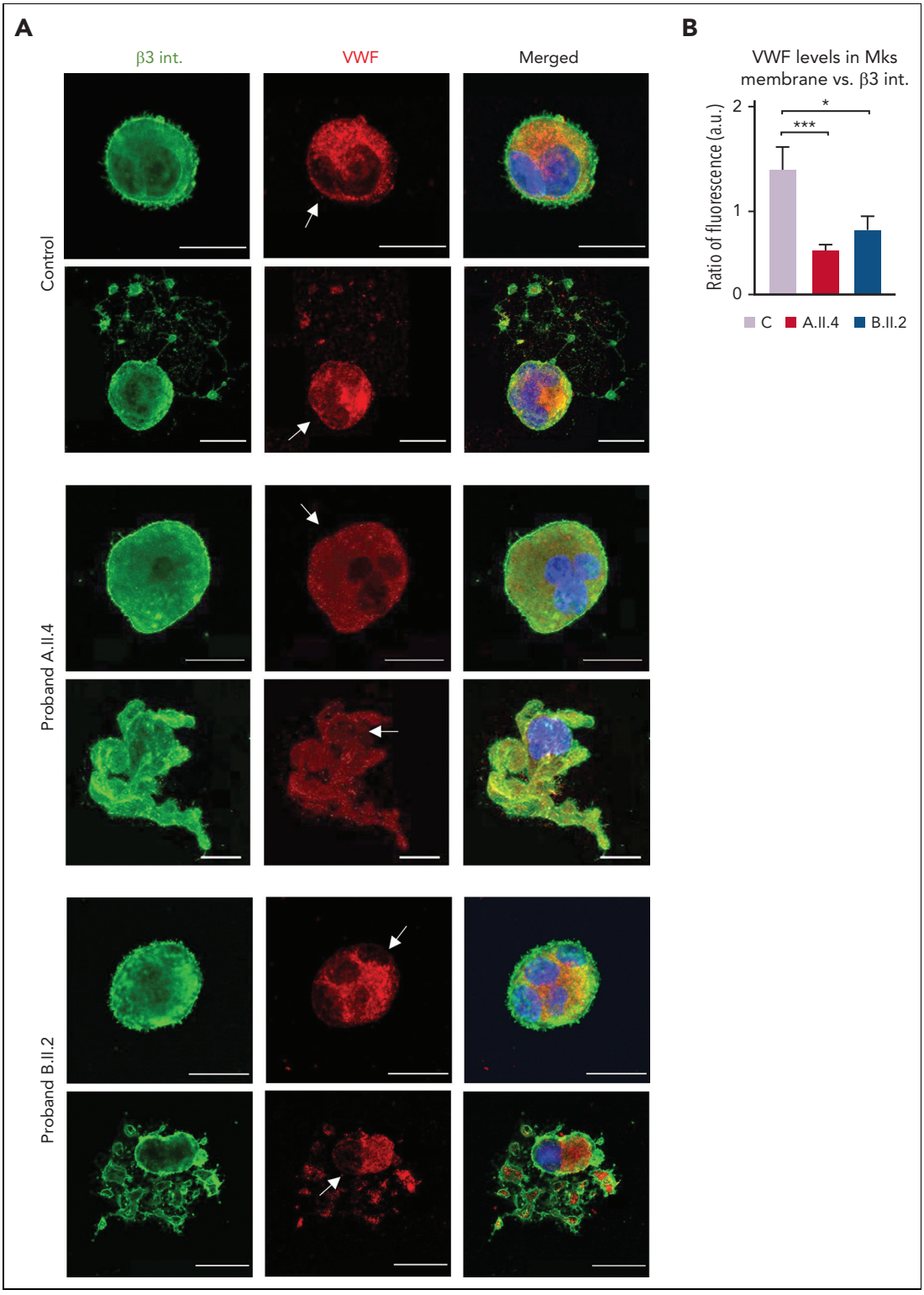

Figure 7. Carriers of GALE p.Lys78ValfsX32, p.Thr150Met, p.Val128Met, and p.Leu223Pro variant displayed VWF delocalization within the MK membrane. MKs were differentiated from peripheral blood progenitor cells (probands A.II.4 and B.II.2) through a 14-days culture, in parallel with healthy controls. (A) Representative image of alpha-granules in mature-polyploid MKs and MK forming proplatelets in control, A.II.4, and B.II.2. Cells were stained with an anti-β3 integrin antibody (green). Alpha-granules were stained with anti-VWF (red). Hoechst (blue) was used for counterstaining nuclei. Arrows indicate the presence of VWF in the control MK membrane, whereas both A.II.4 and B.II.2 had a severely reduced expression of VWF in the membrane. (B) Fluorescence intensity distribution of VWF, relativized with β3 integrin (ratio VWF-β3 integrin), in the MK membrane from healthy controls, A.II.4, and B.II.2. Scale bars, 20 μm. ***$P < .001$; *$P < .05$; 2-tailed Student *t* test.

There is a great controversy not only about the events that give rise to DMS and the subsequent formation of platelets during thrombopoiesis, but also about the role of GPIbα during the DMS and proplatelet formation. The absence of the GPIb-IX-V complex on the MK surface is associated with impaired proplatelet formation, as described in Bernard Soulier syndrome.[56] MKs from *GPIbα*-null mice have a poorly developed DMS and a reduced internal membrane pool,[57] whereas the *GPIbβ*-null mice displayed abnormal DMS formation, and the microtubule bundles at the core of the proplatelet and within the marginal band are measurably thicker than those in wild type platelets, suggesting that GPIbβ could regulate microtubule organization.[58] Indeed, the analysis of the ultrastructure of a patient with Bernard Soulier syndrome uncovered altered DMS, disorganized microtubules, and platelets with sparse or absent granulation.[59,60]

Both probands A.II.4 and B.II.2 showed an unaffected microtubule assembly despite their impaired ability to extend proplatelets, reinforcing the hypothesis that proplatelet formation is contributed by multiple elements. Indeed, impaired filamin A and actin distribution, among the MK cytoskeleton, was observed, which may be expected because GPIbα is not expressed on the MK surface, disrupting the binding to filamin A. It has been previously described in *FLNA*-RD the distribution of both filamin A and actin in patches, as observed here in our patients, MKs containing a large number of granules with a heterogeneous distribution, and a well-developed DMS concentrated in discrete domains.[61] There is increasing evidence that GPIbα–filamin A interaction regulates platelet size. Indeed, the abnormal architecture of giant platelets associated with GPIbα alterations has been proposed to arise from the absence of GPIbα–filamin A interaction.[33,62] It has also been described that the synthesis of mature GPIbα in CHO-GPIbα/β/IX cells is eliminated by brefeldin A, which inhibits the exit from the ER, and it is restored by lactacystin, proving that GPIbα binds to filamin A within the ER and that filamin A binding directs post-ER trafficking of GPIbα to the cell surface.[63] This further supports the evidence that the impaired distribution of filamin A is a consequence of the reduced glycosylation and externalization of the GPIbα in our patients with *GALE*.

Moreover, impaired megakaryopoiesis has been shown in a patient with thrombocytopenia who carried a mutation in the GPIbα interaction site with VWF, and blockade of the extracellular portion of GPIbα was shown to inhibit MK differentiation and proplatelet formation in $CD34^+$ cells.[64,65] It is well known that the interaction between filamin A and GPIbα positively modulates VWF receptor function.[62] The VWF relocation on the MK plasma membrane and binding to GPIbα is crucial for proplatelet formation.[28] In both patients, we observed a reduced expression of VWF on the MK membrane. This alteration is more remarkable in proband A.II.4, whose clinical symptoms are more serious, with even lower levels of GALE, lower platelet counts, decreased GPIb-IX expression in platelets, and decreased granule secretion than B.II.2. Occurance of this severe phenotype could be justified by the fact that patient A.II.4 has a stop codon in *GALE* (p.Lys78ValfsX32 variant).

Reduced levels of GALE in MKs are not only associated with the hypoglycosylation of GPIbα, but also of the β1 integrin. Both are retained in the ER and not delivered to the surface of mature platelets. β1 integrin signaling, which is required for regulating MK function and proplatelet formation,[66,67] was shown to be dependent on LacNac, which is regulated by β-1,4-galactosyltransferase. Alterations at this level has also been associated with thrombocytopenia.[14,44]

In contrast, the expression and/or function of other receptors of the megakaryocytic lineage, such as β3 integrin and TPO receptor, were preserved, thus suggesting that different pathways of glycosylation are likely to be responsible for specific targets. Extending the analysis to additional receptors, such as the transferrin receptor or other crucial proteins whose function depends on glycosylation, may open new paths toward the understanding of the impact of glycosylation in megakaryopoiesis. Importantly, the presence of a fully functional TPO receptor in mutant MKs raises a promising horizon for the treatment of patients with *GALE* with TPO analogues to rescue the severe thrombocytopenia, as an alternative to transfusions.[26] Besides, gene therapy may be a promising approach for the future of these patients.

In summary, we have identified 4 variants in *GALE* that cause syndromic thrombocytopenia. These variants severely reduced the enzymatic activity of UDP-galactose-4-epimerase resulting in impaired platelet glycosylation. In vitro differentiated MKs from patients carrying these *GALE* variants showed reduced externalization of GPIbα and β1 integrin into the cell membrane, likely leading to the impaired proplatelet formation because of the altered distribution of filamin A and actin in MKs. Our new data strongly reinforce the role of *GALE* in glycosylation and unveil novel mechanisms underlying the *GALE*-related thrombocytopenia mediated by defective glycosylation of β1 integrin and the GPIb-IX-V complex.

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Authorship

Contribution: J.M.B., I.G.-T., and A.B. designed the research; A.M.-Q., C.A.D.B., E.V., L.D.-A., J.R., and I.S.-G. performed the functional experiments; C.A.D.B., V.A., and A.M.-Q. performed glycosylation assays; A.M.-Q., C.A.D.B., and P.M.S. performed human megakaryocyte cultures; S.S.-M. and C.M.-G. prepared samples for whole-exome sequencing; A.M.-Q., S.S.-M., C.M.-G., R.B., J.M.H.-R., and J.M.B. conducted whole–exome sequencing analysis and interpretation; P.R.-S. performed enzymatic analysis and interpretation; M.J.P., E.P., and J.R.G.-P. provided clinical information and managed the patients; A.M.-Q., J.R., C.A.D.B., I.G.-T., A.B. and, J.M.B. analyzed and interpreted the results; A.M.-Q. and C.A.D.B. prepared figures and illustrations; A.M.-Q., C.A.D.B., J.R., A.B. and J.M.B. wrote the paper; and all authors reviewed the results and approved the final version of the manuscript.

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Footnotes

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‡On behalf of "Grupo Español de Alteraciones Plaquetarias Congénitas (GEAPC)", Sociedad Española de Trombosis y Hemostasia (SETH).

High-throughput sequencing data are available at Sequence Read Archive database under accession number PRJNA839436.

The online version of this article contains a data supplement.

There is a *Blood* Commentary on this article in this issue.

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REFERENCES

1. Balduini CL, Melazzini F, Pecci A. Inherited thrombocytopenias—recent advances in clinical and molecular aspects. *Platelets*. 2017;28(1):3-13.
2. Pecci A, Balduini CL. Inherited thrombocytopenias: an updated guide for clinicians. *Blood Rev*. 2021;48:100784-100830.
3. Palma-Barqueros V, Revilla N, Sánchez A, et al. Inherited platelet disorders: An updated overview. *Int J Mol Sci*. 2021;22(9):1-31.
4. Bastida JM, Benito R, Janusz K, et al. Two novel variants of the ABCG5 gene cause xanthelasmas and macrothrombocytopenia: a brief review of hematologic abnormalities of sitosterolemia. *J Thromb Haemost*. 2017;15(9):1859-1866.
5. Bury L, Falcinelli E, Gresele P. Learning the ropes of platelet count regulation: inherited thrombocytopenias. *J Clin Med*. 2021;10(3):533-557.
6. Collins J, Astle WJ, Megy K, Mumford AD, Vuckovic D. Advances in understanding the pathogenesis of hereditary macrothrombocytopenia. *Br J Haematol*. 2021;195(1):25-45.
7. Mbiandjeu S, Balduini A, Malara A. Megakaryocyte cytoskeletal proteins in platelet biogenesis and diseases. *Thromb Haemost*. 2021;122(5):666-678.
8. Nurden AT, Nurden P. Inherited thrombocytopenias: history, advances and perspectives. *Haematologica*. 2020;105(8):2004-2019.
9. Freson K. Hemostatic phenotypes and genetic disorders. *Res Pr Thromb Haemost*. 2021;5(5):e12532.
10. Bastida JM, Gonzalez-Porras JR, Rivera J, Lozano ML. Role of thrombopoietin receptor agonists in inherited thrombocytopenia. *Int J Mol Sci*. 2021;22(9):4330-4351.
11. Lee-Sundlov MM, Stowell SR, Hoffmeister KM. Multifaceted role of glycosylation in transfusion medicine, platelets, and red blood cells. *J Thromb Haemost*. 2020;18(7):1535-1547.
12. Grozovsky R, Giannini S, Falet H, Hoffmeister KM. Regulating billions of blood platelets: Glycans and beyond. *Blood*. 2015;126(16):1877-1884.
13. Ma X, Li Y, Kondo Y, et al. Slc35a1 deficiency causes thrombocytopenia due to impaired megakaryocytopoiesis and excessive platelet clearance in the liver. *Haematologica*. 2021;106(3):759-769.
14. Giannini S, Lee-Sundlov MM, Rivadeneyra L, et al. β4GALT1 controls β1 integrin function to govern thrombopoiesis and hematopoietic stem cell homeostasis. *Nat Commun*. 2020;11(1):356-371.
15. Revel-Vilk S, Shai E, Turro E, et al. GNE variants causing autosomal recessive macrothrombocytopenia without associated muscle wasting. *Blood*. 2018;132(17):1851-1854.
16. Futterer J, Dalby A, Lowe GC, et al. Mutation in GNE is associated with severe congenital thrombocytopenia. *Blood*. 2018;132(17):1855-1858.

17. Jones C, Denecke J, Strter R, et al. A novel type of macrothrombocytopenia associated with a defect in α2,3-sialylation. *Am J Pathol*. 2011;179(4):1969-1977.

18. Kauskot A, Pascreau T, Adam F, et al. A mutation in the gene coding for the sialic acid transporter SLC35A1 is required for platelet life span but not proplatelet formation. *Haematologica*. 2018;103(12): e613-e617.

19. Seo A, Gulsuner S, Pierce S, et al. Inherited thrombocytopenia associated with mutation of UDP-galactose-4-epimerase (GALE). *Hum Mol Genet*. 2019;28(1):133-142.

20. Febres-Aldana CA, Pelaez L, Wright MS, et al. A Case of udp-galactose 4′-epimerase deficiency associated with dyshematopoiesis and atrioventricular valve malformations: An exceptional clinical phenotype explained by altered n-glycosylation with relative preservation of the leloir pathway. *Mol. Syndromol*. 2020;11(5-6):320-330.

21. Markovitz R, Owen N, Satter LF, et al. Expansion of the clinical phenotype of GALE deficiency. *Am J Med Genet Part A*. 2021; 185(10):3118-3121.

22. Marín-Quílez A, Vuelta E, Díaz-Ajenjo L, et al. A novel nonsense variant in TPM4 caused dominant macrothrombocytopenia, mild bleeding tendency and disrupted cytoskeleton remodeling. *J Thromb Haemost*. 2022;20(5):1248-1255.

23. Palma-Barqueros V, Bury L, Kunissima S, et al. Expanding the genetic spectrum of TUBB1-related thrombocytopenia. *Blood Adv*. 2021; 5(24):5453-5467.

24. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the american college of medical genetics and genomics and the association for molecular pathology. *Genet Med*. 2015;17(5):405-424.

25. Ko DH, Jun SH, Park KU, et al. Newborn screening for galactosemia by a second-tier multiplex enzyme assay using UPLC-MS/MS in dried blood spots. *J Inherit Metab Dis*. 2011;34(2):409-414.

26. Di Buduo CA, Laurent PA, Zaninetti C, et al. Miniaturized 3d bone marrow tissue model to assess response to thrombopoietin-receptor agonists in patients. *Elife*. 2021;10:e587751.

27. Di Buduo CA, Alberelli MA, Glembostky AC, et al. Abnormal proplatelet formation and emperipolesis in cultured human megakaryocytes from gray platelet syndrome patients. *Sci Rep*. 2016;6:23213-23224.

28. Balduini A, Pallotta I, Malara A, et al. Adhesive receptors, extracellular proteins and myosin IIA orchestrate proplatelet formation by human megakaryocytes. *J Thromb Haemost*. 2008;6(11):1900-1907.

29. Bang YL, Nguyen TTT, Trinh TTB, et al. Functional analysis of mutations in UDP-galactose-4-epimerase (GALE) associated with galactosemia in Korean patients using mammalian GALE-null cells. *FEBS J*. 2009; 276(7):1952-1961.

30. Timson DJ. Functional analysis of disease-causing mutations in human UDP-galactose 4-epimerase. *FEBS J*. 2005;272(23):6170-6177.

31. Broussard A, Florwick A, Desbiens C, et al. Human UDP-galactose 4′-epimerase (GALE) is required for cell-surface glycome structure and function. *J Biol Chem*. 2020;295(5): 1225-1239.

32. Di Buduo CA, Soprano PM, Miguel CP, et al. A gold standard protocol for human megakaryocyte culture based on the analysis of 1,500 umbilical cord blood samples. *Thromb Haemost*. 2021;121(4):538-542.

33. Rosa JP, Raslova H, Bryckaert M, Filamin A. Key actor in platelet biology. *Blood*. 2019; 134(16):1279-1288.

34. Bastida JM, Lozano ML, Benito R, et al. Introducing high-throughput sequencing into mainstream genetic diagnosis practice in inherited platelet disorders. *Haematologica*. 2018;103(1):148-162.

35. Bastida JM, Benito R, Lozano ML, et al. Molecular diagnosis of inherited coagulation and bleeding disorders. *Semin Thromb Hemost*. 2019;45(7):695-707.

36. Ver Donck F, Downes K, Freson K. Strengths and limitations of high-throughput sequencing for the diagnosis of inherited bleeding and platelet disorders. *J Thromb Haemost*. 2020;18(8):1839-1845.

37. Kumar SU, Sankar S, Kumar DT, et al. Molecular dynamics, residue network analysis, and cross-correlation matrix to characterize the deleterious missense mutations in GALE causing galactosemia III. *Cell Biochem Biophys*. 2021;79(2):201-219.

38. Wasilenko J, Lucas ME, Thoden JB, Holden HM, Fridovich-Keil JL. Functional characterization of the K257R and G319E-hGALE alleles found in patients with ostensibly peripheral epimerase deficiency galactosemia. *Mol Genet Metab*. 2005;84(1): 32-38.

39. McCorvie TJ, Timson DJ. In silico prediction of the effects of mutations in the human UDP-galactose 4′-epimerase gene: Towards a predictive framework for type III galactosemia. *Gene*. 2013;524(2):95-104.

40. Openo KK, Schulz JM, Vargas CA, et al. Epimerase-deficiency galactosemia is not a binary condition. *Am J Hum Genet*. 2006; 78(1):89-102.

41. Wohlers TM, Christacos NC, Harreman MT, Fridovich-Keil JL. Identification and characterization of a mutation, in the human UDP-galactose-4-epimerase gene, associated with generalized epimerase-deficiency galactosemia. *Am J Hum Genet*. 1999;64(2):462-470.

42. Fischer A, Steidl C, Wagner TU, et al. Combined loss of Hey1 and HeyL causes congenital heart defects because of impaired epithelial to mesenchymal transition. *Circ Res*. 2007;100(6):856-863.

43. Frieden LA, Townsend TA, Vaught DB, et al. Regulation of heart valve morphogenesis by Eph receptor ligand, ephrin-A1. *Dev Dyn*. 2010;239(12):3226-3234.

44. Di Buduo CA, Giannini S, Abbonante V, et al. Increased B4GALT1 expression is associated with platelet surface galactosylation and thrombopoietin plasma levels in MPNs. *Blood*. 2021;137(15):2085-2089.

45. Li R, Hoffmeister KM, Falet H. Glycans and the platelet life cycle. *Platelets*. 2016;27(6): 505-511.

46. McArthur K, Chappaz S, Kile BT. Apoptosis in megakaryocytes and platelets: the life and death of a lineage. *Blood*. 2018;131(6): 605-610.

47. Edward Quach M, Chen W, Li R. Mechanisms of platelet clearance and translation to improve platelet storage. *Blood*. 2018; 131(14):1512-1521.

48. Bottega R, Marzollo A, Marinoni M, et al. GNE-related thrombocytopenia: evidence for a mutational hotspot in the ADP/substrate domain of the GNE bifunctional enzyme. *Haematologica*. 2021;107(3):750-754.

49. Chang IJ, He M, Lam C. Congenital disorders of glycosylation. *Ann Transl Med*. 2018;6(24): 477-490.

50. Vainchenker W, Raslova H. Megakaryocyte polyploidization: role in platelet production. *Platelets*. 2020;31(6):707-716.

51. Italiano JE, Lecine P, Shivdasani RA, Hartwig JH. Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J Cell Biol*. 1999;147(6): 1299-1312.

52. Patel SR, Richardson JL, Schulze H, et al. Differential roles of microtubule assembly and sliding in proplatelet formation by megakaryocytes. *Blood*. 2005;106(13): 4076-4085.

53. Ghalloussi D, Dhenge A, Bergmeier W. New insights into cytoskeletal remodeling during platelet production. *J Thromb Haemost*. 2019;17(9):1430-1439.

54. Manstein DJ, Meiring JCM, Hardeman EC, Gunning PW. Actin–tropomyosin distribution in non-muscle cells. *J Muscle Res Cell Motil*. 2020;41(1):11-22.

55. Antkowiak A, Viaud J, Severin S, et al. Cdc42-dependent F-actin dynamics drive structuration of the demarcation membrane system in megakaryocytes. *J Thromb Haemost*. 2016;14(6):1268-1284.

56. Balduini A, Malara A, Balduini CL, Noris P. Megakaryocytes derived from patients with the classical form of Bernard-Soulier syndrome show no ability to extend proplatelets in vitro. *Platelets*. 2011;22(4): 308-311.

57. Poujol C, Ware J, Nieswandt B, Nurden AT, Nurden P. Absence of GPIbα is responsible for aberrant membrane development during megakaryocyte maturation: Ultrastructural

study using a transgenic model. *Exp Hematol*. 2002;30(4):352-360.

58. Strassel C, Eckly A, Léon C, et al. Intrinsic impaired proplatelet formation and microtubule coil assembly of megakaryocytes in a mouse model of Bernard-Soulier syndrome. *Haematologica*. 2009;94(6):800-810.

59. Maldonado JE, Gilchrist GS, Brigden LP, Bowie EJ. Ultrastructure of platelets in Bernard-Soulier syndrome. *Mayo Clin Proc*. 1975;50(7):402-406.

60. Mekchay P, Ingrungruanglert P, Suphapeetiporn K, et al. Study of Bernard-Soulier syndrome megakaryocytes and platelets using patient-derived induced pluripotent stem cells. *Thromb Haemost*. 2019;119(9):1461-1470.

61. Nurden P, Debili N, Coupry I, et al. Thrombocytopenia resulting from mutations in filamin A can be expressed as an isolated syndrome. *Blood*. 2011;118(22):5928-5937.

62. Falet H. New insights into the versatile roles of platelet FlnA. *Platelets*. 2013;24(1):1-5.

63. Feng S, Lu X, Kroll MH. Filamin A binding stabilizes nascent glycoprotein Ibα trafficking and thereby enhances its surface expression. *J Biol Chem*. 2005;280(8):6709-6715.

64. Nurden P, Debili N, Vainchenker W, et al. Impaired megakaryocytopoiesis in type 2B von Willebrand disease with severe thrombocytopenia. *Blood*. 2006;108(8):2587-2595.

65. Takahashi R, Sekine N, Nakatake T. Influence of monoclonal antiplatelet glycoprotein antibodies on in vitro human megakaryocyte colony formation and proplatelet formation. *Blood*. 1999;93(6):1951-1958.

66. Kawaguchi T, Hatano R, Yamaguchi K, et al. Fibronectin promotes proplatelet formation in the human megakaryocytic cell line UT-7/TPO. *Cell Biol Int*. 2012;36(1):39-45.

67. Abbonante V, di Buduo CA, Gruppi C, et al. A new path to platelet production through matrix sensing. *Haematologica*. 2017;102(7):1150-1160.