

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 GPIb α and β 1 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 glycoprotein Ib α -IX-V (GPIb α -IX-V) complex and mature β 1 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 GPIb α and β 1 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.

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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 megakaryopoiesis¹² 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 GPIIb α and β 1 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 × 10⁶ 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

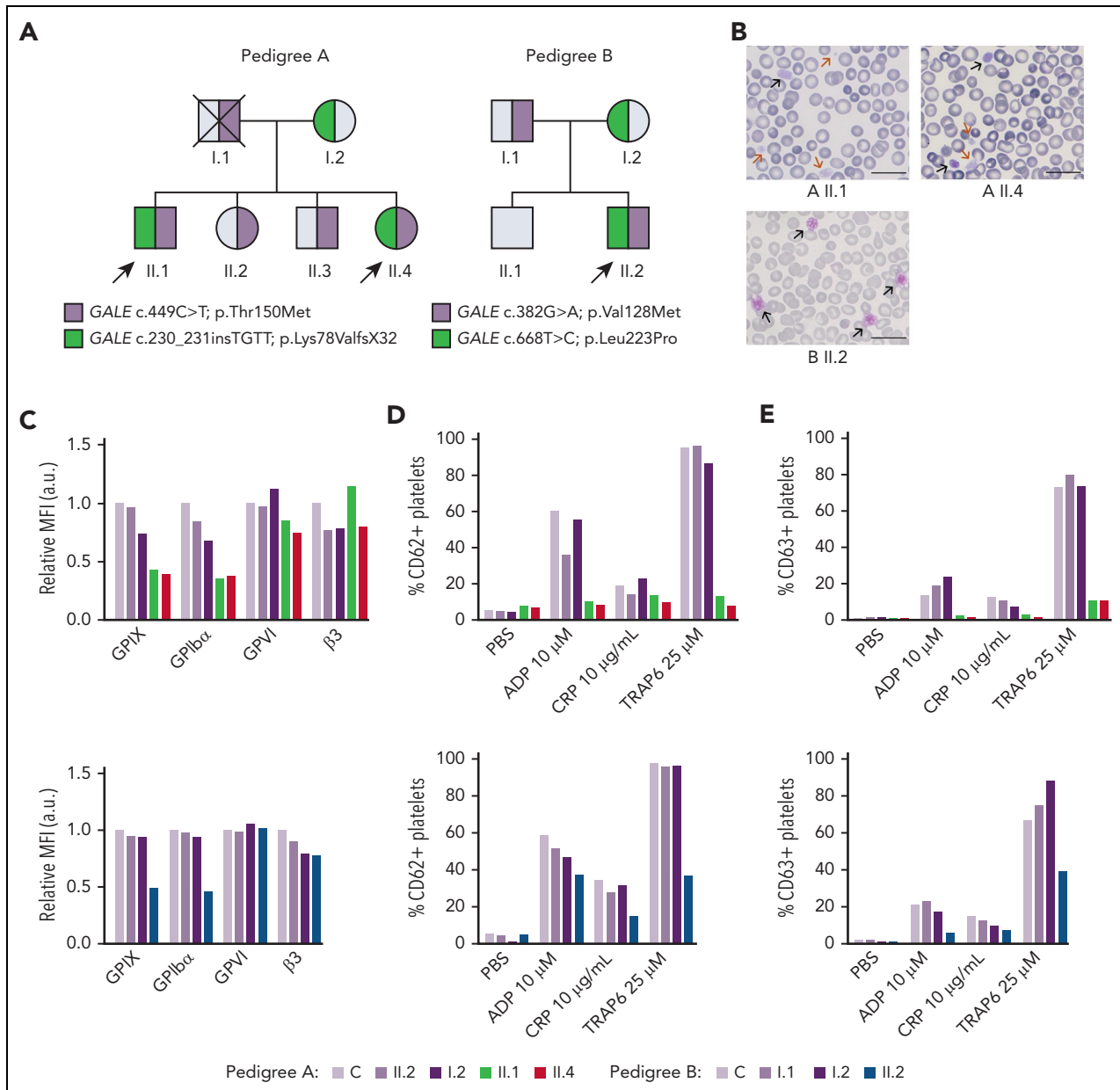


Figure 1. Patients carrying GALE variants showed giant and/or grey platelets in blood films and impaired granules secretion. (A) Family segregation of the variants affecting GALE identified by whole-exome sequencing. Probands are indicated with black arrows. Partially filled symbols in purple or green represent heterozygous status for the indicated GALE variant. (B) Representative peripheral blood films from probands of the 2 pedigrees after May-Grunwald Giemsa staining ($\times 100$). 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 GPIIb α and GPIX levels (GPIIb-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, $3.8 \times 10^9/L$ to $11 \times 10^9/L$)	$9.9 \times 10^9/L$	$3.5 \times 10^9/L$	$8.8 \times 10^9/L$
Neutrophils (N, $1.4 \times 10^9/L$ to $6.5 \times 10^9/L$);	$5.1 \times 10^9/L$	$1.6 \times 10^9/L$	$5.6 \times 10^9/L$
Platelet count (N, $150 \times 10^9/L$ to $450 \times 10^9/L$)	$12.7 \times 10^9/L$	$5.3 \times 10^9/L$	$17 \times 10^9/L$
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 Splenuctomy	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-acetylglucosamine 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-acetylglucosamine 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. Proband 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 GPIIb α and glycosylated β 1 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 GPIIb α and mature/glycosylated β 1 integrin in all probands, whereas the levels of the hypoglycosylated β 1 isoform were similar compared with that in control and healthy members (Figure 3). In contrast, normal levels of β 3 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 GPIIb α (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 GPIIb α (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

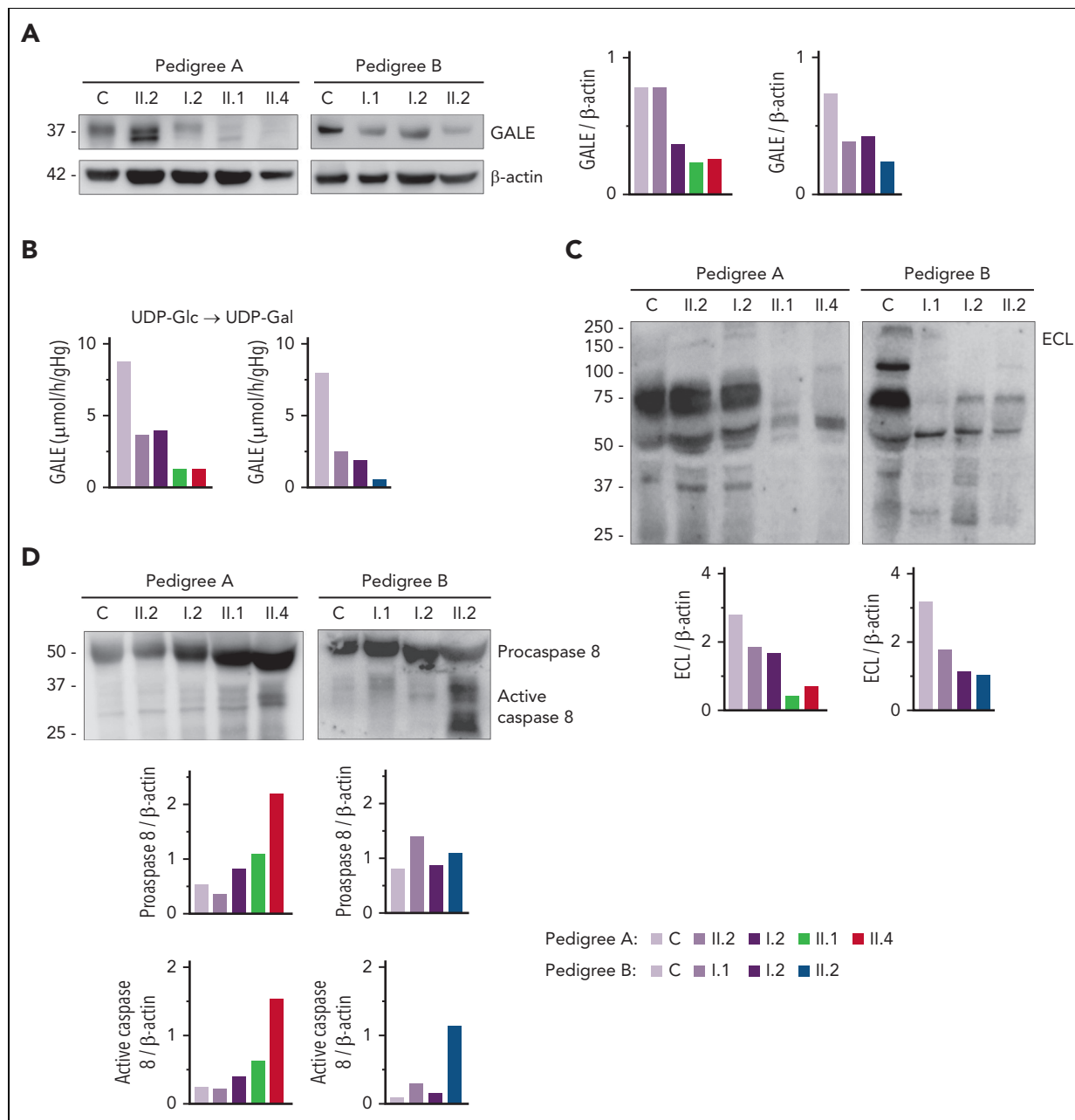
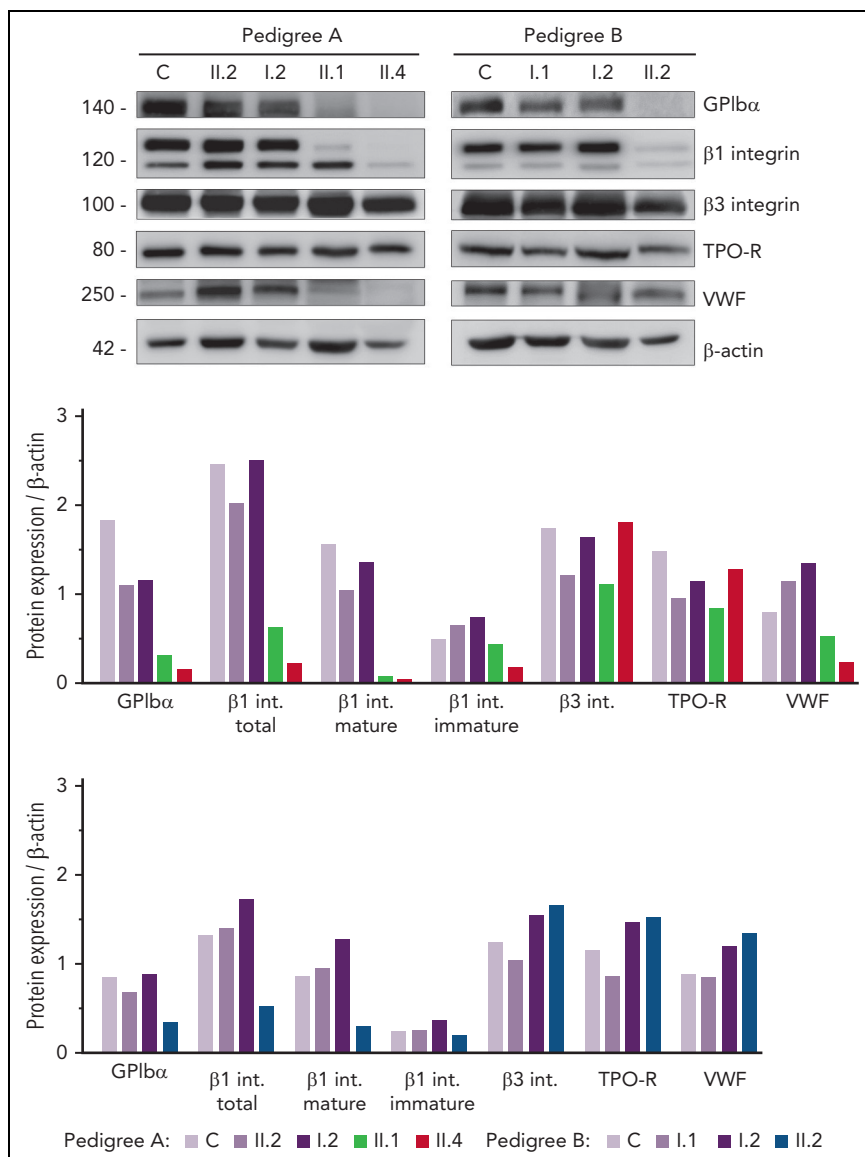


Figure 2. GALE variants associated with reduced GALE protein levels, impaired enzymatic activity, platelet hypoglycosylation, and increased apoptosis. (A) Western blotting from platelet lysates of patients from both pedigrees, unaffected relatives, and controls. Membranes were blotted with anti-GALE 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. Proband 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. Proband 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



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 GPIb α and β 1 integrin glycosylation, externalization, and delivery to platelets

Proband and the healthy controls had a comparable surface expression of the β 3 integrin but reduced GPIb α (A.II.4, 22%; B.II.2, 38% vs the healthy control, 89%) and β 1 integrin (A.II.4, 58%; B.II.2, 33% vs the healthy control, 114%) (Figures 5A). The total levels of GPIb α and β 1 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 GPIb α and β 1 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 GPIb α and β 1 integrin were increasingly associated with calreticulin in probands A.II.4 and B.II.2 vs the healthy control (GPIb α , 61% and 66% vs 28%, respectively; β 1 integrin, 83% and 89% vs 51%, respectively) (Figure 5C). Moreover, GPIb α and β 1 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 GPIb α and β 1

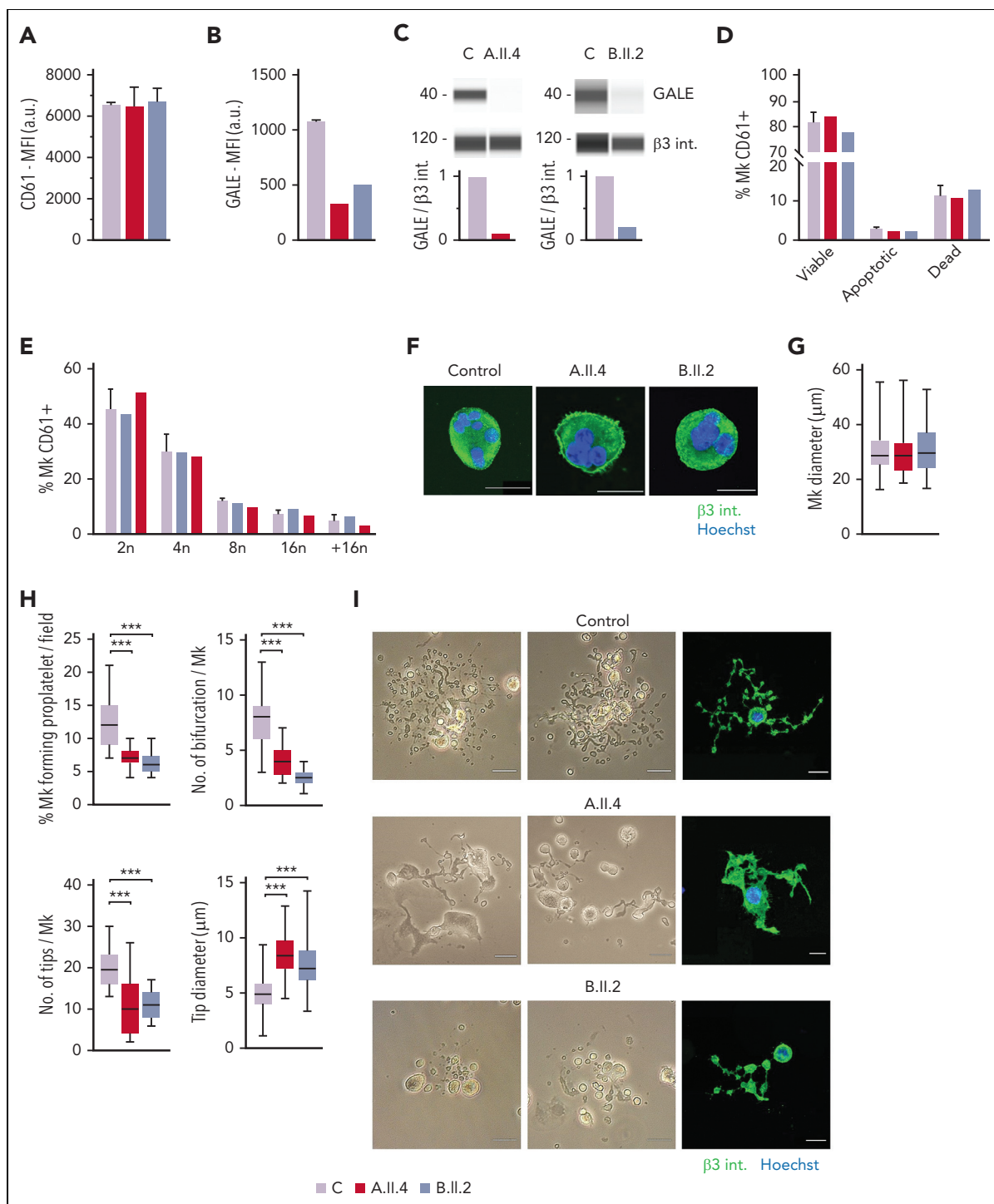


Figure 4. MK culture from probands carrying GALE p.Lys78ValfsX32, p.Thr150Met, p.Val128Met, and p.Leu223Pro variants showed normal polyplodization and maturation but impaired proplatelet formation. MKs were differentiated from peripheral blood progenitor cells from probands A.II.4 and B.II.2 through 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 related 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- $\beta 3$ 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 polyplod MKs after PI labeling. (F) Representative images of mature and polynuclear MKs labeled with an anti- $\beta 3$ integrin antibody (green fluorescence). Hoechst (blue) was used for counterstaining nuclei. Scale bars, 20 μm . (G) Diameter measurement of MK from A.II.4, 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.II.4 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 to 75 is included in the box. Whiskers represent the minimum and maximum values. (I) Representative image of proplatelet formation in optical and immunofluorescence microscope. MKs were plated on fibrinogen-coated coverslips and incubated for 16 hours at 37°C and 5% CO₂. 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.

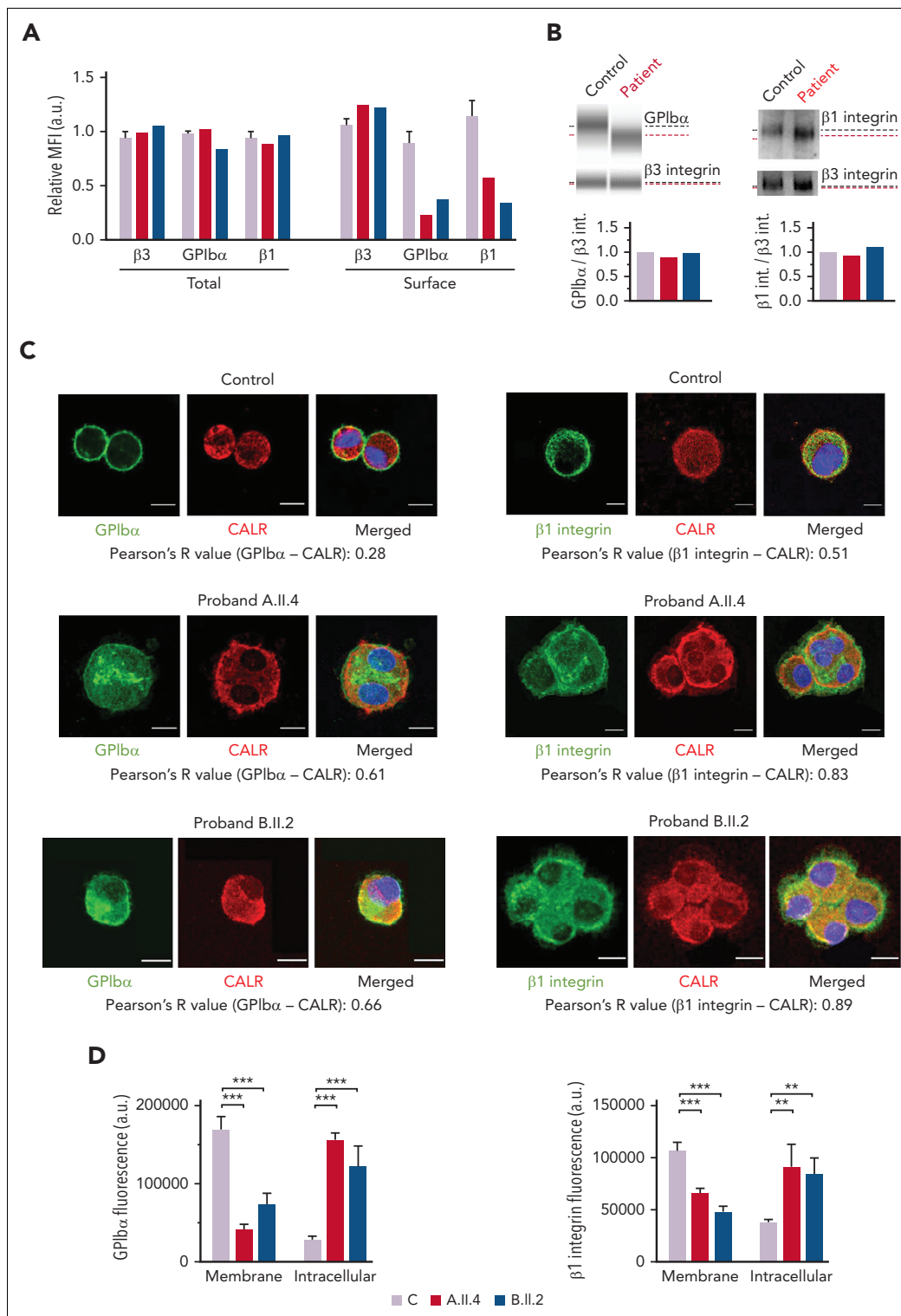


Figure 5. MKs from probands carrying GALE variants displayed impaired GPIIb/IIIa and beta1 glycosylation and externalization. MKs were differentiated from peripheral blood progenitor cells (probands A.II.4 and B.II.2) through a 14-days culture, in parallel with healthy controls. (A) MFI of beta3 integrin (anti-CD61), GPIIb/IIIa (anti-CD42b), and beta1 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 automated immunoblotting of GPIIb/IIIa and Western blotting of beta1 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. beta3 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-GPIIb/IIIa or anti-beta1-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 GPIIb/IIIa or beta1 integrin with calreticulin in control and probands A.II.4 and B.II.2 MKs. (D) Fluorescence intensity distribution of GPIIb/IIIa and beta1 integrin in control vs patients. Control MKs presented a major distribution of GPIIb/IIIa and beta1 integrin in the membrane, with reduced intracellular levels, whereas patients' fluorescence distribution was mainly intracellular. Scale bars, 10 micrometers. *** $P < .001$; ** $P < .01$; 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 GPIIb α 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 variations 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 GPIIb-IX-V complex and almost absence of glycosylated β 1 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 GPIIb α and the β 1 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 β 1 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

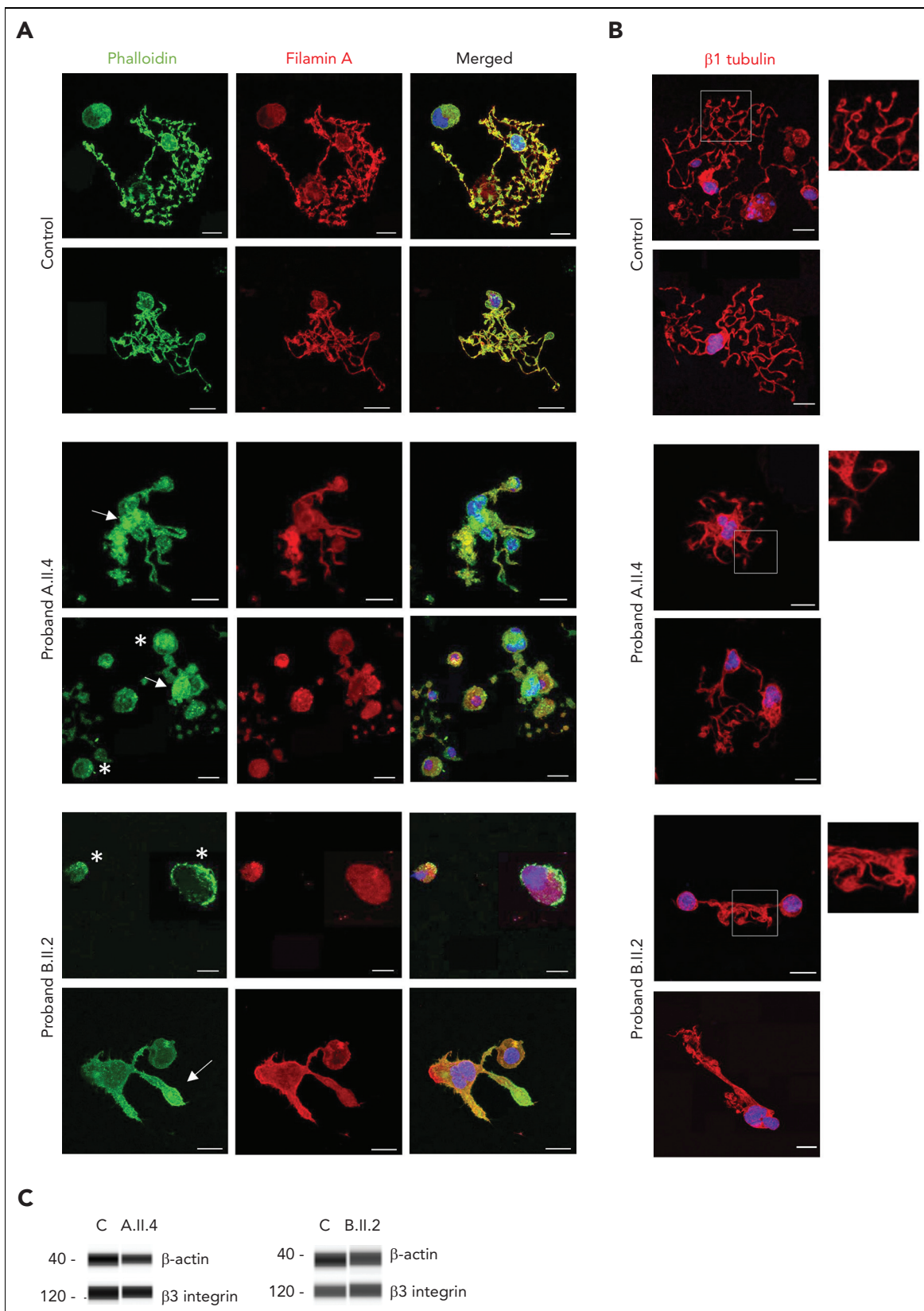


Figure 6. GALE p.Lys78ValfsX32, p.Thr150Met, p.Val128Met, and p.Leu223Pro variants impaired actin and filamin A distribution among the MK cytoplasm. MKs were differentiated from peripheral blood progenitor cells (probands A.II.4 and B.II.2) through a 14-days culture, in parallel with healthy controls. (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- $\beta 1$ -tubulin to investigate microtubule assembly in MKs forming proplatelets. Hoechst (blue) was used for counterstaining nuclei. Scale bars, 20 μ m. (C) Immunoblotting of Mk lysates. Control, A.II.4, and B.II.2 samples were probed with anti- β actin and anti- $\beta 3$ integrin (internal control).

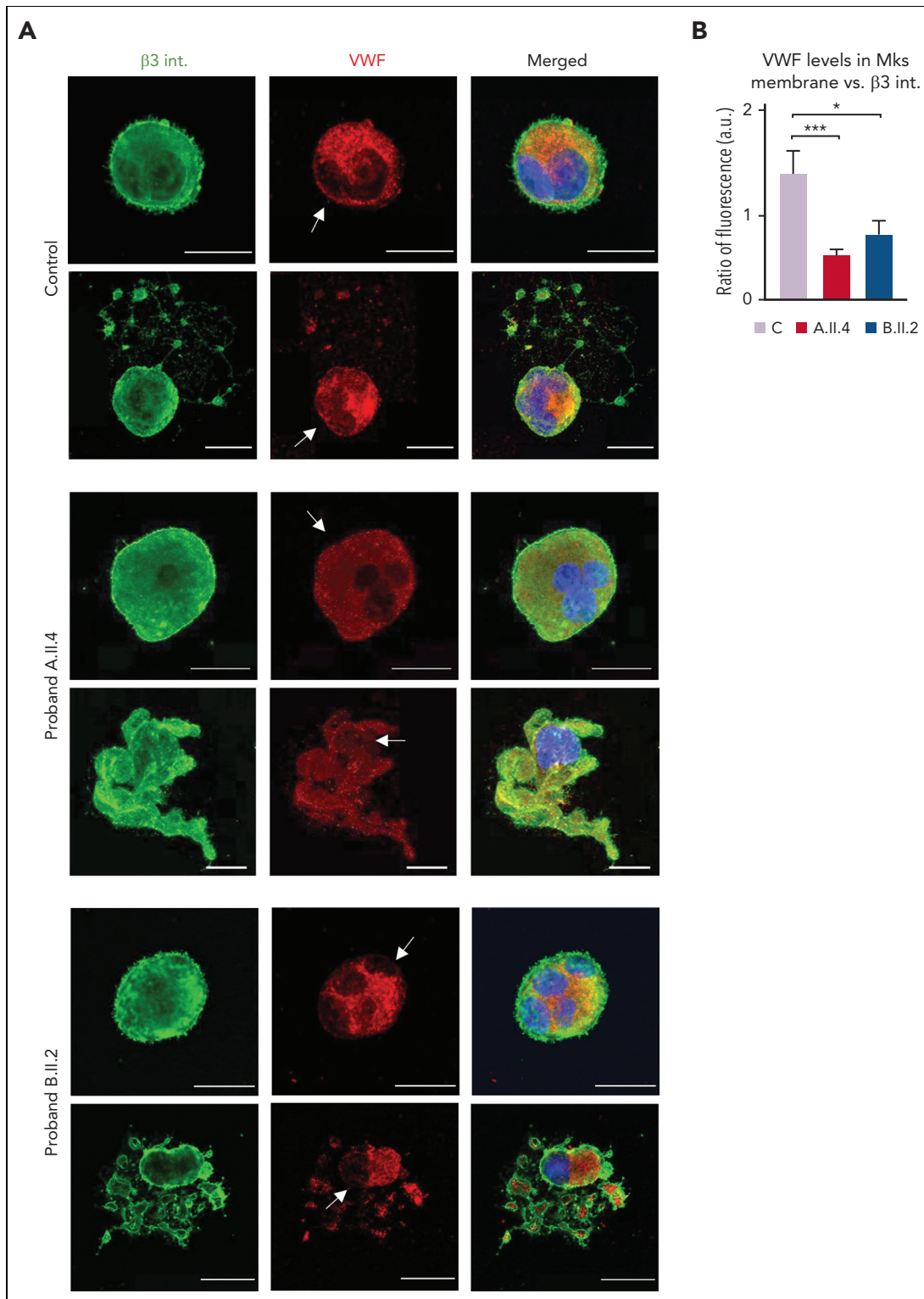


Figure 7. Carriers of GALE p.Lys78ValfsX32, p.Thr150Met, p.Val128Met, and p.Leu223Pro variant displayed VWF delocalization within the MK membrane. MKs were differentiated from peripheral blood progenitor cells (probands A.II.4 and B.II.2) through a 14-days culture, in parallel with healthy controls. (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- $\beta 3$ 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 $\beta 3$ integrin (ratio VWF- $\beta 3$ integrin), in the MK membrane from healthy controls, A.II.4, and B.II.2. Scale bars, 20 μ m. *** $P < .001$; * $P < .05$; 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 GPIb α 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 GPIb α -null mice have a poorly developed DMS and a reduced internal membrane pool,⁵⁷ whereas the GPIb β -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 GPIb β 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 GPIb α 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 GPIb α -filamin A interaction regulates platelet size. Indeed, the abnormal architecture of giant platelets associated with GPIb α alterations has been proposed to arise from the absence of GPIb α -filamin A interaction.^{33,62} It has also been described that the synthesis of mature GPIb α in CHO-GPIb α / β /IX cells is eliminated by brefeldin A, which inhibits the exit from the ER, and it is restored by lactacystin, proving that GPIb α binds to filamin A within the ER and that filamin A binding directs post-ER trafficking of GPIb α 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 GPIb α in our patients with GALE.

Moreover, impaired megakaryopoiesis has been shown in a patient with thrombocytopenia who carried a mutation in the GPIb α interaction site with VWF, and blockade of the extracellular portion of GPIb α 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 GPIb α positively modulates VWF receptor function.⁶² The VWF relocation on the MK plasma membrane and binding to GPIb α 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. Occurrence 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 GPIb α , but also of the β 1 integrin. Both are retained in the ER and not delivered to the surface of mature platelets. β 1 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 β 3 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 GPIb α and β 1 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 β 1 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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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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