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Quentin Kimmerlin, Arnaud Dupuis, Satish Bodakuntla, Claire Weber, Véronique Heim, et al.. Mutations in the most divergent α -tubulin isotype, α 8-tubulin, cause defective platelet biogenesis. *Journal of Thrombosis and Haemostasis*, 2022, 20 (2), pp.461-469. <10.1111/jth.15573>. <hal-04254427>

HAL Id: hal-04254427

<https://hal.science/hal-04254427v1>

Submitted on 15 Nov 2023

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BRIEF REPORT

Mutations in the most divergent α -tubulin isotype, $\alpha 8$ -tubulin, cause defective platelet biogenesis

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Funding Information

This work was supported by the Université de Strasbourg, the Institut Curie, the Agence Nationale de la Recherche ANR-10-IDEX-0001-02, LabEx Cell(n)Scale ANR-11-LBX-0038, the grant ANR-17-CE13-0021. The Fondation pour la Recherche Médicale supported this work with the grants FDT202106013085 to QK, FDT201805005465 to SB, and DEQ20170336756 to CJ.

Abstract

Background: In the panel of genes commonly associated with inherited macrothrombocytopenia, an important fraction encodes key cytoskeletal proteins such as tubulin isotypes, the building blocks of microtubules. Macrothrombocytopenia-causing mutations have been identified in the *TUBB1* and *TUBA4A* genes, emphasizing their importance in the formation of platelets and their marginal band, a unique microtubule ring-like structure that supports the platelet typical disc-shaped morphology. This raised the hypothesis that other tubulin isotypes normally expressed in platelets could play a similar role in their formation.

Objectives: To assess whether tubulin isotype genes other than *TUBA4A* and *TUBB1* could be implicated in inherited macrothrombocytopenia.

Methods: We used high throughput sequencing to screen a cohort of 448 French blood donors with mild thrombocytopenia for mutations in a panel of selected genes known or suspected to be involved in platelet biogenesis.

Results: We identified six distinct novel mutations in *TUBA8*, which encodes the most-divergent α -tubulin, as the causative determinant of macrothrombocytopenia and platelet marginal band defects. Functionally, all *TUBA8* mutations were found to fully or partially inhibit the incorporation of the mutated $\alpha 8$ -tubulin in the microtubule network.

Conclusion: This study provides strong support for a key role of multiple tubulin genes in platelet biogenesis by discovering variants in a tubulin gene that was previously not known to be important for platelets.

KEYWORDS

blood platelets, microtubules, mutation, thrombocytopenia, tubulin

1 | INTRODUCTION

Inherited macrothrombocytopenia is characterized by decreased blood platelet counts and abnormally large platelets. In the panel of genes commonly associated with this condition, a significant proportion encode key cytoskeletal proteins such as myosin (e.g., *MYH9*),¹ actinin (e.g., *ACTN1*),² and tubulin (e.g., *TUBB1*).³ Macrothrombocytopenia-causing mutations have been identified in the *TUBB1* and *TUBA4A* genes,^{3,4} emphasizing their importance in the formation of platelets and their marginal band, a unique microtubule ring-like structure that supports the platelet-typical disc-shaped morphology. This raised the hypothesis that other tubulin isoforms normally expressed in platelets could play a similar role in their formation. Using a high-throughput sequencing approach, we discovered six novel *TUBA8* mutations in individuals with congenital macrothrombocytopenia and marginal band defects, pointing to a role of this α -tubulin isoform in platelet formation. This finding provides new insight into the role of the barely studied $\alpha 8$ -tubulin which, intriguingly, is the most divergent of the α -tubulin family.

2 | MATERIAL AND METHODS

2.1 | Reagents

Polyclonal antiserum against mouse/human $\alpha 4A$ -tubulin (pAb7621),⁴ human $\alpha 8$ -tubulin (pAb7623), and $\beta 1$ -tubulin (pAb5275) were generated by immunizing rabbits against the peptide 76-DEIRNGPYR-84, 32-ADGTFDAQASKIND-45, and 431-VLEEDEEVTEEAEMEPEDKGH-451, respectively.

2.2 | Mutational analysis

A total of 448 blood donors were selected with a platelet count below 150 G/L. Their DNA was extracted from buccal swabs and 17 selected genes suspected to be implicated in thrombocytopenia, including *TUBA8*, were amplified by PCR using amplimers encompassing the coding sequences, intron-exon junctions, and 5' and 3' UTR (ABC study approved by ANSM-French agency for the safety of drugs and health products registered under n° IDRCB: 2014-A00002-45; informed consent was obtained for each individual).⁵ For Case 5 DNA was extracted from whole blood using a QiaAmp DNA kit (Qiagen©) and exons from 72 targeted genes (including *TUBB1*, *TUBA4A*, and *TUBA8*) were sequenced on a MySeq sequencer (Illumina) with a TruSeq Amplicon Custom Low Input Kit (designed with DesignStudio Software Illumina). Variant annotation was performed using SeqNext software (v4.4.0, JSI).

2.3 | Platelet washing procedure

Human washed platelets were prepared as previously described.⁶

Essentials

- Mutations in the *TUBA4A* and *TUBB1* tubulin genes cause inherited macrothrombocytopenia.
- High-throughput sequencing allowed a search for causative mutations in other tubulin genes.
- Mutations in *TUBA8* were identified in individuals with moderate macrothrombocytopenia.
- All identified mutations inhibit the incorporation of $\alpha 8$ -tubulin in the microtubule network.

2.4 | Transmission electron microscopy

Washed platelets were fixed with 2.5% glutaraldehyde, post-fixed with 1% osmium tetroxide, dehydrated with ethanol, and embedded in EPON resin. Then, transversal thin sections (100 nm) were cut, mounted on 200-mesh thin-bar copper grids, and subsequently contrasted with uranyl acetate and lead citrate. Samples were examined at 120 kV under a CM120 TEM (FEI).

2.5 | Isolation and differentiation of human CD34⁺ cells into megakaryocytes

Human CD34⁺ cells were recovered and cultured as previously established.⁷

2.6 | Western blotting

Samples were carefully washed, lysed in Laemmli buffer containing 100 mM dithiothreitol, boiled, separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes (Bio-Rad Laboratories) with the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). Following a blocking step, membranes were incubated with primary antibodies (anti- α -tubulin mouse monoclonal antibody, clone DM1A [1:5000, Merck], pAb7623 [1:1000], pAb7621 [1:1000], or pAb5275 [1:1500]) overnight and horseradish peroxidase (HRP) conjugated-secondary antibodies (Jackson Immuno Research) for 2 h in Tris-buffered saline supplemented with 0.1% Tween 20 and 0.1% bovine serum albumin (BSA). Membranes were revealed with Clarity ECL+ (Bio-Rad Laboratories) for 3 min and visualized with Chemidoc MP (Bio-Rad Laboratories). Quantification was performed by densitometry using the Image Lab software (Bio-Rad Laboratories).

2.7 | Immunostaining of cultured human megakaryocytes and washed platelets

The samples were fixed in 4% paraformaldehyde, cytospun onto poly-L-lysine coated slides, incubated with phosphate-buffered

saline (PBS) supplemented with 100 mM glycine and permeabilized in PBS-0.1% Triton X-100. They were subsequently blocked in PBS supplemented with 10% normal goat serum, 5% BSA, and 5% FcR Blocking Reagent (Miltenyi Biotec). The samples were then incubated with 10 µg/ml of p7623 α -tubulin (IgG purified) or control rabbit IgG, followed by incubation with 4 µg/ml Alexa488-coupled goat anti-rabbit F(ab')₂ secondary antibody (Thermo Fisher Scientific) in combination with 1:150 Alexa647-coupled total α -tubulin antibody (clone DM1A; Sigma Aldrich). The samples were mounted in Mowiol (Thermo Fisher Scientific) and examined under a confocal Leica SP8 inverted microscope and a 63x oil objective. Images were analyzed with ImageJ software (National Institutes of Health).

2.8 | Direct enzyme-linked immunosorbent assay

Platelet lysates were diluted in carbonate buffer (0.1 M, pH 9.6; Merck) and adsorbed overnight at 4°C onto 96 well ELISA plates (MaxiSorp, Starwell, Nunc; Thermo Fisher Scientific). The plates were then washed in PBS-0.05% Tween20, blocked with 200 µL of PBS-0.05% Tween 20-1% BSA for 30 min at 37°C, and incubated with 100 µL of HRP-conjugated anti α -tubulin antibody (1:1000, DM1A clone, ab40742; Abcam) diluted in PBS-0.05% Tween 20-0.1% BSA for 90 min at 37°C. After a washing step, the plates were then incubated with 100 µL of OPD substrate (Thermo Fisher Scientific) for 10 min to detect bound HRP-conjugated antibodies. The reaction was stopped with 50 µL of 3 M HCl, and the optical densities were acquired at 490 nm. The results are expressed as the concentration (in fg) per single platelet, using a standard curve of purified pig brain tubulin (#T240, Cytoskeleton Inc.).

2.9 | TUBA8 mutagenesis, lentivirus production, and transduction

A codon-optimized, coding sequence of human *TUBA8* (accession number NM_018943.3; hTUBA8) was synthesized, cloned into pUC57, and subjected to quick-change site-directed PCR mutagenesis. hTUBA8 variants were then subcloned by sequence- and ligation-independent cloning into a pTRIP lentiviral vector downstream of the EF1- α promoter and green fluorescent protein (GFP) sequence.⁸ Each construct was verified by sequencing. Lentiviral particles were then produced as previously described⁹ and incubated with U2OS cells grown onto glass coverslips. After 48 h, transduced U2OS cells were processed for immunofluorescence microscopy as previously described.¹⁰

2.10 | Ethics statement

Human studies were performed according to the Declaration of Helsinki.

2.11 | Statistical analysis

Results were expressed as the mean (\pm standard error of the mean). Statistical comparisons were performed using one-way analysis of variance followed by Tukey-Kramer post hoc test.

3 | RESULTS AND DISCUSSION

TUBA4A and *TUBB1* show increased transcript expression during megakaryocyte maturation.⁴ Here we confirmed this trend at the protein level and found that another tubulin isotype, *TUBA8*, follows the same dynamics (Figure 1A). In addition, this α 8-tubulin isotype is readily observed in the microtubule network of human cultured megakaryocytes and washed native platelets (Figure 1B). *TUBA8* (NM_018943.3) was therefore included in the panel of genes with potential implication in congenital macrothrombocytopenia.⁵ We performed high throughput sequencing in 448 French blood donors with mild thrombocytopenia and in a patient who was referred to us for a bleeding tendency and the presence of macroplatelets. Six point mutations were identified in *TUBA8* (c.59G>A, c.202A>C, c.640C>T, c.727C>T, c.868G>A, and c.958C>T) that resulted in six different single amino acid substitutions (p. Cys20Tyr, p. Ile68Leu, p. Arg214Cys, p. Arg243Cys, p. Glu290Lys, and p. Arg320Trp; Figure 1B,C). The platelet counts of the six individuals with *TUBA8* mutations ranged from 103 to 147 $\times 10^9/L$, and were thus at the lower limit of the normal values previously established in a control French adult population (171–397 $\times 10^9/L$).¹¹ When available, mean platelet volumes (MPV) ranged from 12.3 to 14 fL, clearly higher than the normal range (7.4–10.8 fL)¹¹ (Figure 1C). For the five blood donors (Cases 1–4 and 6), the defects are not accompanied by known bleeding manifestations, which is the case for most of the individuals reported with *TUBB1* mutations.³ In contrast, the patient with the p. Glu290Lys *TUBA8* (Case 5) had experienced bleeding episodes in the form of menorrhagia. Knowing that menorrhagia can occur independently of a low platelet count and might not be associated with the tubulin defect, this patient might carry other causing mutations that could not be identified by our sequencing gene panel. All the identified *TUBA8* mutations were confirmed by Sanger sequencing and were found to be monoallelic. Database search for common polymorphisms revealed frequencies ranging from 3×10^{-5} to 52×10^{-5} for five out of six *TUBA8* mutations, and no match for one *TUBA8* mutation (p. Cys20Tyr; Figure 1C). The mutated amino acid residues in the α 8-tubulin protein are conserved in every member of the α -tubulin family which, in light of the sequence divergence of α 8-tubulin, suggests an important structural or functional role in tubulins. To be noted, the donor with the p. Arg214Cys mutation (Case 3) was also found to carry a heterozygous c.436G>C mutation in the *TUBB1* gene, which resulted in the p. Gly146Arg amino acid substitution.

Two blood donors (Cases 3–4), and the patient (Case 5) became available for complementary platelet studies. Transmission

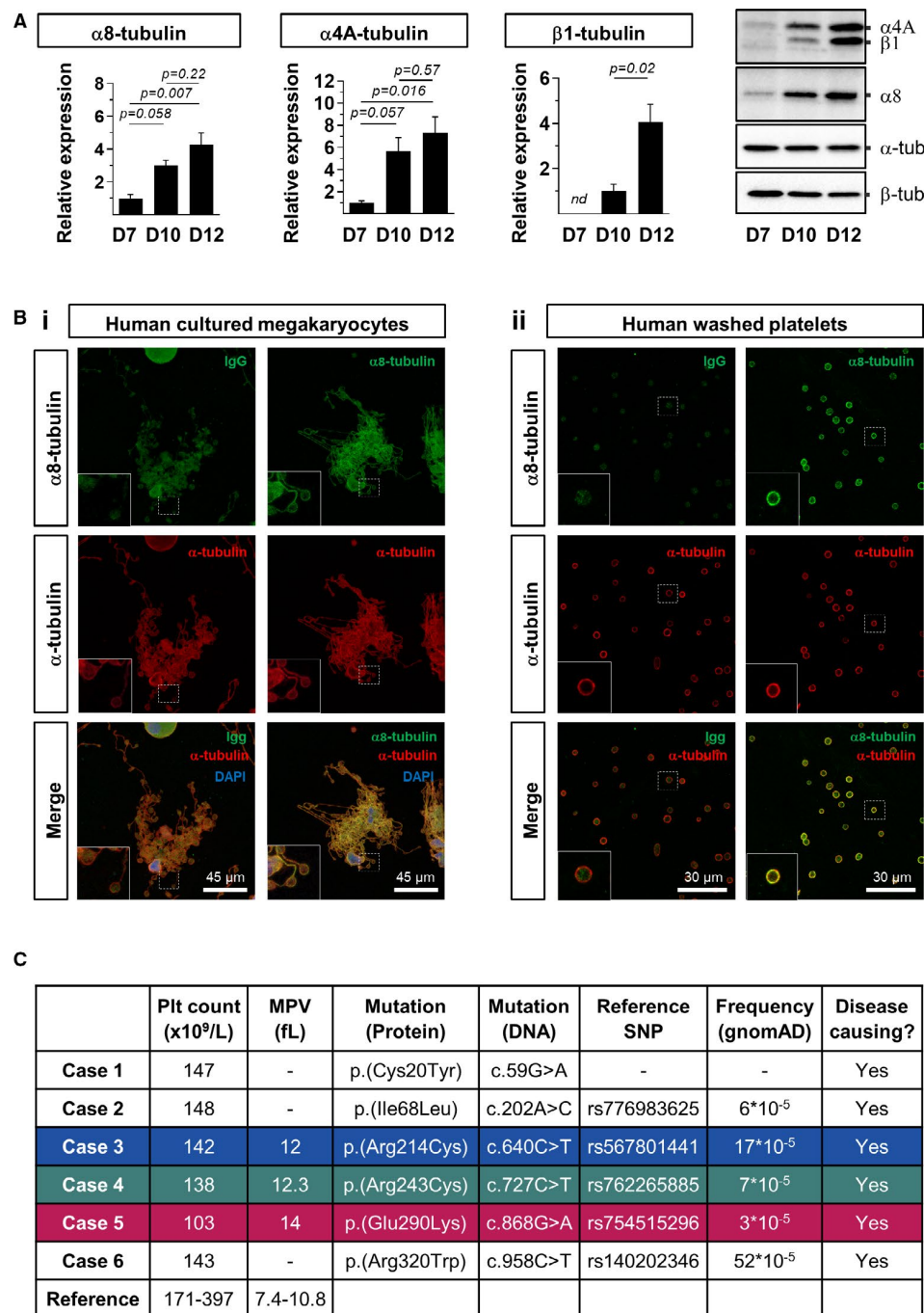


FIGURE 1 Mutations of *TUBA8* in individuals with moderate (macro)thrombocytopenia. **A**, Expression levels of $\alpha 8$ -, $\alpha 4A$ -, and $\beta 1$ -tubulin in human immature megakaryocytes (D7), mature megakaryocytes (D10), and proplatelet-forming megakaryocytes (D12), obtained in culture from CD34⁺ peripheral blood-derived hematopoietic progenitors. The levels of $\alpha 8$ -, $\alpha 4A$ -, and $\beta 1$ -tubulins were estimated by densitometry and normalized to those of total α -tubulin. Results are expressed as the mean \pm standard error of the mean of three independent experiments. Statistical analyses were performed by analysis of variance followed by Tukey post hoc test. **B**, Representative fluorescence micrographs (z-stack, 5 μ m) of $\alpha 8$ -tubulin containing microtubules (green) in human cultured megakaryocytes (i) and human washed platelets (ii). For each panel, the negative IgG control for $\alpha 8$ -tubulin is shown on the left. Microtubules were labelled separately for total α -tubulin (red) and merged images indicate colocalization (yellow). **C**, Main characteristics of the six individuals identified with *TUBA8* mutations. For each, the platelet count and mean platelet volume (MPV), the nucleotide and subsequent amino-acid substitutions, the single nucleotide polymorphism ID and frequency, and the disease-causing potential evaluated with the MutationTaster program are indicated

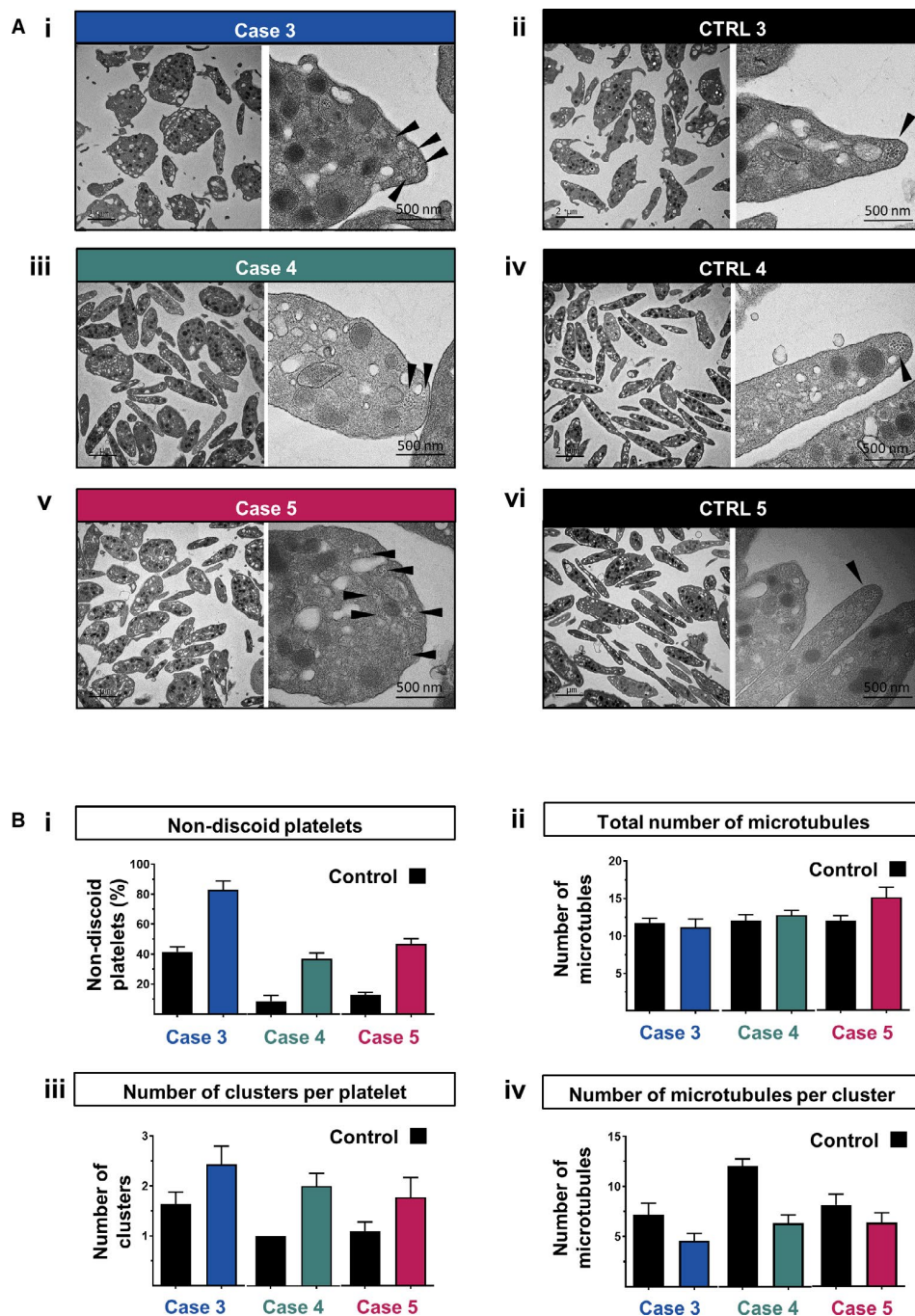


FIGURE 2 Platelet ultrastructural defects in individuals carrying *TUBA8* mutations. A, Representative transmission electron microscopy (TEM) micrographs of washed platelets obtained from the individuals carrying *TUBA8* p.R214C (i; Case 3), p.R243C (iii; Case 4), and p.E290K (v; Case 5) mutations and their respective controls (ii, iv, vi). In close-up views, the arrowheads indicate the multiple disorganized microtubule clusters in the *TUBA8* mutant platelets as opposed to the single cluster located at the platelet periphery for the controls. Please note that slight activation of the platelets of Case 3 and its control occurred during the washing procedure. B, Quantification of (i) the proportion of non-discoid platelets in the *TUBA8* mutant and control individuals. Platelet shape was scored as discoid and non-discoid from the analysis of TEM images. Results represent the mean \pm standard error of the mean (SEM) percentage from 85 to 129 randomly observed platelets; (ii) the total number of microtubules per platelet. For each platelet observed by TEM the number of individual microtubules was counted independent of their proximity. The results represent the mean \pm SEM of 7–15 individual platelets; (iii) the number of microtubule clusters per platelet. Microtubule clusters, illustrated by the arrowheads in (A), were numbered for each platelet analyzed in (ii). Values represent the mean \pm SEM from 7 to 15 individual platelets; (iv) the number of microtubules per cluster. For each platelet observed by TEM in (ii), the number of microtubules within each independent cluster was counted. The results represent the mean \pm SEM of 7–15 individual platelets

electron microscopy analyses of their platelets revealed similar abnormal features for the three individuals compared to their controls (Figure 2A). An abnormal round morphology was observed in 36% and 46% of Case 4 and Case 5 platelets, while only 8% and 12% of their respective control platelets presented this unusual shape. For Case 3, this round morphology was more frequent with 82% of non-discoid platelets, but was also observed for 41% of the respective control platelets (Figure 2Bi). This is likely to be caused by slight platelet activation during the preparation of Case 3 and its control platelets, because shape change represents one of the

most sensitive platelet responses. In addition to their abnormal morphology, platelets of the three individuals showed a severe disorganization of the normally well-structured marginal band. While the total number of microtubules in the marginal bands was similar to that in their respective control (Figure 2Bii), they adopted a scattered distribution with distinct clusters of closely associated microtubules (Figure 2Ai,iii,v-Biii). This stands in strong contrast to control platelets, which typically show a single cluster of microtubules in their submembranous space in transversal sections (Figure 2Aii,iv,vi-Biii). The number of microtubules per

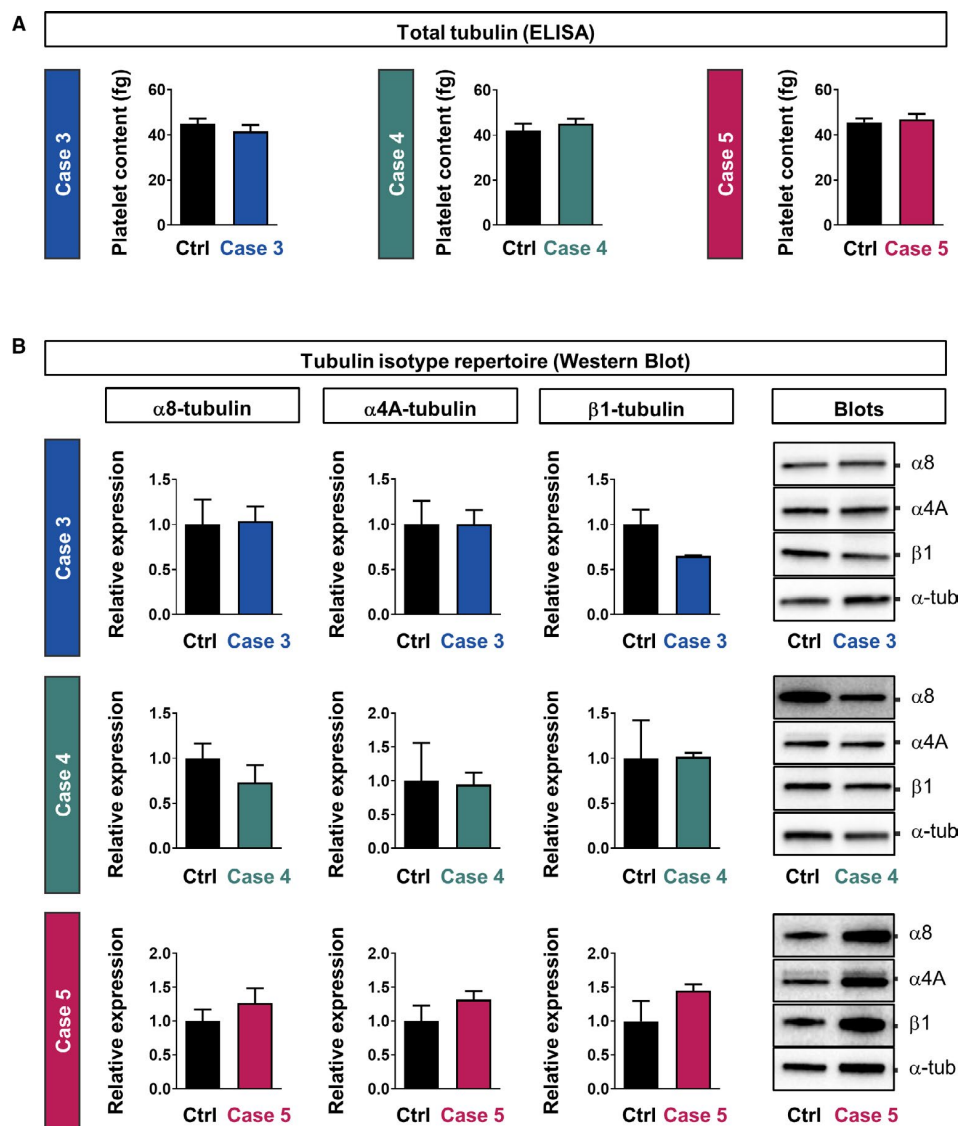
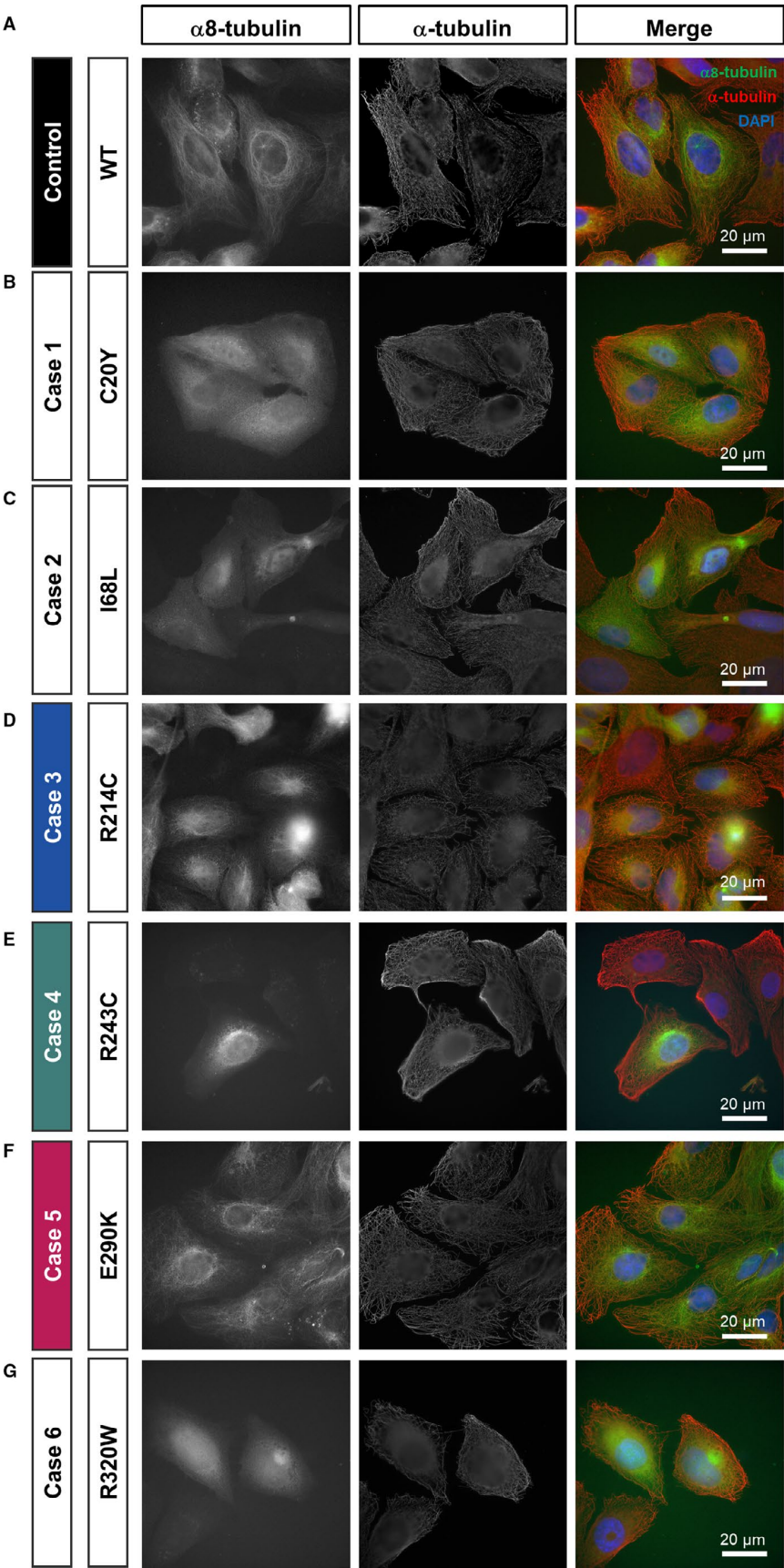


FIGURE 3 Platelet tubulin content in individuals carrying *TUBA8* mutations. A, Quantification by ELISA of the total α -tubulin content in the platelets of the individuals carrying the *TUBA8* p.R214C (Case 3), the p.R243C (Case 4), or the p.E290K (Case 5) mutations and the respective controls. The results are expressed as the concentration (in fg) per single platelet and represent the mean of 3 technical replicates. B, Estimation by western blot of the levels of α 8-, α 4A-, and β 1-tubulin in the platelets of the individuals carrying the *TUBA8* p.R214C (Case 3), the p.R243C (Case 4), or the p.E290K (Case 5) mutations and the respective controls. The expression levels for each isotype, estimated by densitometry, have been normalized to those of total α -tubulin, and the ratio arbitrarily set to 1 for each control sample. Results are expressed as the mean \pm standard error of the mean of three technical replicates

FIGURE 4 Effect of the *TUBA8* mutations on their expression and incorporation into microtubules. **A**, Representative fluorescence micrographs of U2OS cells expressing wild-type N-terminally green fluorescent protein (GFP)-tagged *TUBA8*. **B–G**, Representative fluorescence micrographs of U2OS cells expressing mutant p.C20Y, p.I68L, p.R214C (Case 3), p.R243C (Case 4), p.E290K (Case 5), or p.R320W N-terminally GFP-tagged *TUBA8*. For each condition, microtubules were labelled separately and nuclei were counterstained with DAPI



cluster was therefore diminished in the platelets of the three individuals (Figure 2Biv). Together with the macrothrombocytopenia, these results thus indicate that $\alpha 8$ -tubulin is both important for platelet biogenesis and for the organization of the marginal band and the subsequent platelet shape.

We then proceeded in functional evaluation of the platelets for the three individuals. The levels of the two main functional receptors, $\alpha \text{IIb}\beta 3$ integrin (GPIIb-IIIa) and GPIb, were found to be increased by about 20%–80% (data not shown). This increase is most likely more due to the increased platelet size than to an increased level of activation.^{12,13} Furthermore, p-selectin exposure at the platelet surface in response to the thrombin receptor activating peptide agonist, as well as platelet aggregation in response to a series of agonists, were similar to their respective controls for all three individuals (data not shown). These data therefore indicate that $\alpha 8$ -tubulin, while important for normal platelet production, is not required to sustain the main platelet functions as measured *in vitro*. To evaluate whether the defects in marginal band formation resulted from critically low levels of tubulin, which would impede proper microtubule assembly, we estimated tubulin levels in platelet lysates. The total α -tubulin content was equivalent to the control in the three individuals (Figure 3A). We then evaluated the level of $\alpha 8$ -tubulin relative to total α -tubulin. The $\alpha 8/\alpha \text{tot}$ ratio represented 103%, 73%, and 126% of control for Case 3, Case 4, and Case 5, respectively (Figure 3B). This suggested that the *TUBA8* p. Arg243Cys mutation in Case 4 had a negative effect on the expression or stability of the $\alpha 8$ -isotype in platelets whereas it appeared to be preserved in Cases 3 and 4. In addition, we found that the expression of $\alpha 4A$ - and $\beta 1$ -tubulin were not decreased in the platelets of the three individuals, with the exception of $\beta 1$ -tubulin, which represented 65% of control for Case 3 (Figure 3B). This could be linked to the additional heterozygous *TUBB1* p. Gly146Arg mutation found in this individual. Together, these data suggest that different consequences on the expression and stability of $\alpha 8$ -tubulin might be expected depending on the type of mutation in the six identified individuals.

To understand the mechanism underlying the platelet defects, we evaluated whether the mutant forms of $\alpha 8$ -tubulin can incorporate into microtubules. For this, we transduced the human cell line U2OS with lentivirus encoding the $\alpha 8$ -tubulin variants that are N-terminally tagged with GFP. Following transduction of wild-type $\alpha 8$ -tubulin, a GFP-positive network was observed in all the cells that colocalized with microtubules labelled with an anti-tubulin antibody, indicating incorporation into the cellular microtubules (Figure 4A). A similar, but weaker microtubule localization was also observed with the $\alpha 8$ -tubulin mutations from Case 3 (p. Arg214Cys) and Case 5 (p. Glu290Lys; Figure 4D,F). In contrast, $\alpha 8$ -tubulin variants p. Cys20Tyr, p. Ile68Leu, p. Arg243Cys (Case 4), and p. Arg320Trp did not visibly incorporate into microtubules, and instead showed a diffuse or punctuated labelling (Figure 4B,C,E,G). These results indicate that all six *TUBA8* mutations inhibit, though to variable degrees, their incorporation into

the microtubule network. Such an abnormal incorporation could similarly occur in platelets and affect marginal band formation by directly disrupting its assembly or stability, or indirectly via defective interaction with microtubule-associated proteins involved in its maintenance.

In conclusion, this study provides strong support for a key role of multiple tubulin genes in platelet biogenesis by discovering variants in a tubulin gene that was previously not known to be important for platelets: *TUBA8*. Characterizing platelets from patients carrying these variants provided insight into the role of the divergent and barely studied $\alpha 8$ -tubulin isotype, representing overall another contribution highlighting the relevance of the tubulin code for controlling specialized cellular functions, such as platelet biogenesis. Before its identification in megakaryocytes,⁴ expression of *TUBA8* had been reported in heart, skeletal muscle, testis, and brain;¹⁴ however, little was so far known about its physiological importance. Compared to other tubulin gene variants, *TUBA8* variants were rarely found in brain disorders,¹⁵ and the so-far unique reported case also carries a mutation in *SNAP29*, which prevents a direct conclusion on the role of the *TUBA8* variant in this case.¹⁶ The present report therefore presents the first occurrence of an abnormal biological process directly linked to *TUBA8* mutations. Finally, although patients with platelet tubulinopathies do not classically exhibit bleeding problems, the example of Case 5 reported here raises the possibility that tubulin-related defects could contribute to an increased bleeding in association with another phenotype modifier, as previously documented for *TUBB1*,¹⁷ justifying the current high throughput sequencing programs focusing on hereditary thrombocytopenia.

ACKNOWLEDGMENTS

The authors would like to thank the individuals involved in this study; members of EFS-Grand Est, EFS-Nouvelle Aquitaine, EFS-Bretagne, EFS-Centre Pays de la Loire, for their participation in the ABCT study; Dr. Dominique Desprez for organizing Case 4 and Case 5 blood sampling; Joëlle Dulong, Dr. Benoit Guillet, Nadine Bouvet for organizing Case 3 blood sampling and platelet preparation.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

Q.K. performed platelet and cell biology studies, analyzed data, and co-wrote the paper; A.D. co-designed the ABCT study, organized the clinical part, and collected biological and sequencing data; C.W. and P.G. performed and analyzed molecular experiments; V. Hei. and S.M. performed platelet studies; A.E. performed electron microscopy studies; V. Hen. and S.B. performed molecular biology experiments; S.B. performed transduction and immunofluorescence experiments; C.F. and C.G. organized the clinical study and contributed to intellectual input; C.J. analyzed the data and critically reviewed the paper; F.L. designed the study, analyzed the data, and wrote the paper.

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How to cite this article: Kimmerlin Q, Dupuis A, Bodakuntla S, et al. Mutations in the most divergent α -tubulin isotype, α 8-tubulin, cause defective platelet biogenesis. *J Thromb Haemost*. 2022;20:461-469. doi:[10.1111/jth.15573](https://doi.org/10.1111/jth.15573)