

# A Trp11Arg Substitution in the $\beta 3$ Signal Peptide Prevents Expression of $\alpha \text{IIb}\beta 3$ in Patients with Glanzmann Thrombasthenia

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Glanzmann thrombasthenia (GT) as a rare hereditary bleeding disease is linked to abnormalities in quality or quantity of the  $\alpha \text{IIb}\beta 3$  integrin on the platelet surface.<sup>1</sup> The hallmark of this disease is severely reduced platelet aggregation in response to platelet agonists.<sup>2</sup> Numerous mutations in *ITGA2B* and *ITGB3* genes were identified in patients with GT.<sup>3</sup> For *ITGB3*, gene mutations are categorized in three types: mutations which cause a lack of protein expression (GT type I, <5% of normal), mutations which lead to reduced

expression of functional or nonfunctional  $\alpha \text{IIb}\beta 3$  (GT type II, 5–15% of normal), and mutations which lead to the production of normal amounts of nonfunctional proteins on platelet surface (GT type III).<sup>3,4</sup> Responsible mutations are distributed across all  $\beta 3$  domains.<sup>5–7</sup>

Recently, we described a GT patient who was found to carry two compound heterozygous missense mutations in the *ITGB3* (NM\_000212.2) gene: one mutation c.31T>C (LRG\_481:g.5051T>C) located in exon 1 and one mutation



**Fig. 1** Characterization of c.31A>C mutation on *ITGB3* gene synthesis and expression. (A) Sequencing analysis confirmed the generation of c.31A>C mutation in *ITGB3* exon 1 on expression vector (upper: forward, lower: reverse). (B) Analysis of  $\alpha \text{IIb}\beta 3$  expression in cell lysate from transfected HEK cells. After lysis proteins were separated on SDS-PAGE and blotted, proteins were stained with mab Gi16 or AP3 against  $\alpha \text{IIb}$  or  $\beta 3$ , respectively. GAPDH was detected as internal control. Bound antibodies were detected with HRP-labeled secondary antibody. (C) Surface expression of wild-type or mutant  $\alpha \text{IIb}\beta 3$  integrin on transfected HEK cells was analyzed by flow cytometry using mabs against  $\alpha \text{IIb}$  (mab Gi16),  $\beta 3$  (mab AP3) or  $\alpha \text{IIb}\beta 3$  complex (mab Gi5). Bound antibodies were detected with FITC-labeled anti-mouse secondary antibody. Mouse IgG was used as negative control (upper panel). Bar graphs (lower panel) represent the mean fluorescence intensity in transfected HEK cells. (D) The kinetics of  $\alpha \text{IIb}\beta 3$  mutant (circles) and wild-type (squares) transient surface expression on COS cells were analyzed 48, 72, 98, and 120 hours after transfection using mabs against  $\alpha \text{IIb}$  (mab Gi16) or  $\beta 3$  (mab AP3 and SZ21) (closed symbols) or isotype controls (open symbols). Gray lines indicate wild-type, and black lines represent mutant proteins. (E) COS cells were transfected with wild-type (black bars) or mutant (gray bars)  $\alpha \text{IIb}\beta 3$ . Densitometry analysis of the band showed a higher  $\beta 3$  and  $\alpha \text{IIb}$  expression in wild-type cells which reached a maximum density at 72 hours and dropped to basal level 120 hours after transfection. Analysis of cytoplasmic content of  $\alpha \text{IIb}$  and  $\beta 3$  in transfected cells was performed using ImageJ program normalized to GAPDH. Presented data ( $n = 3$ ) are the mean  $\pm$  SEM. IgG, Immunoglobulin G; SEM, standard error of the mean.

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c.1458C > G in exon 10, which led to amino acid substitutions Trp11Arg (►Fig. 1A) and Cys486Trp (mature Cys460-Trp), respectively.<sup>8</sup> The Cys486Trp mutation is located in the I-EGF1 domain, which is known to harbor distinct amino acids responsible for the formation of HPA-1a epitopes, and was further analyzed in a recent study by some of us.<sup>7</sup> The effect of the first mutation, Trp11Arg, remains unknown.

The first 27 amino acids of  $\beta 3$  are considered to represent the signal peptide by in silico analysis. The c.31T > C muta-

tion has been previously described in GT patients.<sup>5–7</sup> Effects of this mutation on  $\alpha \text{IIb}\beta 3$  membrane expression were investigated using an online predictor mutation taster (<https://www.mutationtaster.org>), suggesting that this mutation changes a splice site and probably affects the protein features. Multiple sequence alignment and phylogenetic tree were done using ClustalW software (<https://www.genome.jp/tools-bin/clustalw>). These results show high evolutionary conservation of Trp11 (►Fig. 2).

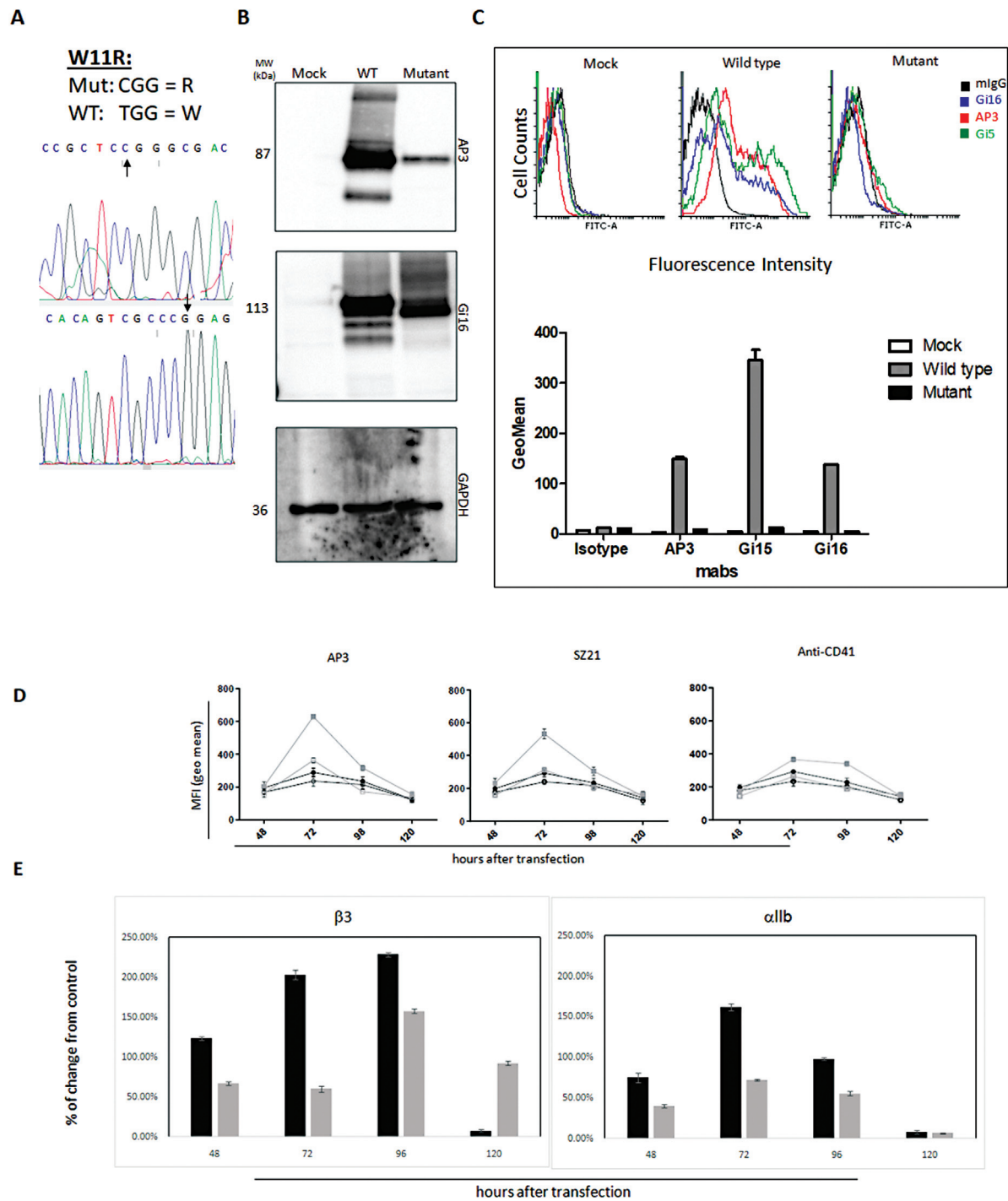


Fig. 2 (A-E) Phylogenetic tree and multiple sequence alignment for first 50 amino acids of  $\beta 3$  integrin across five different species.

To identify the biological effects of the c.31T > C mutation in a previously described patient carrying this mutation in combination with c.1458C > G mutation (compound heterozygote), we used site-directed mutagenesis to induce the mutated 31C in wild-type *ITGB3* cDNA.<sup>8</sup> HEK cells were transfected with the respective wild-type (31T) or mutant (31C) expression vectors, cultured, and lysed. Cell lysates were analyzed by western blot using monoclonal antibodies against  $\beta 3$  (CD61, clone AP3) or  $\alpha \text{IIb}$  (CD41, clone Gi16). Presence of GAPDH was evaluated as an internal reference. In wild-type and mutant lysates, a band of 87 kDa was detected by AP3, indicating presence of  $\beta 3$  protein in both cells. Despite equal concentration of GAPDH, analysis showed a significant decrease in  $\beta 3$  protein in transfected HEK cells expressing 31C when compared with the wild-type. Integrin  $\alpha \text{IIb}$  was expressed in comparable amounts by both, mutant and wild-type cells. However, the pattern was different, and the main band was slightly lighter for the mutant form (►Fig. 1B).

The expression of  $\alpha \text{IIb}\beta 3$  on the surface of HEK cells was evaluated by flow cytometry (►Fig. 1C). Wild-type  $\alpha \text{IIb}$  and  $\beta 3$  proteins were detectable in cytoplasm as well as on the surface of transfected cells, HEK cells. In contrast, analysis of  $\alpha \text{IIb}\beta 3$  protein on the cell surface of transfected cells showed no detectable  $\alpha \text{IIb}\beta 3$  protein on mutant-transfected cells. These observations indicated that despite cytoplasmic presence of both  $\beta 3$  (c.31C variant) and  $\alpha \text{IIb}$ , no mutant  $\alpha \text{IIb}\beta 3$  integrin was transported to the cell surface. Previous analysis of  $\alpha \text{IIb}\beta 3$  expression on platelets surface of GT patients has shown that defects in the  $\beta 3$  gene lead to deficiencies in both  $\alpha \text{IIb}\beta 3$  and  $\alpha \text{v}\beta 3$  proteins.<sup>9</sup> Similarly, in the current study no  $\alpha \text{IIb}$  was expressed on the surface of transfected cells.

To compare the kinetics of  $\alpha \text{IIb}\beta 3$  expression and degradation, COS cells were transiently transfected with wild-type (31T) or mutant (31C) expression vectors, and the presence of  $\alpha \text{IIb}\beta 3$  was evaluated by flow cytometry or western blot at 48, 72, 96, and 120 hours after transfection (►Fig. 1D, E). Both  $\alpha \text{IIb}$  and  $\beta 3$  appeared on the cell surface 72 hours after transfection of wild-type  $\beta 3$ , and were still detectable after 98 hours of transfection. In contrast, we did not find a definite indication for the expression of the mutant (31C) protein at all time points. Analysis of cell lysate evaluated in densitometry demonstrated very weak expression after 48 hours inside the cell, reaching maximum expression after 72 and 96 hours for both wild-type and mutant proteins. Note that mutant  $\beta 3$  was still detectable 120 hours after transfection (►Fig. 1E).

Our data demonstrate that nucleotide 31 in *ITGB3* is part of a regulatory component for gene expression. Mutations in this position affect not only  $\beta 3$  expression but also the expression of  $\alpha \text{IIb}$  as a partner protein. In our homozygous

experimental model, c.31T > C does not affect protein biosynthesis, but affects the transport of the protein to the membrane, leading to GT type I. In our patient, compound heterozygosity was responsible for the presence of low copy numbers of nonfunctional  $\alpha \text{IIb}\beta 3$  on the platelet surface. The c.31T > C mutation was previously reported in a collection of GT patients.<sup>5,6</sup> These authors found c.31T > C in a compound heterozygous German child with severe bleeding symptoms, and they could not assign a biological effect to this mutation. Apparently, c.31C is responsible for the absence of  $\alpha \text{IIb}\beta 3$  from the platelet surface and can be considered causative for GT type I.

#### Author Contributions

B.B. and U.J.S. designed the experiments, analyzed the data, and wrote the manuscript. Y.W. and M.A. performed the experiments and analyzed the data. G.B. helped analyzing the data and writing the manuscript.

#### Conflict of Interest

None declared.

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