Defective binding of ETS1 and STAT4 due to a mutation in the promoter region of *THPO* as a novel mechanism of congenital amegakaryocytic thrombocytopenia

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Abstract

Congenital amegakaryocytic thrombocytopenia (CAMT) is a recessive disorder characterized by severe reduction of megakaryocytes and platelets at birth, which evolves toward bone marrow aplasia in childhood. CAMT is mostly caused by mutations in *MPL* (CAMT-MPL), the gene encoding the receptor of thrombopoietin (THPO), a crucial cytokine regulating hematopoiesis. CAMT can be also due to mutations affecting the *THPO* coding region (CAMT-THPO). In a child with the clinical picture of CAMT, we identified the homozygous c.-323C>T substitution, affecting a potential regulatory region of *THPO*. Although mechanisms controlling *THPO* transcription are not characterized, bioinformatics and *in vitro* analysis showed that c.-323C>T prevents the binding of transcription factors ETS1 and STAT4 to the putative *THPO* promoter, impairing *THPO* expression. Accordingly, in the proband the serum THPO concentration indicates defective THPO production. Based on these findings, the patient was treated with the THPO-mimetic agent eltrombopag, which induced a significant increase in platelet count and stable remission of bleeding symptoms. Herein, we report a novel pathogenic variant responsible for CAMT and provide new insights into the mechanisms regulating transcription of the *THPO* gene.

Introduction

Congenital amegakaryocytic thrombocytopenia (CAMT) is an autosomal recessive disease characterized by thrombocytopenia with severely reduced or absent megakaryocytes in the bone marrow and progression toward trilineage bone marrow aplasia. Most affected individuals have mutations in *MPL*, the gene encoding for the receptor of thrombopoietin (THPO),[1] and are referred to as having CAMT-MPL.[2] THPO is a crucial regulator of hematopoiesis, being required for both the survival of multipotent hematopoietic progenitors and their differentiation into megakaryocytes, explaining the clinical picture of CAMT.[3]

Indeed, a few homozygous mutations, (p.Arg38Cys, p.Arg99Trp, p.Arg119Cys, and Arg157*), all affecting the coding region of the *THPO* gene, have recently been identified in families with severe amegakaryocytic thrombocytopenia (CAMT-THPO[2]), in some cases associated with multilineage bone marrow failure.[4-6] Moreover, monoallelic mutations (p.Arg31*, p.Arg99Trp, p.Glu204Glyfs*123, and p.Leu269Profs*58) of the same gene cause a dominant form of thrombocytopenia with incomplete penetrance characterized by mild reduction in platelet count and normal platelet size.[7,8] Consistent with a haploinsufficiency effect, mild thrombocytopenia is one of the features of the syndromic disorders associated with microdeletions of chromosome 3 containing the *THPO* gene.[4,9,10] Therefore, there is a growing body of evidence regarding the role of *THPO* alterations as the cause of both autosomal recessive and dominant thrombocytopenia.

Here, we report a patient with severe amegakaryocytic thrombocytopenia caused by a homozygous mutation (c.-323C>T) in the promoter region of the *THPO* gene. Functional studies demonstrated that the mutation affects the binding of two transcription factors, ETS1 and STAT4, to the *THPO* promoter, significantly reducing the expression of THPO. Consistent with these findings, the affected in-

dividual was successfully treated with the THPO receptor agonist eltrombopag.[11]

Methods

Patient

This study was conducted in accordance with the guidelines of the local Helsinki Committee.

Exome sequencing

Whole exome sequencing was performed using the Twist Human Core Exome Plus Kit (Twist Bioscience, San Francisco, CA, USA) as the gene capture kit, and the enriched libraries were sequenced on a NovaSeq 6000 sequencing machine (Illumina, San Diego, CA, USA). For each sample, paired end reads (2×100 bp) were obtained and processed. The Illumina Dragen Bio-IT Platform version 3.8 was used to align reads to the human reference genome (hg38) based on the Smith-Waterman algorithm,[12] as well as to call variants based on the GATK variant caller version 3.7.[13] Additional variants were called with Freebayes version 1.2.0.[14] Variant annotation was performed using KGG-Seq version 1.2.[15] Further annotation and filtration steps were performed by in-house scripts using various additional public and private datasets such as the Human Gene Mutation Database,[16] ClinVar,[17] gnomAD[18] and the Sheba Medical Center's database of all variants from previously sequenced samples. Final variant analysis was only performed on rare mutations with an allele frequency of <0.05 in gnomAD and the in-house database, and that were annotated as protein changing and protein-damaging based on KGG-Seq, in addition to all variants that were previously reported as pathogenic or likely pathogenic in the HGMD or ClinVar datasets.

Plasmids

pGL3-THPO reporter constructs were obtained by cloning each *THPO* promoter into the pGL3-Basic construct (Promega, Madison, WI, USA) plasmid, upstream to a Firefly luciferase reporter gene, between the KpnII and HindIII restriction sites (New England Biolabs). The Renilla luciferase which is used to normalize luciferase activity is expressed by a pRL-CMV plasmid. The promoter sequences of *THPO* were obtained from Ensembl (*http://www.ensembl.org/index.html*) and UCSC (*http://genome.ucsc.edu/cgibin/* hgGateway) databases and amplified from control genomic DNA with AccuPrime™ Taq DNA Polymerase High Fidelity (Invitrogen™, #12346086), following the manufacturer's instructions.

In the pGL3-THPO reporter the c.-323C>T was introduced using a Phusion™ Site-Directed Mutagenesis Kit (ThermoScientific, F541), following the manufacturer's instructions.

Statistical analyses and reproducibility

At least three independent replicates were performed for each of the experiments. All graphs present single data point mean ± standard error of mean. Statistical tests were performed using GraphPad Prism 8. *P* values were obtained using a two-tailed Student unpaired parametric *t* test. Reported blots and micrographs are representative of three independent experiments. This study does not include any data deposited in external repositories.

Other standard methods

Methods for standard procedures, including cell culture and transfection, RNA extraction and quantitative real-time polymerase chain reaction, chromatin immunoprecipitation, western blotting and co-immunoprecipitation analysis, dual luciferase assays, and proximity ligation assay are reported in the *Online Supplementary Methods*.

Results

Clinical features of the proband and his family members

The proband (II-3) (Figure 1A) was a 3.5-year-old boy when he presented to our hospital with easy bruising and thrombocytopenia (platelet count: 21x10^9/L) with a normal mean platelet volume. He had a lifelong history of frequent bruising but no other bleeding symptoms. On a blood count shortly after birth, his platelet count had been 70x10^9/L but no further workup was done until the age of 2 years, when he was diagnosed with urethral stenosis. At that time, he showed severe thrombocytopenia (platelet count: 20x10^9/L) and received his only platelet transfusion prior to meatotomy to correct the stenosis. Revision of the blood counts performed prior to our evaluation did not reveal other cytopenias aside from mild occasional neutropenia. Bone marrow examination from two different biopsies showed a normocellular marrow with severe hypoplasia of the megakaryocytes, with a few small immature forms seen, and mild dyserythropoiesis, consistent with amegakaryocytic thrombocytopenia. Chromosomal breakage analysis did not reveal increased breakage in the presence of diepoxybutane, ruling out Fanconi anemia. Moreover, chromosomal analysis on the bone marrow showed a normal 46,XY karyotype and fluorescence *in situ* hybridization studies did not reveal deletion of chromosomes 5, 7, 5q, 7q, or 20q.

The proband was born to Muslim Palestinian parents who are first cousins to one another. His mother (I-2) has a history of mild thrombocytopenia that worsened during each pregnancy; her platelet counts are now stable around 80x10^9/L, and her mean platelet volume is normal. She has no history of bleeding symptoms. The proband's younger sibling (II-4) had a platelet count of 130x10^9/L around birth but several weeks later a repeat platelet

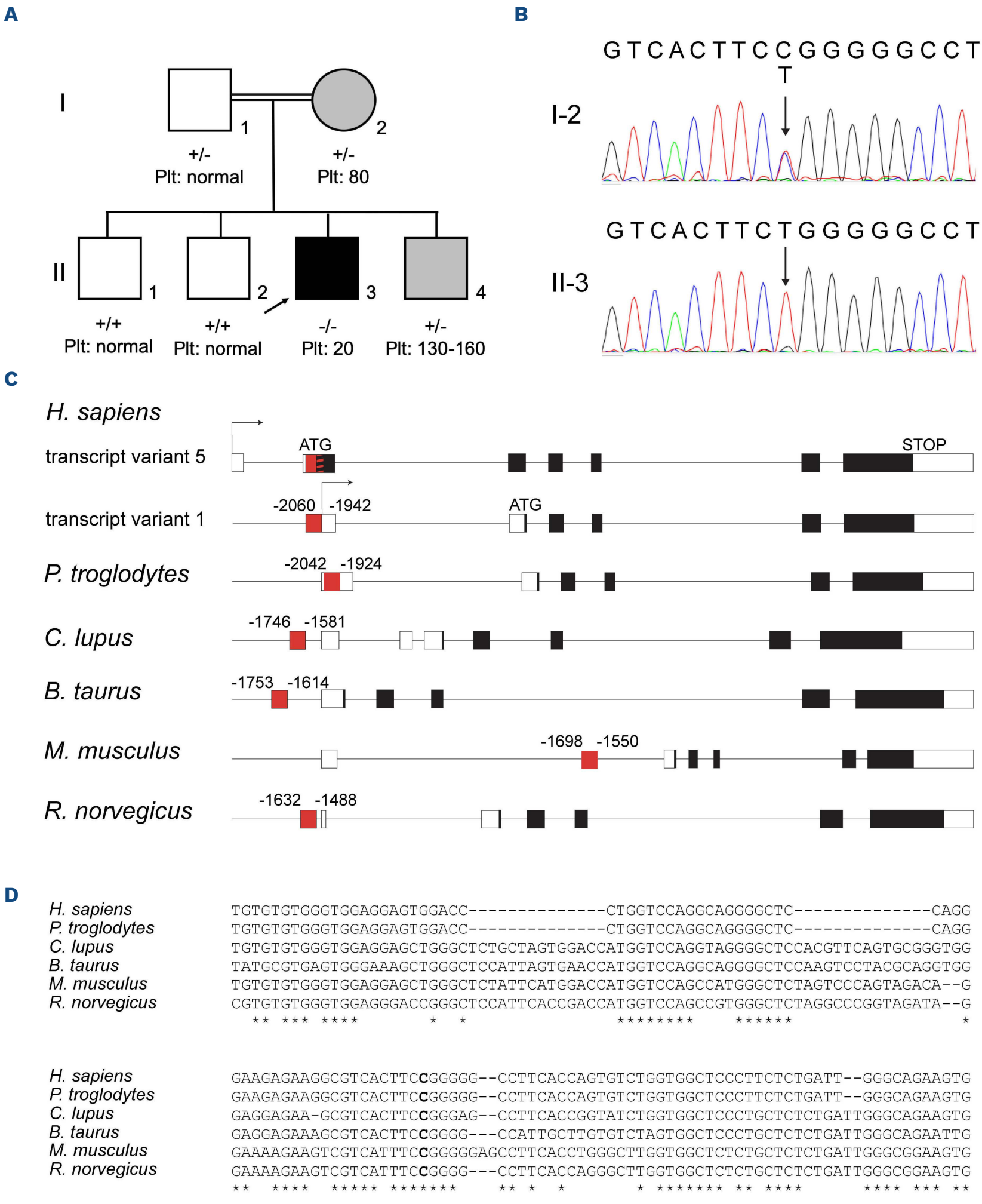

Figure 1. Identification of c.-323C>T in the promoter region of the *THPO* gene in a patient with congenital amegakaryocytic thrombocytopenia. (A) Pedigree of the family. The black symbol indicates the subject carrying the homozygous c.-323C>T (NM_000460.2) mutation with severe thrombocytopenia, and the gray symbols the carriers with mild thrombocytopenia. (B) Electropherograms of the *THPO* gene showing the c.-323C>T mutation in the proband and in his mother. (C) Genomic structure of the human *THPO* gene and its transcript variants 5 and 1. The genomic structures of the gene in different species are also reported for evolutionary comparison. The black boxes represent the open reading frame. The red boxes show the regions orthologous to the 119 bp human region surrounding the c.-323C nucleotide aligned in (D). The relative genomic positions of the orthologous regions upstream of the ATG translation start site are also indicated. (D) Multiple alignments of the red squared regions indicated in (C) from different species. The c.-323C nucleotide (bold case), as well as the surrounding positions, are well conserved among species. The asterisks indicate conserved nucleotides. Plt: platelet count x 10^9/L.

count was 160x10^9/L. There are no other cytopenias in any family members.

Identification of c.-323C>T in the promoter region of the *THPO* gene

Whole-exome sequencing allowed us to identify a homozygous nucleotide substitution (chr3:g.184096040G>A from GRCh37) within the *THPO* gene in the proband (Figure 1B). The human *THPO* gene is transcribed from two different promoters, generating two major mRNA, one of 2186 bp (transcription variant 5) and the other of 1918 bp (transcription variant 1) originated from different transcription start sites (Figure 1C). Variant 5 (NM_001290003.1) is constituted of seven exons with the first ATG in exon 2 and a putative open reading frame of 493 codons. The chr3:g.184096040G>A substitution would affect nucleotide C at position 98 (c.98C>T; p.Pro33Leu). However, this putative 493 amino acid isoform has never been reported, likely because its translation is inhibited by a series of translational start codons (uAUG) located in the 5'-UTR.[19]

The transcription variant 1 (NM_000460.4) consists of six exons with the first ATG in exon 2 and an open reading frame of 353 codons (Figure 1C).[20,21] Bioinformatics analysis showed that in this transcription variant, the chr3:g.184096040G>A substitution would affect the nucleotide at position 45 upstream of the transcription start site (or nucleotide 323 upstream of the ATG; c.-323C>T). Considering that the only protein so far detected is the 353 amino acid isoform translated by variant 1, we focused on the c.-323C>T substitution, hypothesizing that it impairs *THPO* basal promoter activity.

Consistent with consanguinity, segregation analysis revealed that the parents and the younger sibling of the proband (II-4) were heterozygous for c.-323C>T whereas the other two healthy siblings were homozygous for the wild-type allele (Figure 1A). The variant is very rare, reported in the dbSNP (rs1208732776) but not in GnomAD. Moreover, multiple alignment of orthologous regions containing the c.-323C nucleotide from different species shows high sequence homology for a stretch of 25 nucleotides, suggesting an evolutionary conserved function of this region (Figure 1D).

No additional variant was detected in other genes causative of amegakaryocytic thrombocytopenias, such as *MPL*, *MECOM*, *HOXA11* or *RBM8A*, nor in those associated with Fanconi anemia or responsible for other inherited bone marrow failure syndromes (*Online Supplementary Table S1*).

c.-323C>T prevents STAT4 and ETS1 binding and strongly reduces *THPO* expression

Considering that the mechanisms regulating expression of the *THPO* gene are still elusive, we inspected the region upstream of the transcription start site, looking for putative binding sites of transcription factors. Nucleotide c.-323C was predicted to be within the consensus sequence for the binding of c-ETS-1 (here after ETS1) and STAT4 (Figure 2A). Of note, these putative binding sites were not recognized when the c.-323C>T substitution was included in a query sequence.

To evaluate its potential pathogenic role, we cloned the wild-type and c.-323C>T region (spanning 450 bp upstream of the transcription start site) of the *THPO* promoter upstream of the luciferase reporter gene. Transfecting HEK293T cells, which constitutively express THPO,[20] we observed that c.-323C>T significantly reduced (by almost 80%) the reporter activity as compared to the wild-type counterpart (Figure 2B).

To determine whether ETS1 and STAT4 regulate *THPO* transcription, we performed the same assay as above after silencing the two transcription factors, either alone or in combination, and we observed a significant reduction of the luciferase activity when the reporter gene was under the control of the wild-type promoter (Figure 2C). Of note, the luciferase activity was almost absent when the reporter gene was regulated by the mutant promoter, independently of ETS1 and STAT4 downregulation, further supporting the concept that c.-323C>T prevents recruitment of the transcription factors on the *THPO* regulatory sequences.

Since biological samples from the proband were unavailable, we developed a transient *in vitro* chromatin immunoprecipitation assay[22] to determine whether ETS1 and STAT4 bind the *THPO* promoter directly. Using the reporter constructs in HEK293T cells, we observed that both ETS1 and STAT4 bound strongly to the synthetic wild-type *THPO* promoter. Binding of the transcription factors to the mutant promoter was lower, though with different affinity (Figure 2D). Taken together, these data suggest that ETS1 and STAT4 are able to bind the *THPO* promoter and that this interaction is significantly impaired, at least for STAT4, in the presence of c.-323C>T.

STAT4, ETS1 and STAT3 regulate THPO expression in a liver cell line

Since THPO is mainly produced in the liver,[3] we evaluated THPO expression levels by quantitative real-time polymerase chain reaction analysis and western blotting in HepG2 cells. Knocking down ETS1 and STAT4 alone or in combination strongly reduced THPO at both mRNA and protein levels (Figure 3A, B), demonstrating that ETS1 and STAT4 regulate its endogenous expression in a hepatocyte cell model.

To determine whether STAT4 and ETS1 interact with each other, we carried out proximity ligation assays, revealing the presence of complexes between STAT4 and ETS1, whose formation was reduced upon STAT4 knockdown (Figure 3C). The interaction of the two transcription fac-

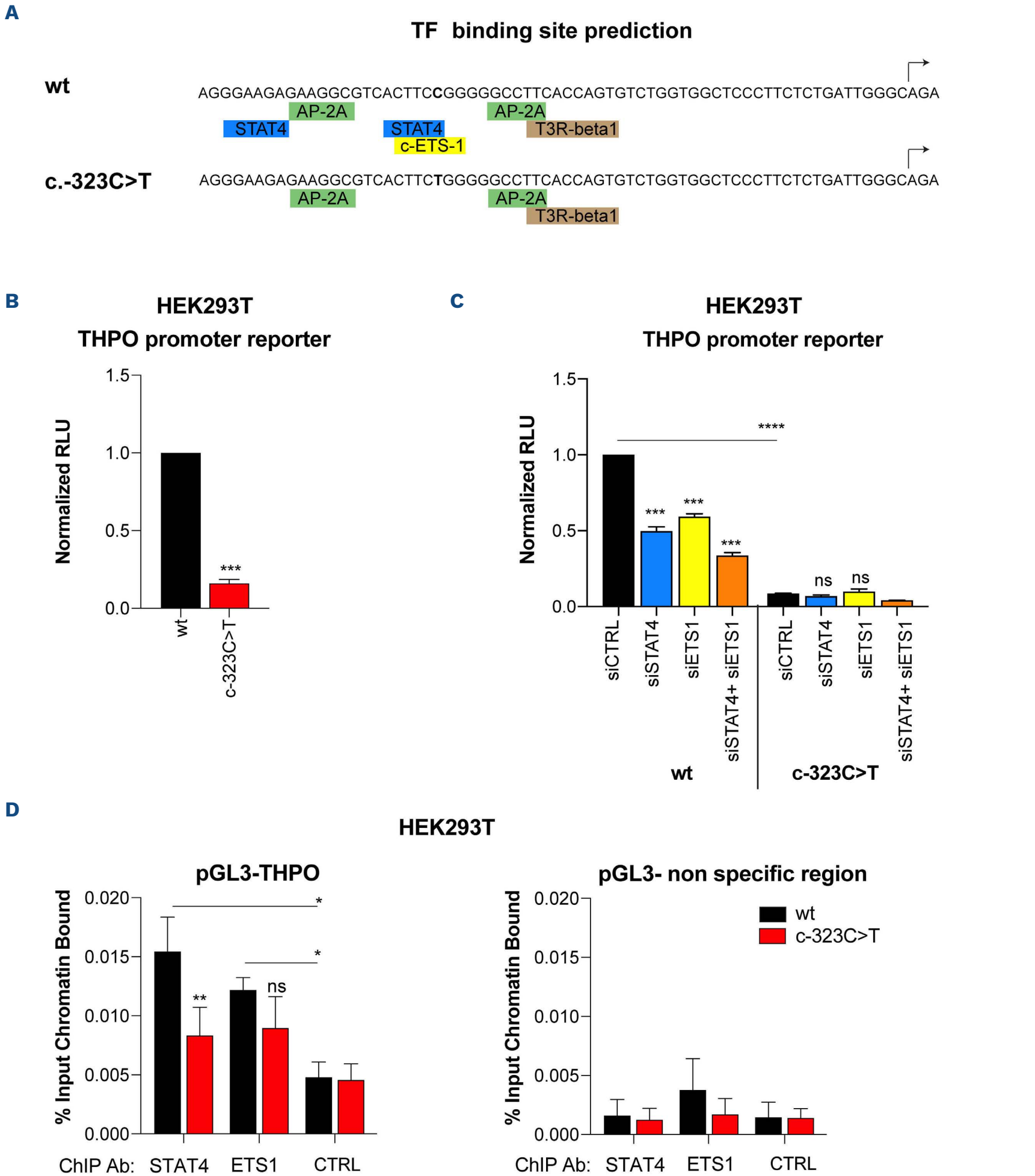

Figure 2. Variant c.-323C>T prevents STAT4 and ETS1 binding on the *THPO* promoter. (A) Schematic representation of the putative binding sites for transcription factors in the regions (wild-type and mutant form of transcript variant 1) upstream of the transcription initiation site (arrow). The c.-323C>T substitution is in bold case. (B) Luciferase assays were performed in HEK293T cells with pGL3-THPO in either the wild type or c.-323C>T forms. Renilla luciferase co-transfected with the reporter was used to normalize for transfection efficiency. (C) Luciferase assays were performed in HEK293T cells upon silencing endogenous STAT4 and ETS1 alone or in combination with specific short interfering RNA for 48 h. (D) Lysates of HEK293T cells transfected with pGL3-THPO either wild-type or c.-323C>T were subjected to chromatin immunoprecipitation analysis with antibodies recognizing STAT4 or ETS1, or with Protein A/G PLUS-Agarose as a negative control. Binding to the *THPO* promoter region was calculated as the fraction of the input chromatin bound. Binding to non-specific chromatin is shown in the right panel. wt: wild-type; TF: transcription factor; RLU: relative luminescence units; ns: not significant;si: short interfering; ChIP Ab: chromatin immunoprecipitation antibody; CTRL: control.

tors was confirmed by co-immunoprecipitation of the STAT4 and ETS1 proteins from lysates of HepG2 cells (Figure 3D), suggesting that they form a transcriptional complex on THPO regulatory sequences, promoting *THPO* transcription.

Considering that the expression of THPO in HepG2 cells is induced by JAK2/STAT3 upon uptake of desialylated platelets,[23] we further explored the mechanism controlling the transcription of the gene to determine any role of STAT3. PROMO *in silico* analysis of the *THPO* promoter did not find any consensus for the binding of STAT3 within the 1,500 bp region upstream of the transcription start site, which includes the c.-323C nucleotide affected by the mutation. Although no binding was expected, we knocked down STAT3 and STAT4 alone or in combination in HepG2 cells. We observed a reduction of THPO expression at both mRNA and protein levels (*Online Supplementary Figure S1A, B*), confirming a role of STAT3 in the regulation of the transcription of *THPO* independently of its binding to the basal promoter.

Serum THPO level was lower than expected in the proband

Since the variant c.-323C>T affects the transcriptional regulation of THPO, we measured the THPO level in the serum of the proband. The THPO concentration was 126 pg/mL (laboratory reference range 15-80 pg/mL for children <15 years old), a value within the normal range when obtained in healthy individuals, and slightly high for children.[2,24] Since serum THPO concentration is negatively regulated by the total megakaryocyte and platelet mass,[25,26] the THPO level is expected to be very much

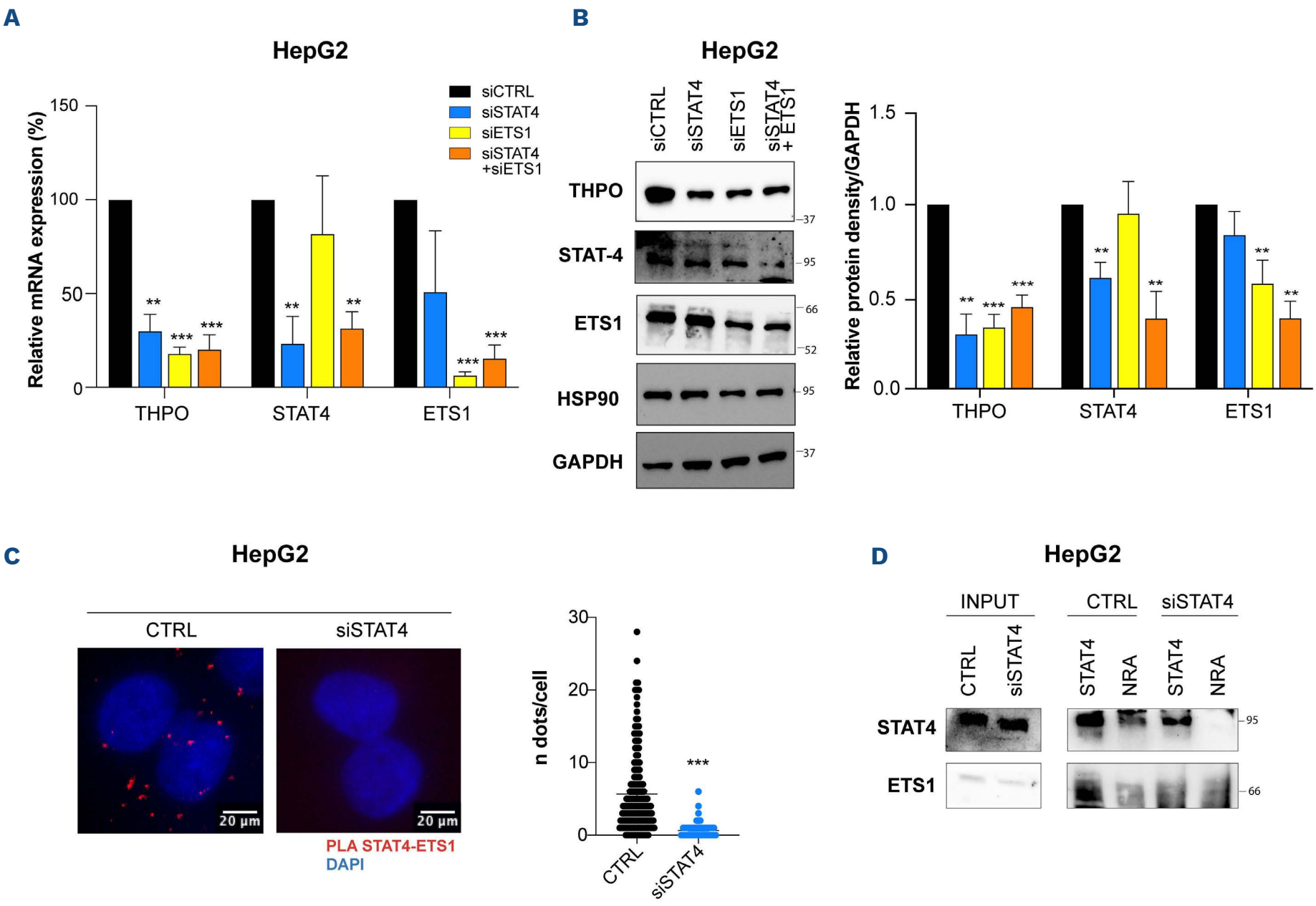

Figure 3. STAT4 and ETS1 regulate THPO expression. (A) *THPO*, *STAT4* and *ETS1* expression was evaluated by real-time quantitative polymerase chain reaction, normalized to β-actin RNA expression level. (B) Western blot analysis of THPO, STAT4, and ETS1 expression, using HSP90 and GAPDH as a loading control. Right: the graph shows the quantification of western blot bands measured by densitometry, normalized to GAPDH. (C) Proximity ligation assay (PLA) with primary antibodies against STAT4 and ETS1. (D) Lysates of HepG2 cells were subjected to co-immunoprecipitation analysis with antibodies recognizing STAT4 or IgG as a negative control. All the above experiments were performed in HepG2 cells upon silencing of endogenous STAT4 and ETS1, as indicated with specific short interfering (si) RNA for 48 h. Graphs present the mean ± standard error of mean of three independent experiments. Blots are representative of three biological replicates. *P* values were calculated by a two-tailed unpaired Student *t* test: **P*<0.05, ***P*<0.01,****P*<0.001.

higher than normal in amegakaryocytic thrombocytopenia, as well as in other forms of bone marrow aplasia/hypoplasia.[27-29]

Effective response to eltrombopag

Based on our overall findings, we considered it reasonable to treat the proband with a THPO-receptor agonist.[11] He was therefore started on eltrombopag 1.4 mg/kg/day, leading to an increase in his platelet count and then stabilization around $40x10^9$/L after 1 month. Since this was deemed an inadequate response, the dose was increased to 2.8 mg/kg/day. The patient's platelet count continues to increase gradually after 6 months of treatment at this dose; at the latest analysis, his platelet count was $125x10^9$/L. At present, he is clinically well and no longer has easy bruising.

Discussion

THPO is a critical cytokine that binds to its receptor MPL and stimulates expansion, differentiation, and maturation of megakaryocyte progenitors.[30] Additionally, it is essential for the survival of the multipotent hematopoietic stem cell compartment.[3] The clinical picture of CAMT reflects these non-redundant roles of the THPO/MPL axis. In fact, patients with CAMT present at birth with amegakaryocytic thrombocytopenia, which progresses to pancytopenia and bone marrow aplasia during childhood. CAMT is caused by biallelic mutations affecting the open reading frame, mainly of *MPL* but also of the *THPO* gene, referred to as CAMT-MPL and CAMT-THPO, respectively.[1,2,4,31,32]

In this paper, we report one patient with a clinical picture of CAMT characterized by severe congenital thrombocytopenia and marked hypoplasia of megakaryocytes but without pancytopenia. Whole exome sequencing analysis revealed a homozygous (c.-323C>T) substitution in a potentially regulatory region of the *THPO* gene that could explain the disease. Of interest, the proband's mother (II-2) and one sibling (II-4), heterozygous for c.-323C>T, have asymptomatic mild thrombocytopenia, which is consistent with the slightly reduced platelet count observed in individuals with monoallelic loss-of-function variants of *THPO*.[4-6]

Considering that the c.-323C>T substitution was at first regarded as a variant of uncertain significance, we carried out *in vitro* functional studies to determine its potential effect on *THPO* expression. We found that the promoter region carrying the variant strongly reduced reporter gene activity, supporting the hypothesis that transcription of the gene and consequent expression of the THPO cytokine are significantly impaired.

Consistent with these findings, the serum THPO concentration in the proband was only slightly above the normal range, despite the severely reduced megakaryocyte and platelet mass. Indeed, serum concentration of THPO is finely regulated by a mechanism of receptor-mediated clearance by megakaryocytes and platelets.[25,33] Therefore, the THPO level is always considerably increased in all forms of amegakaryocytic thrombocytopenia or bone marrow aplasia/hypoplasia.[2,27-29] For instance, in a recently published series of 56 patients with CAMT due to *MPL* mutations, the median serum THPO concentration was 1,493 pg/mL.[1] The only known exception to this rule is CAMT due to defective THPO production, in which the level of THPO is unexpectedly within the "normal" range, as in our patient but also in those carrying p.Arg38Cys or p.Arg119Cys mutations of THPO, which impair the trafficking of the mutant protein and prevent its secretion.[4-6]

Hematopoietic stem cell transplantation is the cornerstone of treatment for CAMT-MPL and for other forms of inherited bone marrow failure syndrome. However, transplantation is expected to be ineffective in CAMT-THPO. In fact, hematopoietic stem cell transplantation led to poor and often tragic outcomes in individuals with *THPO* variants.[5] In contrast, these patients showed very good responses to romiplostim, which were characterized not only by increases in platelet count but also improvements of other cytopenias, when present.[5,6] Supported by our data, we treated the proband with eltrombopag, another THPO-mimetic drug that was preferred to romiplostim because of its oral daily instead of weekly subcutaneous administration. This is the first report of eltrombopag therapy in a patient with a *THPO* mutation. The therapy led to a significant and stable improvement of thrombocytopenia and complete remission of bleeding symptoms. We hypothesize that, if untreated, the patient would have progressed to develop multilineage marrow aplasia, as described in the other CAMT patients with homozygous *THPO* mutations.[4,6] Extended follow-up is therefore required to ascertain whether eltrombopag is able to prevent such an evolution in this case.

Overall, these findings underline the fundamental importance of distinguishing patients with CAMT due to *THPO* mutations from those with mutations in *MPL* variants or with other inherited bone marrow failure syndromes. Therefore, molecular analysis of the *THPO* gene, including its regulatory regions, should be routinely included in the diagnostic workup of patients with a clinical suspicion of CAMT. Moreover, in view of the current evidence, we suggest that measurement of serum THPO concentration could be a valuable screening tool to recognize patients with *THPO* mutations. The finding of a normal serum THPO level in the context of an amegakaryocytic thrombocytopenia should strongly suggest an alteration of *THPO*.

Little is known about the mechanisms controlling transcription of the *THPO* gene. In the liver, the main site of production of THPO, the cytokine is expressed constantly, with no transcriptional or post-transcriptional regulation yet identified with the exception of limited evidence suggesting that

circadian rhythm and inflammation might influence its expression.[34,35] Although *THPO* transcription initiates at two alternative promoters, and multiple alternative splicing events occur at the 5'-UTR, only transcript variant 1 is efficiently translated into THPO.[19] The *THPO* promoter structure and activity have been studied and debated extensively, but no motifs, such as TATA-box, GC- and CAAT-boxes, or transcription factors regulating THPO expression have been characterized upstream of the transcription start site. The only exception is C/EBPdelta binding at approximately 800 bp upstream of the transcription start site, whose role in *THPO* regulation has yet to be defined.[19,36]

Our study identified for the first time a regulatory region of *THPO* transcription, in which ETS1 and STAT4 interact with each other in a complex and bind to consensus sequences regulating the level of expression of the gene. This activity is reduced in the presence of c.-323C>T or by silencing the two transcription factors, either alone or in combination. The effect of ETS1 and STAT4 knockdown on THPO expression was evident in a hepatocyte cell model both at the mRNA and protein levels. Of note, this promoter region is evolutionarily conserved among species, suggesting a conserved mechanism of THPO transcriptional regulation.

ETS1 is a transcription factor belonging to the large family of the ETS domain transcription factors, mainly known for its role in immune homeostasis regulation and in cancer development.[37,38] However, as supported by our data, it also plays an important role in megakaryopoiesis, as a multilayer regulator.[39] STAT4 belongs to the STAT family of transcription factors, whose members are involved in inflammatory responses and in autoimmune/inflammatory diseases.[40] Nothing is known about its role in megakaryopoiesis. However, STAT4 is part of a feedback loop circuitry with interleukin-6 (IL6), as IL6 phosphorylates STAT4, which in turn upregulates IL6.[41] Of relevance, IL6 enhances *THPO* mRNA expression, causing inflammatory thrombocytosis,[34] supporting a potential role of STAT4 in controlling megakaryopoiesis.

Of note, another member of the STAT family (STAT3) induces *THPO* transcription upon the binding of desialylated platelets to the Ashwell-Morell receptor[23,42] but how STAT3 controls this process is – at least to our knowledge - unknown. Using *in silico* analysis to explore this mechanism did not reveal any potential direct binding of STAT3 on the *THPO* promoter. However, we confirmed that it controls the expression level of *THPO*, which is reduced when STAT3 is silenced. Since STAT3 and STAT4 share upstream activator signals and interact in an heterodimeric complex,[43] we speculate that the ETS1/STAT4 complex, directly binding the *THPO* promoter, induces basal expression of the *THPO* gene; then, upon different stimuli (e.g., a signal from sialylated platelets), the interplay of STAT3 with STAT4 increases *THPO* expression. However, further studies are needed to verify this intriguing hypothesis and understand the complex mechanisms controlling the level of expression of *THPO*.

In conclusion, we identified a family with CAMT caused by the c.-323C>T mutation, which affects the regulatory region of the *THPO* gene. In patients with a clinical picture of CAMT, the molecular basis of the disease is not always ascertained, although a precise diagnosis is essential for their appropriate management. Measurement of serum THPO concentration could represent a useful screening tool to recognize patients with defective THPO production, who can be treated effectively with THPO-mimetic drugs, avoiding hematopoietic stem cell transplantation.[5] Moreover, through the identification of ETS1 and STAT4 as transcription factors recruited on the *THPO* promoter, we provide novel insights into the mechanisms controlling the expression of the *THPO* gene.

Disclosures

No conflicts of interest to disclose.

Contributions

VC designed and performed the experiments, analyzed the data, and wrote the manuscript. IBF performed next-generation sequencing analysis. MF interpreted the mutational screening data. EA collected hematologic and clinical data, and wrote the manuscript. AP interpreted genetic and clinical data, and edited the manuscript. AS supervised the project, designed experiments and wrote the paper. All authors critically revised the paper and approved the final version.

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Data-sharing statement

All data relevant to the study are included in the manuscript or are available upon request to the corresponding author.

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