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Brief Report



PLATELETS AND THROMBOPOIESIS

# CALR mutant protein rescues the response of MPL p.R464G variant associated with CAMT to eltrombopag

Francesca Basso-Valentina,[1] Gabriel Levy,[2] Leila N. Varghese,[2] Myriam Oufadem,[3] Benedicte Neven,[4,5] Charlotte Boussard,[4] Nathalie Balayn,[1] Caroline Marty,[1] William Vainchenker,[1] Isabelle Plo,[1] Paola Ballerini,[3] Stefan N. Constantinescu,[2] Remi Favier,[1,3,*] and Hana Raslova[1,*]

[1]INSERM, Unité Mixte de Recherche (UMR) 1287, Gustave Roussy, Université Paris Saclay, Equipe Labellisée Ligue Nationale contre le Cancer, Villejuif, France; [2]Ludwig Institute for Cancer Research, de Duve Institute, Université Catholique de Louvain, Brussels, Belgium; [3]Assistance Publique–Hôpitaux de Paris (AP-HP), Hôpital Armand Trousseau, Centre de Réfrence des Pathologies Plaquettaires, Paris, France; [4]Pediatric Hematology-Immunology and Rheumatology Department, Hôpital Necker-Enfants Malades, AP-HP Centre Université de Paris, Paris, France; and [5]Université de Paris, Imagine Institute, Laboratory of Immunogenetics of Pediatric Autoimmunity, INSERM UMR 1163, Paris, France

KEY POINTS

- A novel MPL$^{R464G}$ variant induces CAMT due to defective trafficking to the cell surface.
- The CALR mutant renders MPL$^{R464G}$ responsive to ELT.

**Congenital amegakaryocytic thrombocytopenia (CAMT) is a severe inherited thrombocytopenia due to loss-of-function mutations affecting the thrombopoietin (TPO) receptor, MPL. Here, we report a new homozygous MPL variant responsible for CAMT in 1 consanguineous family. The propositus and her sister presented with severe thrombocytopenia associated with mild anemia. Next-generation sequencing revealed the presence of a homozygous *MPL*$^{R464G}$ mutation resulting in a weak cell-surface expression of the receptor in platelets. In cell lines, we observed a defect in MPL$^{R464G}$ maturation associated with its retention in the endoplasmic reticulum. The low cell-surface expression of MPL$^{R464G}$ induced very limited signaling with TPO stimulation, leading to survival and reduced proliferation of cells. Overexpression of a myeloproliferative neoplasm–associated calreticulin (CALR) mutant did not rescue trafficking of MPL$^{R464G}$ to the cell surface and did not induce constitutive signaling. However, it unexpectedly restored a normal response to eltrombopag (ELT), but not to TPO. This effect was only partially mimicked by the purified recombinant CALR mutant protein. Finally, the endogenous CALR mutant was able to restore the megakaryocyte differentiation of patient CD34$^+$ cells carrying MPL$^{R464G}$ in response to ELT.**

## Introduction

Congenital amegakaryocytic thrombocytopenia (CAMT; OMIM 604498) is a rare autosomal-recessive bone marrow failure disorder that can evolve into severe aplastic anemia and leukemia.[1] Usually, patients with CAMT exhibit high thrombopoietin (TPO) serum levels, and a majority of cases carry heterozygous compound or homozygous nonsense, missense, and splicing mutations in the *MPL* gene encoding the TPO receptor.[2,3] The cell-surface expression of mature MPL necessary for TPO-induced signaling is mainly achieved through the conventional endoplasmic reticulum (ER)-Golgi pathway dependent on MPL association with JAK2 as a chaperone protein.[4] However, an unconventional, autophagy-dependent manner of delivery of immature MPL to the cell surface has also been described.[5] TPO/MPL signaling controls hematopoietic stem cell survival and megakaryocyte (MK) differentiation.[6] Interestingly, TPO and MPL play a more important role in humans than in mice. Indeed, *Thpo*[7] and *Mpl*[8] knockout mice are viable, with a residual platelet production preventing hemorrhage, and do not develop bone marrow failure.

## Study design

### Patients

Blood samples from patients and healthy subjects were collected after informed written consent and obtained in accordance with the Declaration of Helsinki. The study was approved by the Comité de Protection des Personnes CPP N°2020T2-02.

### Samples and cell lines

Peripheral blood CD34$^+$ cells, megakaryoblastic UT-7 cells, HEK293T cells, and Ba/F3 cells were transduced, sorted, and cultured as described in supplemental Methods (available on the *Blood* Web site).

### Statistics

Statistical analyses were performed using PRISM software (GraphPad). Statistical significance was determined using a Mann-Whitney test or 2-way analysis of variance with Bonferroni posttest. Differences were considered significant at $P < .05$.

More details are provided in supplemental Methods.

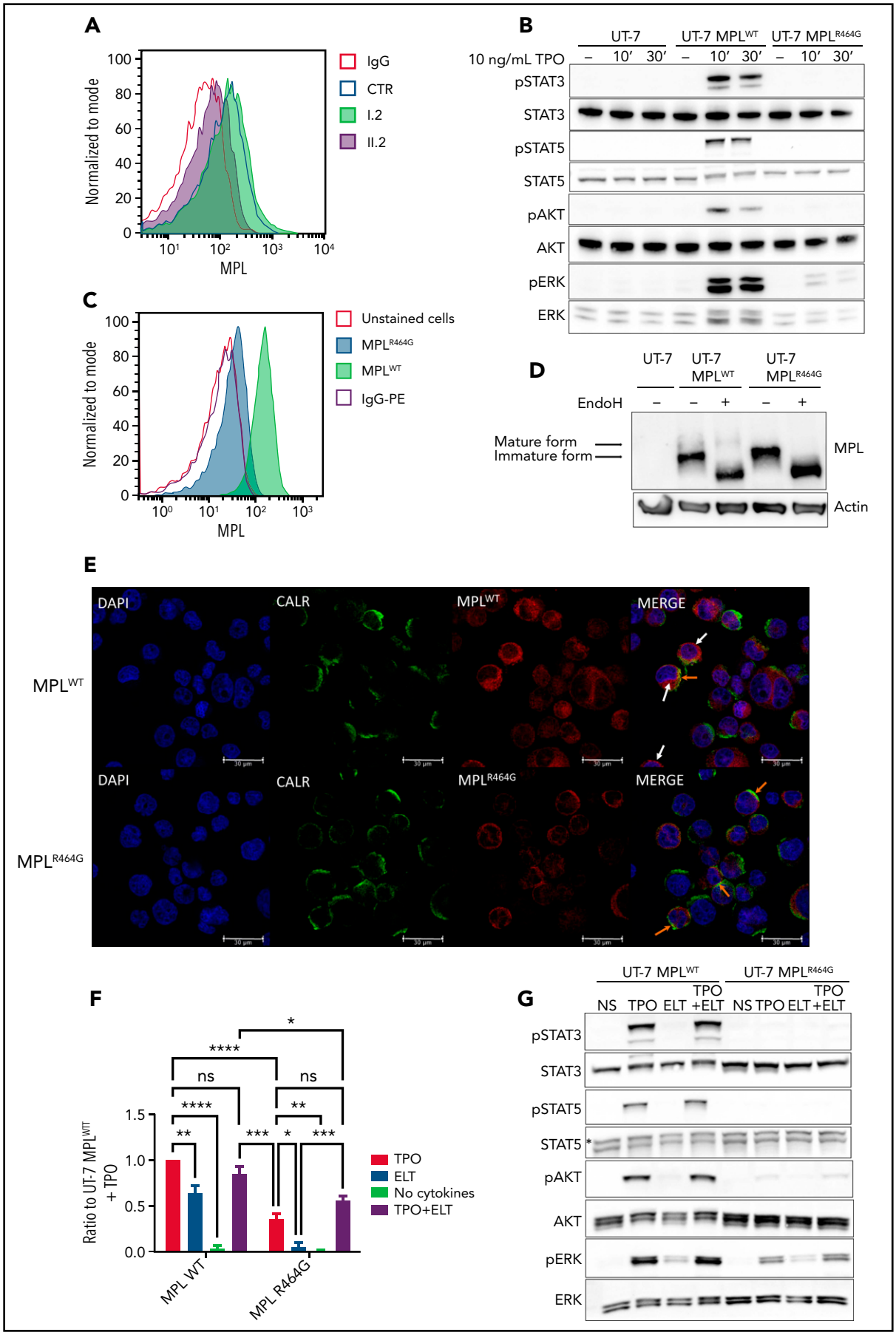


Figure 1.

## Results and discussion

We report here 1 French family (supplemental Figure 1A) with a novel homozygous *MPL* mutation in 2 children with severe thrombocytopenia and high TPO levels. The propositus is a 3-year-old female (II.3) born to first-cousin parents of Turkish origin and diagnosed with a severe thrombocytopenia ($34 \times 10^9$ platelets per L) at the age of 3 months. At the age of 2 years, due to the decrease in platelet counts ($<20 \times 10^9$/L) associated with a slight anemia, she was first unsuccessfully treated with romiplostim, then with eltrombopag (ELT) with only a weak response. Her 8-year-old sister (II.2) was also severely thrombocytopenic ($39 \times 10^9$ platelets per L), while both parents had normal platelet counts (supplemental Figure 1B).

Next-generation sequencing identified *MPL* p.R464G (variant allele frequency [VAF], 100%) and *ZFPM1* p.A137G (VAF, 50%) variants for the propositus and *MPL* p.R464G (VAF, 100%) variant for her sister. The *MPL* p.R464G mutation at a VAF of 50% in both parents was confirmed by Sanger sequencing (data not shown). Interestingly, the same variant was recently reported in 1 unrelated heterozygous CAMT patient.[9] The R464 residue is located on the extracellular domain of MPL.

To tease out the mechanism by which this mutant could lead to severe thrombocytopenia, we first investigated its expression on the surface of platelets from the propositus sister (II.2), presenting higher platelet levels throughout the follow-up. As shown in Figure 1A, an almost-complete absence of MPL at the platelet surface was observed.

Next, we overexpressed the wild-type (WT) and mutant $MPL^{R464G}$ in both the human granulocyte-macrophage colony-stimulating factor (GM-CSF)–dependent UT-7 and the interleukin-3–dependent murine Ba/F3 cell lines. As expected, in UT-7 cells, TPO stimulation of STAT3, STAT5, AKT, and ERK pathways was observed only when $MPL^{WT}$ was expressed. In both UT-7 and Ba/F3 cells overexpressing $MPL^{R464G}$, TPO induced a slight but significant activation of ERK and a barely detectable phosphorylation of STAT and AKT that were not observed in the absence of cytokines (Figure 1B; supplemental Figure 2A-B). No defect in signaling was detected in presence of GM-CSF (supplemental Figure 2C). Similarly, a dual luciferase assay in HEK293T cells for STAT5 activity showed a discrete response to TPO (supplemental Figure 3), suggesting that the mutation affects either the interaction with TPO or the cell-surface expression of MPL. Low levels of $MPL^{R464G}$ were detected at the cell surface of Ba/F3 cells by flow cytometry (Figure 1C), and even lower levels were found on the membrane of UT-7 cells (not shown). Western blot analysis showed that $MPL^{R464G}$ was in an immature, incompletely glycosylated form, but total $MPL^{R464G}$ compared with $MPL^{WT}$ cell amounts were not different (Figure 1D). A defect in trafficking, rather than an increased degradation, was corroborated by the colocalization of $MPL^{R464G}$ with the calreticulin (CALR) ER chaperone, suggesting its retention in the ER (Figure 1E; supplemental Figure 4). The proliferation rate of UT-7 $MPL^{R464G}$ cells in the presence of TPO was profoundly decreased compared with UT-7 $MPL^{WT}$ cells (Figure 1F). A complete absence of proliferation was detected in the presence of ELT, a small-molecule agonist of MPL that binds in proximity to the mutation[10] (Figure 1F). Furthermore, the combination of TPO and ELT did not achieve a significant synergism on proliferation and signaling (Figure 1F-G; supplemental Figure 5). Overall, these results indicate that due to a trafficking defect, only a small amount of $MPL^{R464G}$ reaches the cell surface, inducing weak signaling in response to TPO stimulation, as previously described for cell lines expressing limited levels of $MPL^{WT}$.[11] CAMT can be classified in 2 groups; type I presents a more severe phenotype and a total loss of cell-surface MPL expression due to deletions and nonsense and frameshift mutations, whereas the less severe type II may have residual receptor function due to missense MPL mutations.[12] Our results indicate the $MPL^{R464G}$ variant belongs to the type II CAMT.

Trafficking of the previously described type I CAMT–associated $MPL^{R102P}$ is totally blocked in the ER; however, both its traffic and signaling can be restored by the expression of 2 CALR mutants, $CALR^{del52}$ and $CALR^{ins5}$, identified in myeloproliferative neoplasms.[13] We investigated whether a similar rescue was possible for $MPL^{R464G}$. As shown in Figure 2A, $CALR^{WT}$ overexpression in UT-7 $MPL^{R464G}$ cells was not able to induce constitutive signaling or signaling in the presence of ELT. In contrast, a slight constitutive signaling was detected with $CALR^{del52}$ overexpression, and, unexpectedly, this signaling was enhanced by ELT, but not by TPO (Figure 2A; supplemental Figure 6). However, $CALR^{del52}$ was not able to rescue the trafficking defect of $MPL^{R464G}$ (Figure 2B). $CALR^{del52}$ has been shown to activate $MPL^{WT}$,[14-16] but it only slightly activates $MPL^{R464G}$. Interestingly, $CALR^{del52}$ renders $MPL^{R464G}$ cells responsive to ELT and increases the response of $MPL^{WT}$ cells to ELT (Figure 2A; supplemental Figure 6), suggesting that $CALR^{del52}$ may induce conformation changes in MPL and a better accessibility of the ELT-binding site or/and improve MPL dimerization. In contrast, the interaction between $CALR^{del52}$ and $MPL^{R464G}$ on the cell surface may prevent TPO binding and may explain the modest additional effect of TPO on signaling in $CALR^{del52}$-expressing cells (Figure 2A). In agreement with these results, the proliferation of UT-7 $MPL^{R464G}$ cells expressing

**Figure 1. $MPL^{R464G}$ is only weakly expressed on the cell surface and induces very weak signaling compared with $MPL^{WT}$.** (A) Flow cytometric analyses of MPL expression on platelets from 1 patient homozygous for $MPL^{R464G}$ (II.2), her heterozygous mother (I.2), and 1 healthy control (CTR). (B) Western blot analysis of TPO-induced signaling in UT-7 cells without MPL expression (UT-7), overexpressing the WT form of MPL (UT-7 $MPL^{WT}$), and mutant MPL (UT-7 $MPL^{R464G}$). Cells were starved overnight and stimulated with 10 ng/mL TPO for 10 or 30 minutes. No signal is detected in parental UT-7 cells, and a weak ERK signal is detected in the presence of $MPL^{R464G}$ in UT-7 cells. (C) Flow cytometric analyses of MPL expression on the surface of Ba/F3 cells overexpressing $MPL^{WT}$ and $MPL^{R464G}$. (D) Western blot analysis of the mature (85 kDa) and immature (80 kDa) forms of MPL. The mature form of MPL that is resistant to endoglycosidase H (Endo H) digestion and able to reach the cell membrane is not detectable in UT-7 cells overexpressing $MPL^{R464G}$. (E) Immunofluorescence staining for the expression of MPL and CALR in UT-7 cells overexpressing $MPL^{WT}$ or $MPL^{R464G}$. The orange arrows indicate MPL colocalization with CALR in the ER, and the white arrows indicate diffuse cytoplasmic/cell-surface MPL expression. Scale bars, 30 μm. An anti-MPL coupled with phycoerythrin (PE; A,C) and uncoupled anti-MPL antibodies (D-E) were used. (F-G) Proliferation curves and western blot analysis of signaling of UT-7 cells overexpressing $MPL^{WT}$ and $MPL^{R464G}$ in presence of TPO (10 ng/mL), ELT (2 μg/mL), TPO (10 ng/mL) + ELT (2 μg/mL), or without cytokines (no cytokines). (F) $5 \times 10^4$ cells were plated in triplicate in 24-well plates and counted every day for 4 days. The counts were reported to the condition of UT-7 $MPL^{WT}$+TPO (in blue color). Shown are averages of 3 independent experiments at day 4, each performed in triplicate ± standard error of the mean (error bars). *$P < .05$; ns, not significant; Mann-Whitney unpaired, nonparametric 1-tailed test. (G) Cells were starved overnight and stimulated for 10 minutes. A weak ERK signaling is detected in the presence of $MPL^{R464G}$ in all 3 conditions (TPO, ELT, and TPO + ELT). No signaling is detected in nonstimulated (NS) cells. **$P < .01$; ***$P < .005$; ****$P < .001$.

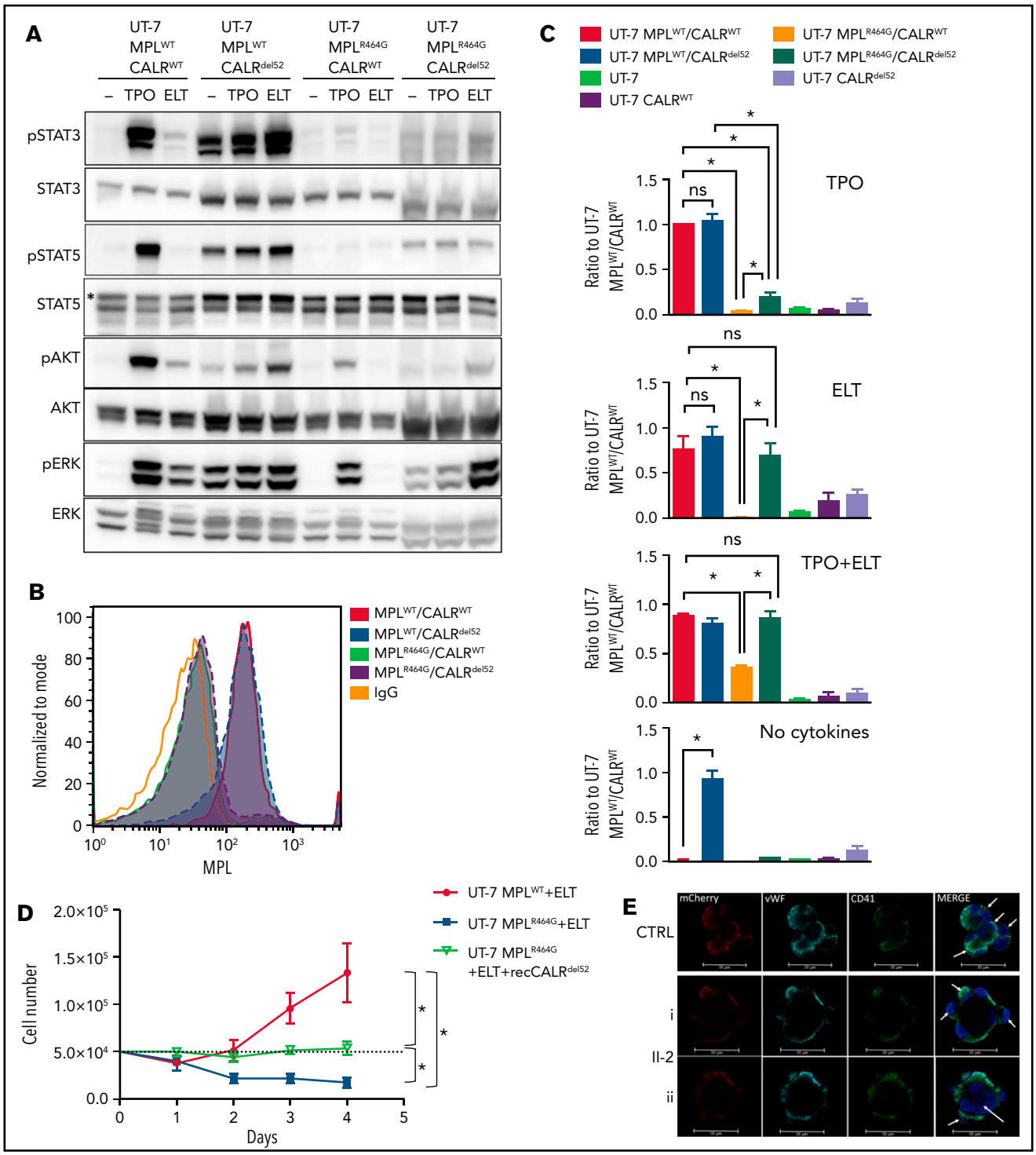


**Figure 2. CALR mutant (CALR$^{del52}$) restores the response of MPL$^{R464G}$ to ELT.** UT-7 and Ba/F3 cells were transduced with retroviruses to overexpress WT MPL (MPL$^{WT}$) or mutant MPL (MPL$^{R464G}$) together with GFP, and retroviruses overexpressing WT CALR (CALR$^{WT}$) or mutant CALR (CALR$^{del52}$) with mCherry, and sorted for GFP$^+$mCherry$^+$ cells. Parental UT-7 cells were used as negative control. (A) Western blot analysis of signaling induced by TPO or ELT. UT-7 cells were cultured in presence of GM-CSF, starved overnight, and stimulated with TPO (10 ng/mL) or ELT (2 μg/mL) for 10 minutes. CALR$^{del52}$ enhances signaling in presence of ELT both in MPL$^{WT}$- and MPL$^{R464G}$- expressing cells. (B) Flow cytometric analysis of MPL expression on Ba/F3 cells using an allophycocyanin-coupled MPL antibody. CALR$^{del52}$ does not induce MPL$^{R464G}$ traffic to the cell membrane. IgG, immunoglobulin G. (C) Proliferation assay in the presence of TPO, ELT, or TPO + ELT or without cytokines (no cytokines). (D) Proliferation assay in presence of ELT and recombinant CALR$^{del52}$ (recCALR$^{del52}$) (20 μg/mL). In panels C and D, 5 × 10$^4$ UT-7 cells were plated in triplicate in 24-well plates and counted every day for 4 days. Shown are averages of 3 independent experiments, each performed in triplicate ± standard error of the mean (error bars). In panel C, the counts are reported for the condition UT-7 MPL$^{WT}$/CALR$^{WT}$ (purple) + TPO and are shown only at day 4 of culture. *$P < .05$, Mann-Whitney unpaired, nonparametric 1-tailed test. (E) Control (CTRL) and patient CD34$^+$ cells (II-2) isolated from peripheral blood were transduced with retrovirus encoding *CALR$^{del52}$*, sorted at day 2 on CD34$^+$, and cultured in presence of stem cell factor and ELT for 12 days. At least 20 mCherry$^+$ and 20 mCherry$^-$ cells were analyzed. All mCherry$^+$ cells expressed MK markers, while no mCherry$^-$ cells differentiated into MKs (not shown). Representative pictures of mCherry$^+$ CTRL and II-2 MK (CD41$^+$ and von Willebrand factor [vWF]$^+$) are shown. White arrows indicate individual MKs; 2 different plans of the same view are shown (i,ii). Scale bars, 30 μm.

CALR$^{del52}$ was almost completely restored in the presence of ELT, but not TPO, which also did not increase the effects of ELT. This is consistent with our previous findings that CALR$^{del52}$ protein binds to MPL and sterically hinders the TPO-binding site,[15] although the TPO-binding site per se is not required for activation by CALR mutants of MPL.[14] In contrast, overexpression of CALR WT elicited a synergism between TPO and ELT (Figure 2C). Furthermore, proliferation was completely dependent on JAK2 activation, suggesting a normal interaction between MPL$^{R464G}$ and JAK2 (supplemental Figure 7). As CALR$^{del52}$ activates MPL on the cell surface,[13] and since secreted soluble CALR$^{del52}$ was reported to potentially act as a ligand for cell-surface MPL[17] (S.N.C., manuscript in preparation), we investigated the effects of exogenously added soluble CALR$^{del52}$ protein on the proliferation of MPL$^{R464G}$-expressing UT-7 cells. As shown in Figure 2D, the soluble CALR$^{del52}$ only partially restored the response to ELT of UT-7 MPL$^{R464G}$ cells, suggesting that endogenous CALR$^{del52}$ may bind more efficiently to MPL$^{R464G}$ than exogenous CALR$^{del52}$. Lastly, we wondered whether CALR$^{del52}$ could restore MK differentiation of patient CD34$^{+}$ cells in vitro. To this end, the II.2 patient CD34$^{+}$ cells were transduced with a CALR$^{del52}$ retrovirus and cultured in presence of stem cell factor and ELT (supplemental Figure 8). As expected, only the cells expressing CALR$^{del52}$ were able to differentiate into MKs (Figure 2E).

Overall, these data demonstrate that CALR$^{del52}$/MPL$^{R464G}$ complex leads to a conformation that is very permissive to the activating effect of ELT, which binds at the outset of the transmembrane domain (H499). CALR$^{del52}$ binds to the extracellular domain of MPL involving glycosylated N117 in D1, likely exposing the H499-containing region where ELT binds. The combination of TPO and ELT seems to have an effect similar to that observed with exogenous CALR$^{del52}$ and ELT. Taken together, these data may reveal novel approaches to modify the response of MPL to ELT and suggest that a dual activation of MPL may be efficient in cases of low MPL trafficking to the cell membrane.

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## Authorship

Contribution: F.B.-V., G.L., and L.N.V. designed and performed experiments, analyzed data, and contributed to the manuscript draft; M.O., P.B., and N.B. performed experiments and analyzed data; B.N. provided clinical and biological follow-up of patients; S.N.C. provided bacterial recombinant CALR$^{del52}$; C.M., I.P., W.V., and S.N.C. discussed results and contributed to manuscript editing; R.F. and H.R. designed and supervised the work and wrote the paper; and all authors contributed to the final approval of the manuscript.

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ORCID profiles: M.O., 0000-0001-8927-0346; W.V., 0000-0003-4705-202X; I.P., 0000-0002-5915-6910; H.R., 0000-0001-8846-5299.

Correspondence: Hana Raslova, INSERM UMR 1287, Gustave Roussy, 114 rue Edouard Vaillant, 94805 Villejuif, France; e-mail: hana.raslova@gustaveroussy.fr.

## Footnotes

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*R.F. and H.R. contributed equally to this study.

For original data, e-mail the corresponding author.

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## REFERENCES

1. Freedman MH, Estrov Z. Congenital amegakaryocytic thrombocytopenia: an intrinsic hematopoietic stem cell defect. *Am J Pediatr Hematol Oncol*. 1990;12(2):225-230.
2. Ihara K, Ishii E, Eguchi M, et al. Identification of mutations in the c-mpl gene in congenital amegakaryocytic thrombocytopenia. *Proc Natl Acad Sci USA*. 1999;96(6):3132-3136.
3. Ballmaier M, Germeshausen M, Schulze H, et al. c-mpl mutations are the cause of congenital amegakaryocytic thrombocytopenia. *Blood*. 2001;97(1):139-146.
4. Royer Y, Staerk J, Costuleanu M, Courtoy PJ, Constantinescu SN. Janus kinases affect thrombopoietin receptor cell surface localization and stability. *J Biol Chem*. 2005;280(29):27251-27261.
5. Cleyrat C, Darehshouri A, Steinkamp MP, et al. Mpl traffics to the cell surface through conventional and unconventional routes. *Traffic*. 2014;15(9):961-982.
6. Alexander WS, Roberts AW, Nicola NA, Li R, Metcalf D. Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl. *Blood*. 1996;87(6):2162-2170.
7. de Sauvage FJ, Carver-Moore K, Luoh SM, et al. Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. *J Exp Med*. 1996;183(2):651-656.
8. Gurney AL, Carver-Moore K, de Sauvage FJ, Moore MW. Thrombocytopenia in c-mpl-deficient mice. *Science*. 1994;265(5177):1445-1447.
9. Germeshausen M, Ballmaier M. CAMT-MPL: congenital amegakaryocytic thrombocytopenia caused by *MPL* mutations—heterogeneity of a monogenic disorder—comprehensive analysis of 56 patients [published online ahead of print 23 July 2020]. *Haematologica*. doi:10.3324/haematol.2020.257972.
10. Kim MJ, Park SH, Opella SJ, et al. NMR structural studies of interactions of a small, nonpeptidyl Tpo mimic with the thrombopoietin receptor extracellular juxtamembrane and transmembrane domains. *J Biol Chem*. 2007;282(19):14253-14261.

11. Millot GA, Vainchenker W, Duménil D, Svinarchuk F. Distinct effects of thrombopoietin depending on a threshold level of activated Mpl in BaF-3 cells. *J Cell Sci*. 2002;115(Pt 11):2329-2337.

12. Germeshausen M, Ballmaier M, Welte K. MPL mutations in 23 patients suffering from congenital amegakaryocytic thrombocytopenia: the type of mutation predicts the course of the disease. *Hum Mutat*. 2006;27(3):296.

13. Pecquet C, Chachoua I, Roy A, et al. Calreticulin mutants as oncogenic rogue chaperones for TpoR and traffic-defective pathogenic TpoR mutants. *Blood*. 2019; 133(25):2669-2681.

14. Chachoua I, Pecquet C, El-Khoury M, et al. Thrombopoietin receptor activation by myeloproliferative neoplasm associated calreticulin mutants. *Blood*. 2016;127(10): 1325-1335.

15. Marty C, Pecquet C, Nivarthi H, et al. Calreticulin mutants in mice induce an MPL-dependent thrombocytosis with frequent progression to myelofibrosis. *Blood*. 2016; 127(10):1317-1324.

16. Araki M, Yang Y, Imai M, et al. Homomultimerization of mutant calreticulin is a prerequisite for MPL binding and activation. *Leukemia*. 2019;33(1): 122-131.

17. Pecquet C, Balligand T, Chachoua I, et al. Secreted mutant calreticulins as rogue cytokines trigger thrombopoietin receptor activation specifically in CALR mutated cells: perspectives for MPN therapy [abstract]. *Blood*. 2018;132(suppl 1). Abstract 132.