

Calreticulin mutants in mice induce an MPL-dependent thrombocytosis with frequent progression to myelofibrosis

Running title: MPL requirement for CALR mutant-driven MPN

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Key points

- Calreticulin type I and type II mutants are drivers of the disease as they induce thrombocytosis in a retroviral mouse model.
- The thrombopoietin receptor MPL is required for calreticulin mutants to induce an Essential Thrombocythemia phenotype in transplanted mice.

Abstract

Frameshift mutations in the calreticulin (*CALR*) gene are seen in about 30% of essential thrombocythemia and myelofibrosis patients. To address the contribution of the *CALR* mutants to the pathogenesis of myeloproliferative neoplasms (MPNs) we engrafted lethally irradiated recipient mice with bone marrow cells transduced with retroviruses expressing these mutants. In contrast to wild-type *CALR*, *CALRdel52* (type I) and, to a lesser extent, *CALRins5* (type II) induced thrombocytosis due to a megakaryocyte (MK) hyperplasia. Disease was transplantable into secondary recipients. After six months, *CALRdel52*-, in contrast to rare *CALRins5*-transduced mice, developed a myelofibrosis associated with a splenomegaly and a marked osteosclerosis. Monitoring of virus-transduced populations indicated that *CALRdel52* leads to expansion at earlier stages of hematopoiesis than *CALRins5*. However, both mutants still specifically amplified the MK lineage and platelet production. Moreover a mutant deleted of the entire exon 9 (*CALRdelex9*) did not induce a disease suggesting that the oncogenic property of *CALR* mutants was related to the new C-terminus peptide. To understand how the *CALR* mutants target the MK lineage, we used a cell line model and demonstrated that the *CALR* mutants, but not *CALRdelex9*, specifically activate the thrombopoietin (TPO) receptor (MPL) to induce constitutive activation of JAK2 and STAT5/3/1. We confirmed in *c-mpl*- and *tpo*-deficient mice that expression of *Mpl*, but not of *Tpo*, was essential for the *CALR* mutants to induce thrombocytosis *in vivo*, although *Tpo* ligand contributes to disease penetrance. Thus, *CALR* mutants are sufficient to induce thrombocytosis through MPL activation.

Introduction

Classical myeloproliferative neoplasms (MPNs) include Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF).¹ To date, recurrent mutations in three genes have been identified as phenotypic drivers.² The mechanism of action of activating mutations in the *JAK2* and the thrombopoietin receptor (*MPL*) genes has been extensively studied and their effect on the disease have been attributed to a constitutive activation of the JAK2/STAT signaling axis. More recently, two groups reported new somatic mutations in the gene encoding calreticulin (*CALR*) in approximately 30% of *JAK2*- and *MPL*-negative ET and PMF patients.^{2,3}

CALR is a chaperone protein of the endoplasmic reticulum (ER) lumen that controls proper folding of neo-synthesized glycoproteins and retains calcium in the ER.^{4,5} The *CALR* mutations occur exclusively in the last exon (exon 9) of the gene. They are insertions and/or deletions that result in a frame-shift to a specific alternative reading frame, resulting in a novel C-terminal sequence in the mutants. *CALRdel52* and *CALRins5* are the two most prevalent mutations found in ET and PMF patients, with *CALRdel52* being more frequent than *CALRins5* in PMF.⁶ Here, using a retroviral mouse model we demonstrate that both mutants induce a human ET-like disease with major differences in the severity of thrombocytosis, the evolution into fibrosis and the level of clonal amplification. *CALR* mutants are drivers of the disease and are capable to constitutively activate the JAK/STAT signaling pathway through *MPL*. *MPL* is required to induce spontaneous megakaryocytic progenitor growth and thrombocytosis in mice.

Methods

Retrovirus

CALRdel52, *CALRins5*, *CALRwt* and *CALRdelex9* lacking the entire exon 9 were inserted into a pMSCV-IRES-GFP retroviral vector. Control was the empty virus. Vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped viral particles were produced into 293EBNA cells.

Bone marrow transduction procedure

All procedures were approved by the Gustave Roussy ethics committee (protocol

2012_061). Bone marrow (BM) cells were collected from two month-old C57BL/6J mice (Janvier, Le Genest, France) four days after 5-fluorouracil injection (150 mg/kg), and Lineage-negative (Lin⁻) cells were purified and transduced with the different retrovirus. Finally, 0.4×10^6 transduced cells/animal were intravenously injected into lethally irradiated (9.5 Gy for wild-type and 8 Gy for *c-mpl* and *tpo* knock-out mice) recipient mice. For secondary transplantation, BM cells collected from ten-week-old primary recipients were injected (2×10^6 cells/animal) into irradiated mice.

Analysis of mouse blood and content in progenitor and precursor cells

Peripheral blood cells, BM and spleen. Platelets, red blood cell (RBC) and white blood cell (WBC) counts were determined using an automated counter (MS9; Schloessing Melet, Cergy-Pontoise, France). BM cells were removed by flushing the femurs and tibias. Spleens were weighed and single-cell suspensions were prepared. Total hematopoietic cell number per mouse was calculated assuming that the cells in two femurs and tibias represent 25% of the total BM and from the total number of cell isolated from the spleen.

Flow cytometry. LSR II and FACSCanto I (BD Biosciences Le Pont de Claix, France) were used to determine the percentages of GFP-positive (GFP⁺) infected cells and cell content of BM and spleen. Lin⁻ cells were negatively selected with APC-conjugated anti-CD3, B220, Gr-1, Mac-1 and Ter-119 rat anti-mouse antibodies (Abs). For analysis of Lin⁻Sca-1⁺c-Kit⁺ (LSK) progenitor cell-enriched fraction, Lin⁻ cells were stained with Abs against mouse Sca-1 (PE-Cy7) and c-Kit (PerCP-Cy5.5). LSKCD150⁺CD48⁻ SLAM hematopoietic stem cell-enriched population was isolated with Pacific Blue anti-mouse CD48 and Brilliant Violet 510 anti-mouse CD150 Abs. All Abs were from eBiosciences (Ozyme, Saint Quentin en Yvelines, France). BM and spleen precursor cells were also analyzed with fluorochrome-conjugated Abs directed against mouse CD41 and CD42 (megakaryocytic lineage), Ter-119 and CD71 (erythroid lineage), Mac-1 and Gr-1 (granulocytic lineage), B220 and CD3 (lymphoid lineage).

Progenitor analysis in semi-solid cultures. BM and spleen nucleated cells were plated in duplicate in methylcellulose MethoCult 32/34 (Stemcell Technologies, Grenoble, France) in presence of cytokines (SCF, IL-6, IL-3, TPO, EPO) and erythroid

(BFU-E, burst forming unit-erythroid) and granulo-monocytic (CFU-GM, colony forming unit-granulocyte macrophage) progenitors were counted seven days later. BM nucleated cells were plated in duplicate in serum-free fibrin clot assays with SCF, IL-6 and no or increasing doses of TPO to quantify megakaryocytic progenitor (CFU-MK)-derived colonies at day seven by acetylcholinesterase staining.

Histology. Tibia and spleen sections were fixed in 4% formaldehyde, decalcified and paraffin embedded. Sections were stained with hematoxylin/eosin/safran for cytology and megakaryocytes were reliably identified with von Willebrand factor stain. Reticulin fibers were revealed by Gordon and Sweet's silver staining

Viability and proliferation of Ba/F3 cell models

Ba/F3 cells were transduced to express human MPL (pMX-IRES-CD4), human EPOR (pMX-IRES-GFP) or the human G-CSFR (pMX-IRES-GFP) and the various human CALR (pMSCV with an IRES-GFP/mCherry) constructs. To determine long-term cytokine independence, Ba/F3 cells were plated at a density of 250,000 cells/mL, and total live cells were manually counted after ten days of culture. The Premix WST-1 Cell Proliferation Assay System (Clontech) was used to measure dose-dependent cell proliferation to TPO.

Western blot analysis

Sorted GFP⁺ Lin⁻ spleen cells were analyzed by Western blot to assess the relative expression levels of CALRdel52 and CALRins5. CALR mutants were detected using a monoclonal rabbit Ab directed against the novel C-terminal tail of the CALR mutants (A. Vannucchi, Florence, Italy). Signaling studies and protein expression levels were performed on Ba/F3 cell lines using polyclonal Abs against the phosphorylated forms of JAK2 (Tyr1007/1008), TYK2 (Tyr1054/1055), STAT1 (Tyr701), STAT3 (Tyr705), STAT5 (Tyr694), ERK1/2 (Thr202/Tyr204) and AKT (Ser473) and against the pan proteins (Cell Signaling Technology, Ozyme). MPL was revealed with an Ab from Upstate (Millipore, Molsheim, France). Monoclonal anti- β -Actin Ab was from Sigma (Saint Quentin Fallavier, France) and was used to check the loading

Results

***CALRdel52* and *CALRins5* mice induce thrombocytosis in mice.**

To study the *in vivo* effect of the CALR mutants in mice, lineage-negative (Lin⁻) bone marrow (BM) cells transduced with retroviruses expressing human *CALRdel52*, *CALRins5*, wild-type *CALR* (*CALRwt*), *CALRdelex9* (lacking the entire exon 9) or empty vector (Mock) were engrafted into lethally irradiated recipient mice. Results from two independent experiments were pooled.

The CALR mutant-expressing mice rapidly developed thrombocytosis compared to *CALRwt* or Mock mice and at higher levels in the presence of *CALRdel52* than in the presence of *CALRins5* (Fig. 1A-C). In contrast, white blood cell (WBC) and red blood cell (RBC) counts were not impacted until six months after bone marrow transplantation (BMT). To verify that the differences in platelet levels between the two mutants were not due to differences in protein levels, GFP-positive (GFP⁺) Lin⁻ cells sorted from spleen of ten-month-old mice were examined by Western blot. Both mutated CALR proteins were equally expressed (Fig. 1D). Finally, the disease was transplantable into secondary hosts with features identical to the primary recipients (Fig. S1A-C), showing that it is a cell-intrinsic disorder. It is noteworthy that even if *CALRdelex9* protein was less abundant than the CALR mutants (Fig. S1D), *CALRdelex9* mice similarly to the two controls (*CALRwt* and Mock) did not develop a disease, suggesting that the novel C-terminal sequence, rather than the absence of the wild-type tail, plays a central role in the driver properties of the CALR mutants (Fig. 1A-C).

***CALRdel52* mice develop myelofibrosis.**

Platelet and RBC counts decreased in the *CALRdel52* mice six months after transplantation (Fig. 1A and B). This was associated with a time-dependent increase in spleen weight and decrease in BM nucleated cells (Fig. 2A and B). After ten months, histologic examination of *CALRdel52* mice revealed expanding osteogenesis in BM with punctuations of newly formed bone (Fig. 2C). Abundant clusters of megakaryocytes (MKs) were seen in the BM, but also in the spleen, which was partially disorganized (Fig. 2C). Silver staining showed thickening of the reticulin fiber network in both BM and spleen. Interestingly, a year post-engraftment, slight spleen fibrosis was observed in one

out of ten *CALRins5* mice that were analyzed while all *CALRdel52* mice had developed fibrosis within six months (Fig. S1D).

***CALRdel52* and *CALRins5* mutations differentially impact the stem cell compartment, but both amplify the MK lineage.**

Based on the use of a bicistronic GFP-expression retroviral vector, we determined that around 50% of engrafted cells were retrovirally-transduced. Virus-infected (GFP⁺) cells were analyzed in blood, BM and spleen at different levels of differentiation and time points after transplantation. The percentages of GFP⁺ cells within the early hematopoietic progenitors and hematopoietic stem cells (Lin⁻Sca1⁺c-Kit⁺ (LSK) and Lin⁻Sca1⁺c-Kit⁺CD48⁻CD150⁺ (SLAM) populations, respectively) at three months post-engraftment were similar to those found in the initial graft (Fig. 3A) for all mice, except for *CALRdel52* mice in which the percentage of GFP⁺ cells increased from ~50% to ~100% from three to six months. These results revealed that *CALRdel52* expression, in contrast to *CALRins5*, provides an advantage to hematopoietic stem cells (HSC) that completely out-compete the non-transduced cells at ten months after engraftment leading to WBCs of almost exclusively virus-infected origin (Fig. S2A and B). Moreover, this clonal advantage leads to a significant increase in the frequency of LSKs and SLAMs in BM and spleen in *CALRdel52* mice ten months after engraftment (Fig. 3B).

***CALRdel52* and *CALRins5* amplify the MK lineage.**

Dominance of both the *CALRdel52* and the *CALRins5* cells was revealed in late stage of megakaryopoiesis, in BM and spleen MKs (Fig. 4A), leading to an early rise in the percentage of GFP⁺ platelets in both mutant-expressing mice (Fig. 4B). Thus, both *CALRdel52* and *CALRins5* provide a competitive advantage to the MK lineage. As expected, the frequency of MKs increased in the BM and spleen of both *CALRdel52* and *CALRins5* mice (Fig. 4C). The cumulative numbers of MKs was significantly higher in *CALRdel52* and *CALRins5* mice than in *CALRwt* mice (Fig. 4D) without significant differences in erythroblasts, granulocytic and lymphoid precursors (Fig. S2C). We found that MK progenitors (CFU-MKs) expressing the CALR mutants displayed a hypersensitivity to thrombopoietin (TPO), maybe explaining this proliferative advantage

of the MK lineage (Figure 4E). Taken together, one of the differences between the two CALR mutants resides in the level of initial cell amplification: HSC *versus* MK lineage for the CALRdel52 and CALRins5 mutations, respectively (Fig. 4F).

CALR mutants induce MPL activation and JAK/STAT signaling.

The development of thrombocytosis in mice, combined with the previous observation that expression of CALRdel52 induces a constitutive activation of the JAK/STAT signaling,⁷ could suggest that the oncogenic activity of CALR mutant is mediated *via* MPL, the key receptor of the MK lineage. Ba/F3 cells were transduced to express CALRwt, CALRdelex9 or CALR mutants. CALR mutants, in contrast to CALRwt and CALRdelex9, induced consistent cytokine-independent growth of Ba/F3 cells expressing MPL, but not of the cells that did not express MPL (data not shown). Interestingly, only MPL, but neither EPOR nor the G-CSFR maintained long-term cytokine-independent cell growth in the presence of both CALR mutants (Fig. 5A). These results were confirmed by a cell proliferation assay in response to increasing doses of TPO showing that Ba/F3-MPL cells in presence of CALRdel52 or CALRins5 are nearly completely independent of TPO (Fig. 5B), mimicking the TPO hypersensitivity of mouse CFU-MKs observed previously (Fig. 4E). In Ba/F3-MPL cells, we confirmed that expression of the CALR mutants induced a strong constitutive phosphorylation of STAT5/3/1 and an apparent weaker phosphorylation of JAK2 due to the decrease level of the protein (Fig. 5C). In contrast, the mitogen-activated protein kinase (ERK) and the phosphoinositide 3-kinase (PI3-K)/AKT pathways were only weakly activated. TPO addition provided only a minor increase in the phosphorylation of these molecules compared to controls. While JAK2, CALRwt and CALR mutants were found to co-immunoprecipitate with MPL in Ba/F3-MPL cells, the active phosphorylated form of JAK2 only bound to the MPL-containing complex in cells expressing the CALR mutants (Fig. 5D). These data demonstrate that the CALR mutants interact with MPL and activate JAK2 and the STATs independently of TPO.

The thrombopoietin receptor MPL is crucial in CALRdel52-induced thrombocytosis in mice.

We verified that CALR mutants-induced phenotype in mice was dependent on the presence of MPL. We found that CFU-MKs from wild-type C57B/L6 mice expressing the CALR mutants exhibited an increased growth in the absence of TPO in comparison to those expressing CALRwt (Fig. 4E and Fig. 6A). This was not the case when CALRdel52 was expressed in Lin⁻ progenitors from *c-mpl* knock-out (*c-mpl*^{-/-}) mice (Fig. 6A), indicating that amplification of CFU-MKs by CALR mutants requires Mpl.

Moreover, to confirm the involvement of MPL and of its ligand TPO *in vivo*, we transplanted lethally irradiated *c-mpl*^{-/-} mice with *mpl*-deficient Lin⁻ cells transduced to express CALRwt or CALRdel52 (~60% GFP⁺ cells). Similarly, CALRwt- and CALRdel52-transduced wild-type Lin⁻ cells were engrafted in *tpo*^{-/-} mice and wild-type mice as controls (~60% GFP⁺ cells). Seven weeks post-BMT, percentages of GFP in blood were still around 60%, and the absence of Mpl led to the inhibition of CALRdel52-induced thrombocytosis (Fig. 6B). Interestingly, in a *Tpo*-deficient setting, mice developed a phenotype, but which was less penetrant than the wild-type mice. These results suggest that TPO is not absolutely required for the induction of an ET phenotype by CALR mutants *in vivo*.

Discussion

This study demonstrates that ectopic expression of the two most common MPN-associated CALR mutants in hematopoietic stem and progenitor cells is sufficient to induce a MPN phenotype mimicking human ET in mice and that constitutive activation of MPL and hypersensitivity to TPO may explain this phenotype. Moreover, CALRdel52 mice developed a “post-ET” myelofibrosis with associated decrease in platelet count, anemia, splenomegaly, BM hypocellularity and marked osteosclerosis. TPO-high expressing mice⁸ and *MPLW515L* retrovirally-transduced mice⁹ also recapitulate a similar disease with thrombocytosis progressing to myelofibrosis. This study contrasts with the various *jak2V617F* mouse models that progress into myelofibrosis *via* a PV-like stage.¹⁰⁻¹⁴ These differences may be related to the specificity of MPL activation by the CALR mutants in comparison to JAK2V617F.

The diseases generated by the two types of CALR mutations differ with respect to the levels of thrombocytosis and kinetics and frequency of myelofibrosis transformation. This

suggests that, in mice, the ability of *CALRins5* to promote pathologic MK proliferation is comparatively milder than that of the *CALRdel52* mutant. Moreover, *CALRdel52* mice developed fibrosis with a much higher frequency than *CALRins5* mice in agreement with observations made in patients where *CALRdel52* is more frequent in myelofibrosis than *CALRins5* (70% compared to 13%).^{15,6} Moreover, in patients the frequency of post-ET myelofibrosis is much higher for *CALRdel52* (around 12% at 10 years) than for *CALRins5* or even for *JAK2V617F* ETs.¹⁶ This also suggests that *CALRins5* mutation might be of low penetrance for myelofibrosis progression.

While both *CALR* mutations are acquired by HSCs, the phenotypic differences may be explained by the level of initial clonal amplification: HSC and MK for *CALRdel52* and *CALRins5* mutations, respectively. *CALRdel52* mutation seems to play an important role in HSC clonal dominance whereas the role of *JAK2V617F* in HSC remains controversial.^{17,14} This is illustrated by the low variant allele frequency for *JAK2V617F* in comparison to *CALR* mutants in ET. Further study will be necessary to understand these differences in the mechanism of clonal amplification induced by the two *CALR* mutants.

Interestingly, the *CALRdelex9* mice, expressing a *CALR* lacking the entire exon 9, do not develop thrombocytosis suggesting that, besides differences in protein expression levels, the presence of the new mutant protein tail, rather than the absence of the wild-type C-terminal sequence, seems determinant for the phenotype. The main biochemical difference between *CALRdel52* and *CALRins5* mutants concerns the number of the negatively charged amino acids lost in comparison to *CALRwt*. Thus, the length and/or the ratio of the positive to negative charges on the C-terminal tail of the *CALR* mutant might play an important role in modulating its oncogenic strength.

Using a cell line model, we demonstrate that *CALR* mutants specifically activate the MPL/JAK2 axis to the same extent. The specificity of activation of MPL among several other cytokine receptors tested was reported in details by the companion paper.¹⁸ In addition, the phenotypic difference observed between *CALRdel52* and *CALRins5* mice is thus probably not directly linked to the activation of JAK/STAT pathway but rather to differences in calcium signaling, as suggested by a recent study.¹⁶ However, using a genetic approach we could demonstrate, as observed for the activation of JAK2/STAT in Ba/F3 cell line, that MPL was indispensable for the induction of thrombocytosis by

CALRdel52. Indeed, in the absence of Mpl the animals remain thrombocytopenic. In contrast, after Tpo ablation, the animals could develop a thrombocytosis but with an important variability suggesting that TPO may play a role in the penetrance of the disease. This result further underscores the great similarities in the mechanism of thrombocytosis and disease induction between JAK2V617F and CALR mutants. In both cases MPL but not TPO is indispensable, TPO only increasing the disease phenotype.¹⁹ Moreover, in a companion paper, the requirement of MPL and of JAK2 in the TPO-independent growth of primary megakaryocyte progenitors from human patients was confirmed using specific shRNAs.¹⁸

Elucidating the mechanism of action of CALR mutants has profound implications for the therapy of *CALR*-positive patients. Our results predict that the JAK2 inhibitors currently used in clinical trials could be effective in *CALR*-positive patients, as already observed in two patients with PMF.²⁰

In addition, we could show in cell lines that CALR mutants only slightly activate the PI3-K pathway. These data have important implications for HSCs where constitutive activation of the PI3-K pathway by *pten* knock-out has been shown to reduce self-renewal capacities,²¹ suggesting that the stem cells with *CALR* mutation would achieve clonal dominance much faster than those with JAK2 mutations. This could be one of the mechanism explaining the higher CALR mutant allelic burden compared to that of JAK2V617F in ET patients as underscored above.²²

Moreover, we show an association between CALRwt or CALR mutants and JAK2 and MPL in Ba/F3-MPL cells while the constitutive active phosphorylated form of JAK2 was only detected in presence of the CALR mutants. A study shows that the interaction between CALR and MPL is mediated *via* the N-glycosylation of asparagine residues in the extracellular domain of MPL and suggests that it might stabilize an intermediate active conformation of MPL.¹⁸ Further biochemical studies will be important to determine how CALR mutants can specifically activate MPL and what are the mechanisms involved in the differences between CALRdel52 and CALRins5, which seem not to be only explained by the level of JAK2/MPL axis activation.

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Authorship Contributions

S.N.C., I.P. and W.V. conceived and designed the study, interpreted the data and wrote the paper. C.M. designed and performed experiments, analyzed the data, prepared the figures and wrote the paper. C.P., H.N. and M.E. designed, performed experiments and analyzed the data. I.C. contributed to the *c-mpl*^{-/-} mouse and cell lines studies. M.T. gave inputs in histopathological analysis and J.L.V. and H.R. designed experiments analyzed the data and wrote the paper. R.K. analyzed the data and provided essential reagents.

Disclosure of Conflict of Interest

The authors declare that there are no conflicts of interest.

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Figures Legends

Figure 1. CALR mutants induce an ET-like phenotype *in vivo* in mice. Primary *CALRdel52* (n=22), *CALRins5* (n=20), *CALRdelex9* (n=10), *CALRwt* (n=22) and Mock (n=10) recipient mice were analyzed every three weeks after BM transplantation (BMT), over a one-year-period. Graphs show **(A)** platelets with a smaller scale on the right, **(B)** red blood cell (RBC) and **(C)** white blood cell (WBC) counts as mean $\pm$ SEM. Bonferroni's multiple comparison test: *p<0.05, **p<0.001, ***p<0.0001. Arrows indicate six months post-transplantation. **(D)** Spleen GFP⁺ Lin⁻ cells were examined by Western blot for CALR protein levels using an antibody specifically directed against the novel C-terminal tail of the mutants (CALRmut). « ns » shows a non-specific band. An antibody directed against the whole CALR protein (CALRtot) was also used. β -actin serves as a loading control. Blots show representative results.

Figure 2. BM and spleen features shows evolution of the disease to myelofibrosis for all *CALRdel52* mice and one *CALRins5* mouse. **(A)** Spleen weight and **(B)** BM cellularity (n=3-5), expressed as mean $\pm$ SEM. t-test: *p<0.05, **p<0.001. **(C)** Hematoxylin-eosin-safran (HES), von Willebrand factor staining (vWF) and reticulin silver silver stain (RET) of ten-month-old mouse BM and spleen of *CALRdel52* mice show clusters of MKs, osteosclerosis and a progressive thickening of the reticulin network. Images were obtained using a DM2000 Leica microscope and a DFC300FX Leica camera with Leica Application Suite v.2.5,OR1 acquisition software (2.5X and 25X magnifications).

Figure 3. Different amplification of early hematopoietic stem and progenitor cells in *CALRdel52* and *CALRins5* mice. **(A)** Evolution of the percentage of GFP⁺ cells in BM and spleen LSK and SLAM cell compartments (n=3 to 5). **(B)** Frequency of BM and spleen LSK and SLAM cells of *CALRwt*, *CALRdel52* or *CALRins5* origin after ten months transplantation (n=5). Results are means $\pm$ SEM. t-test: *p<0.05, **p<0.001, ***p<0.0001.

Figure 4. Amplification of the megakaryocytic lineage in *CALRdel52* and *CALRins5* mice. **(A)** Percentage of GFP⁺ megakaryocytes (MKs) (CD41⁺CD42⁺ cells) in BM and spleen in ten months post-BMT mice (n=3). **(B)** Evolution of the percentage of GFP⁺ platelets in blood (n=20-22). Bonferroni's multiple comparison test: **p<0.001, ***p<0.0001 **(C)** Frequency of BM and spleen MKs in *CALRwt*-, *CALRdel52*- and *CALRins5*-transduced ten-month-old mice (n=3). **(D)** Cumulative numbers in BM plus spleen of MKs from *CALRwt*-, *CALRdel52*- and *CALRins5*-transduced ten-month-old mice (n=3). **(E)** Percentages of BM megakaryocytic progenitor (CFU-MKs) colonies growing in the presence of SCF, IL-6 and increasing concentrations of TPO. Values were pooled for three, six and ten months post-BMT mice (n=13-15). Results are means $\pm$ SEM. t-test: *p<0.05, **p<0.001, ***p<0.0001. **(F)** Schematic representation of the difference in clonal amplification between *CALRdel52*- and *CALRins5*-transduced mice. Both *CALRdel52* and *CALRins5* mutations lead to a specific amplification of MKs and platelets in transduced mice. *CALRdel52* induces an earlier amplification of hematopoietic cells (HSC compartment) than the *CALRins5* mutation (CFU-MK level) leading to an overall greater production of platelets than the *CALRins5* mutation.

Figure 5. CALR mutants induce a specific activation of MPL. **(A)** Ba/F3 cells transduced to express Mock or *CALRwt* or mutants and MPL, EPOR or G-CSFR were cultured for ten days in the absence of any cytokines and their number was measured by a cell counter (Beckman Coulter). **(B)** Proliferation was assessed 72 hours after culturing Ba/F3-MPL cells expressing Mock, *CALRwt* or the *CALR* mutants in the absence or in the presence of increasing doses of TPO (0.005, 0.015, 0.05, 0.15, 0.5, 1.5 and 5 ng/mL) by WST-1 proliferation assay. Dose-response curves are means (in percent of viability of the maximum growth value) $\pm$ SEM (n=3 in triplicate). Bonferroni's multiple comparison test: *p<0.05, **p<0.001. **(C)** Ba/F3-MPL cells expressing different *CALR* constructs was examined by Western blotting for the presence and phosphorylation status of various signaling molecules. Cells were serum- and cytokine-starved for five hours prior to ten minutes stimulation with 10 ng/mL TPO. Expression of β -actin was used as loading control. **(D)** Immunoprecipitation (IP) using anti-FLAG

antibody from lysates of Ba/F3 cells expressing a FLAG-tagged MPL and transduced with respective *CALR* constructs followed with Western blotting.

Figure 6. Mpl is required in CALR mutant-induced spontaneous growth of megakaryocytic progenitor and thrombocytosis in mice. (A) Lin⁻ cells were purified from C57BL/6 (n=3) and *c-mpl*^{-/-} mice (n=3) and transduced with retrovirus expressing CALRwt or CALRdel52. GFP⁺ Lin⁻ cells were sorted two days later and plated in a fibrin clot assay in presence of SCF, IL-6 and increasing doses of TPO. Curves are means of CFU-MK frequency ± SEM. Bonferroni's multiple comparison test: *p<0.05, **p<0.001, ***p<0.0001 and « ns » for non significant. **(B)** Lin⁻ cells purified from C57BL/6 and *c-mpl*^{-/-} mice were transduced to express CALRwt and CALRdel52 and engrafted in wild-type (n=4-5), *c-mpl*^{-/-} (n=5-6) and *tpo*^{-/-} (n=3-4) mice. Platelet counts were determined seven weeks after engraftment. Results show individuals and the means ± SEM. t-test: *p<0.05.

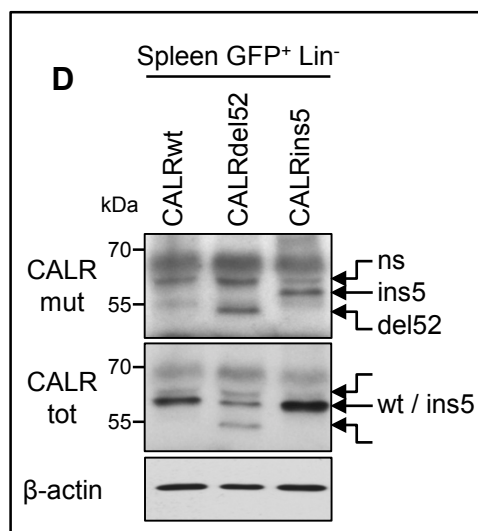
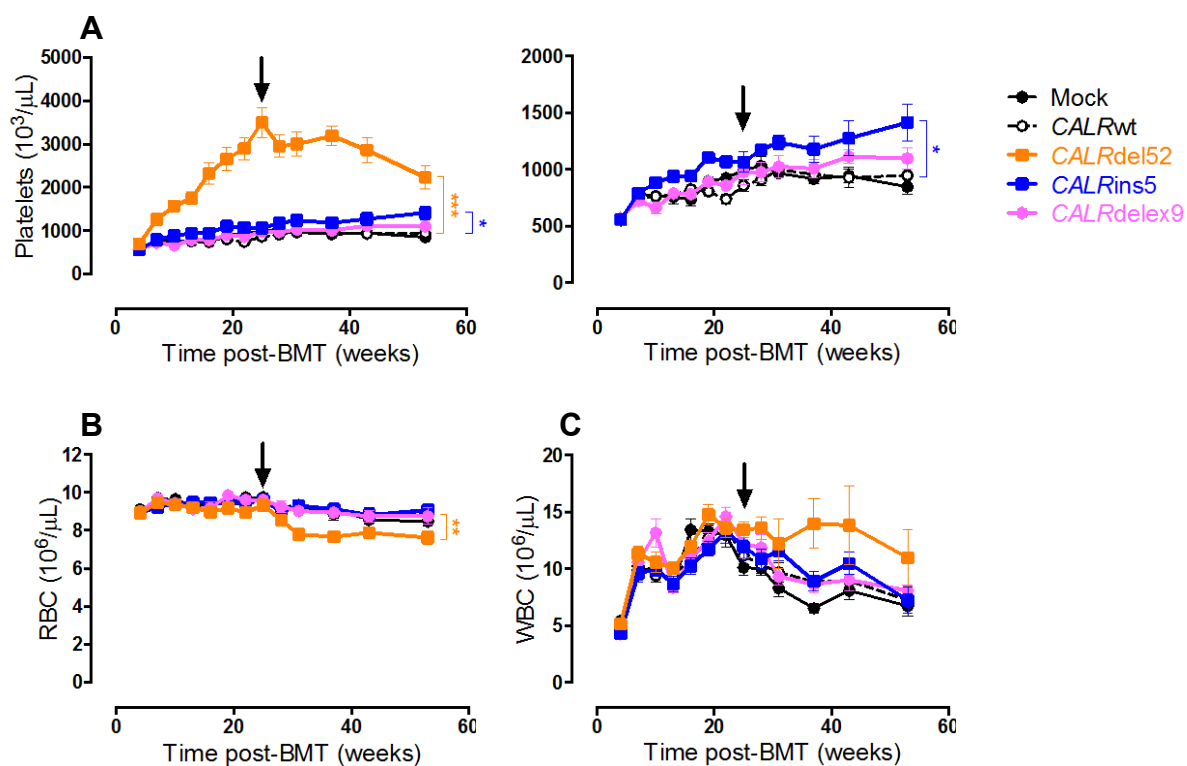


Figure 1

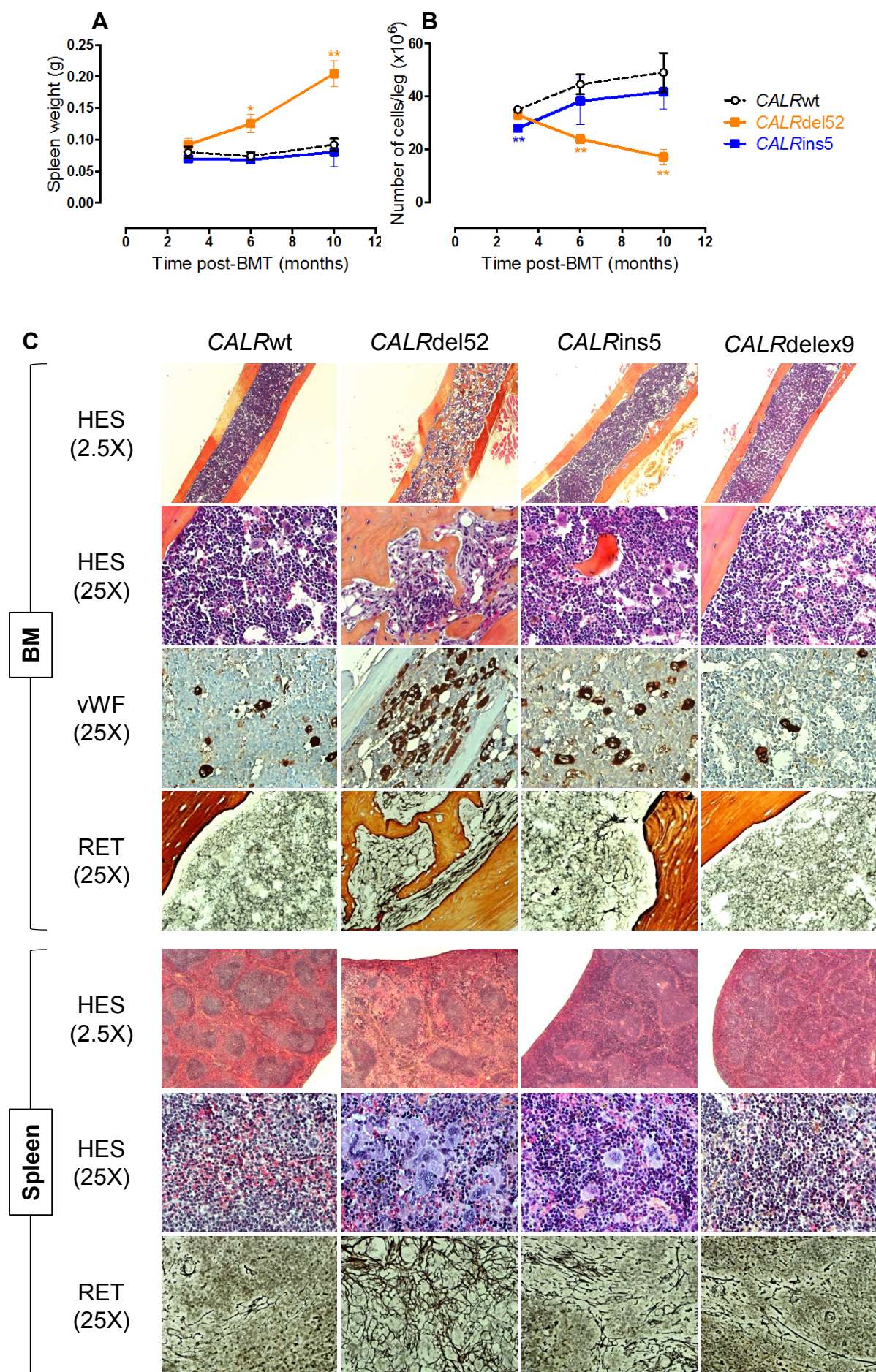
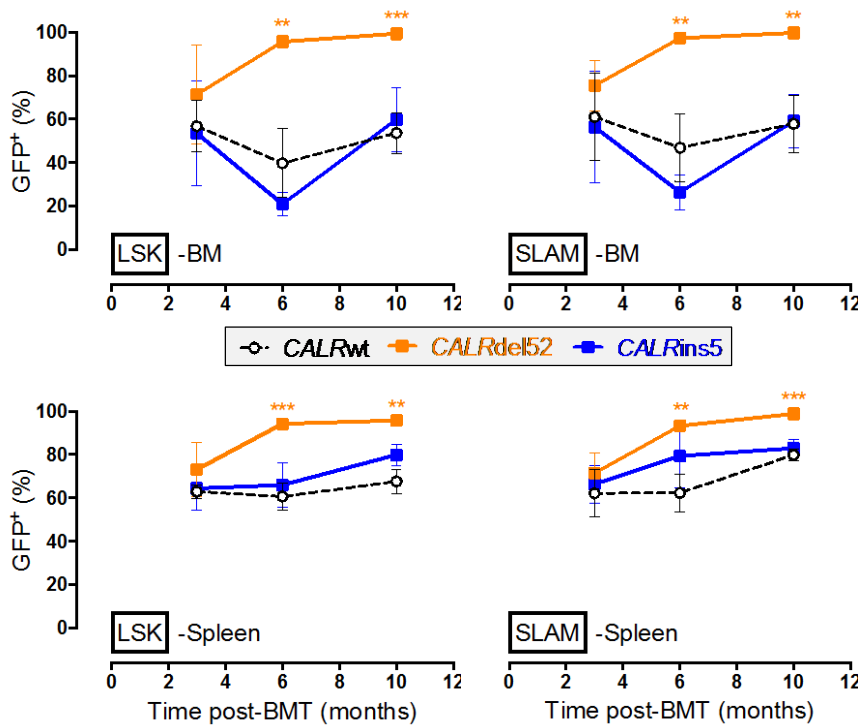
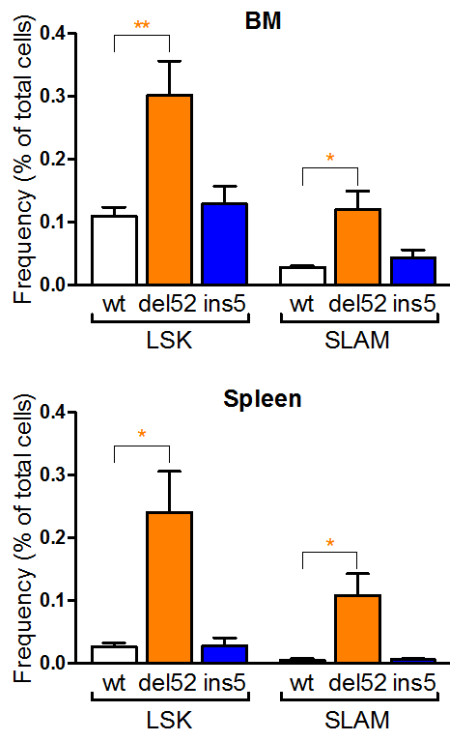


Figure 2

A**B****Figure 3**

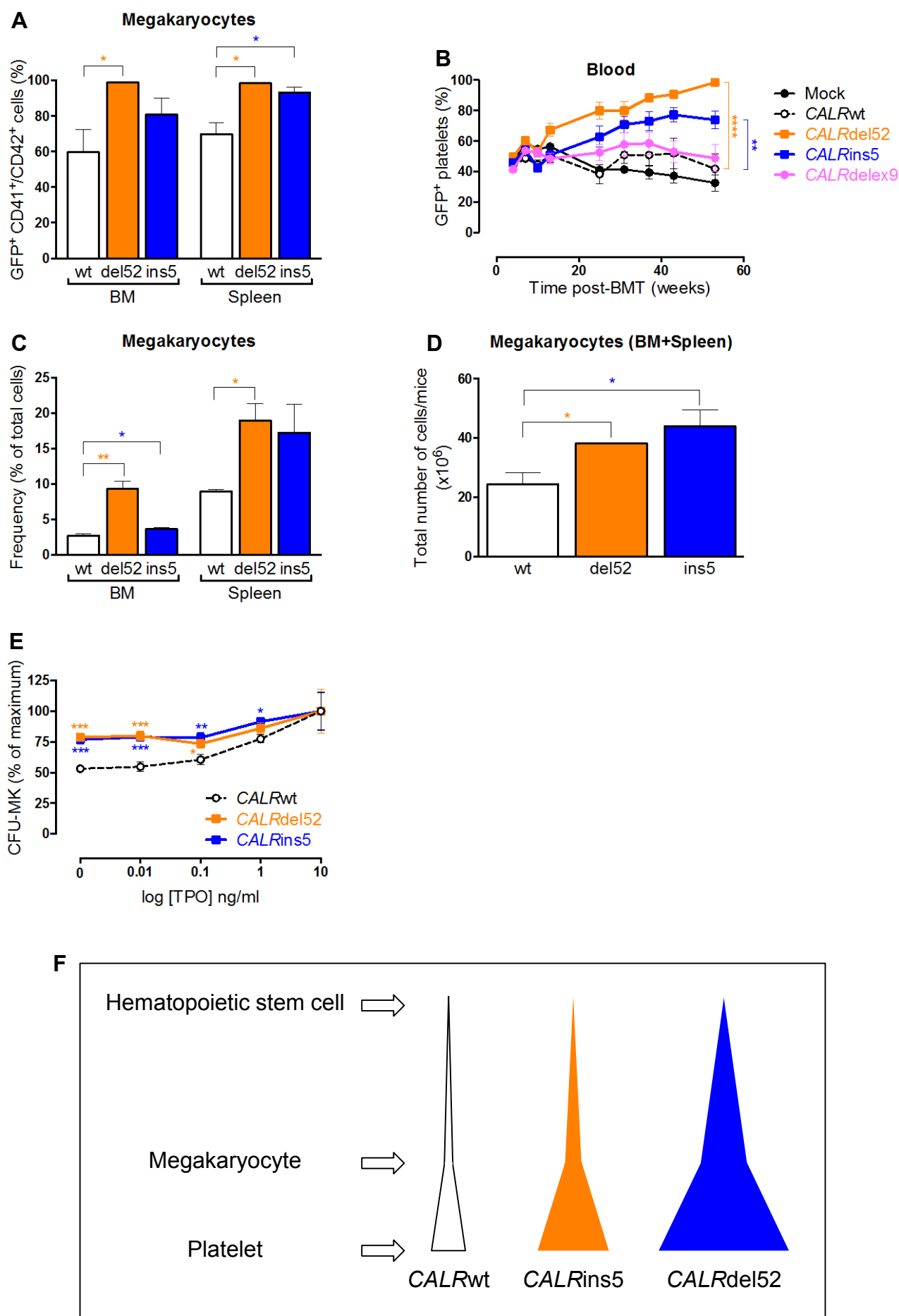


Figure 4

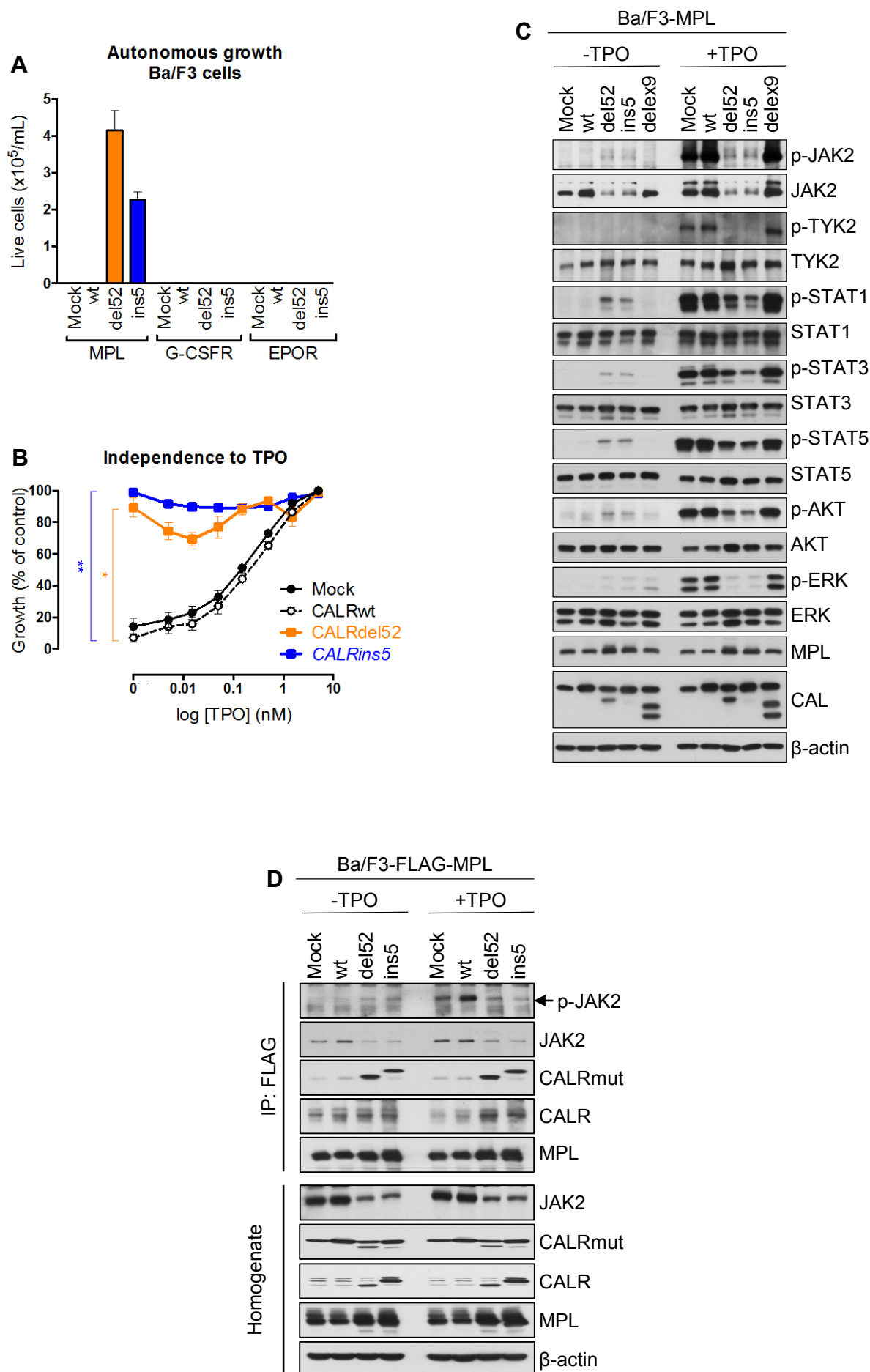


Figure 5

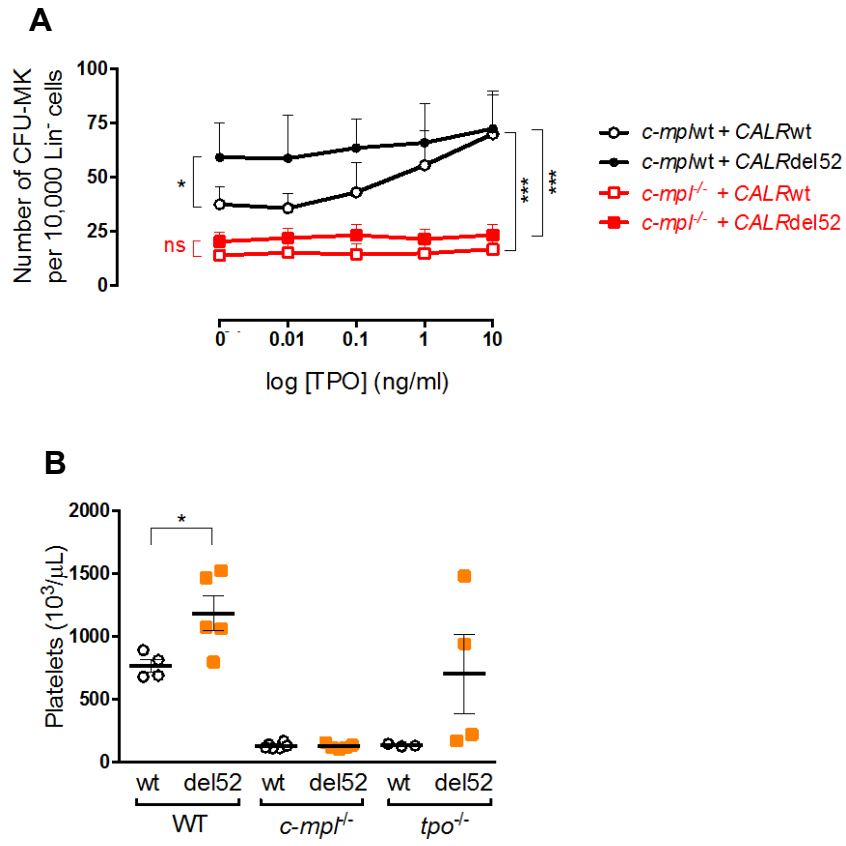


Figure 6



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Calreticulin mutants in mice induce an MPL-dependent thrombocytosis with frequent progression to myelofibrosis

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