Secreted Mutant Calreticulins As Rogue Cytokines in Myeloproliferative Neoplasms

Supplemental Methods

Patient samples and allele burdens.

Plasma samples (148 MPN patients and 11 healthy controls) were collected through an international collaboration of clinicians from Austria, Italy and Japan after obtaining Ethical approval for each center. Mutated *CALR* allele burdens were evaluated by PCR fragment analysis. Statistical analyses were performed using GraphPad (GraphPad Software Inc., La Jolla, CA).

Human samples for CFU-Mk assays were obtained after written informed consent was obtained from patients in accordance with the Declaration of Helsinki and the study was approved from Comité de Protection des Personnes (CPP) Ile de France IV- Institutional review board (agreement from US Department of Health and Human Services (n°IRB 00003835- Protocol 2015/59-NICB) and Commission Nationale de l'Informatique et des Libertés (CNIL) (authorization #915663).

Production of recombinant proteins

Recombinant human CALR wild-type and CALR del52 contain a N-terminal His tag sequence (MGSHHHHHHGSSG) that replaces the CALR signal peptide sequence (aa1-17). Cys 163 was mutated to serine. The proteins were produced and purified as described previously [1].

Flow Cytometry

For assessment of CALR del52 cell surface localization, HEK293T were transiently transfected with CALR del52 species and full length human TpoR

(hTpoR) or an empty vector 48h prior to the experiment. Cells were detached without trypsination and stained with anti-FLAG antibody or IgG control (primary) and APC coupled Goat anti-mouse antibody (secondary). Cells transfected with single fluorescence vector (GFP or mCherry) or compensation beads stained with APC-coupled antibody were used for compensation controls. 30,000 events of cells co-transfected with the two constructs were acquired for each condition on BD LSRFortessa™ Cell Analyzer. Post-acquisition analysis was performed with FlowJo. For assessment of TpoR mutant cell surface localization, HEK293T were transfected with indicated mutant or an empty control vector. 48h post-transfection, cells were processed as described above and stained with anti-HA coupled to APC antibody. 30,000 events of transfected cells were acquired for each condition on using a FACSVerse™ and the percentage of positive cells for HA-APC staining was determined amongst the transfected cells.

Purified protein quality analysis by thermal shift

The graphs represent the 350/330 nm intrinsic fluorescence from Trp and Tyr residues at different temperatures. The S-shaped curve is typical of well-folded proteins as the accessibility of Tyr and Trp residues gradually increases upon temperature increases and denaturation. The Tm is computed as the temperature at which half of the proteins in the sample are denatured. Thermal shift was performed on purified samples with the Tycho system (NanoTemper).

Production of recombinant proteins

Recombinant human CALR wild-type and CALR del52 contain a N-terminal His tag sequence (MGSHHHHHHGSSG) that replaces the CALR signal peptide sequence (aa1-17). Cys 163 was mutated to serine. The proteins were produced and purified as described previously [1].

Antibodies

Phospho-specific antibodies and antibodies against receptors and signaling proteins were described in [2]. Polyclonal antibodies (SAT602) targeting the mutant CALR were from MyeloPro (Vienna, Austria). Monoclonal antibody

targeting the CALR mutant C-terminus (clone 11) was produced in-house by rat immunization with the peptide CAAEKQMKDKQDEEQRTRRMMRTKMRM. Monoclonals antibodies were produced and purified from hybridomas supernatant. Antibody targeting the N-terminus of CALR (#ab195511) was from Abcam (Cambridge, MA). Anti-human TFRC antibody was commercially available (Cell Signaling, D7G9X).

Cell lines and constructs

Ba/F3 cells, HEK293T and UT-7/Tpo cells were maintained as described [2]. The CRISPR/Cas9 engineered Ba/F3 cells were described in [3]. UT-7/Tpo were described in [4]. They maintained in DMEM culture medium supplemented with 10% FBS, 5ng/ml human GM-CSF and 1% Pennicilin-Streptomycin. Primary cells were harvested from the bone marrow of a Knock-in *Calr* $^{del52/WT}$ mouse model developed in our lab using a CRISPR/Cas9 approach [5].

The TFRC-mcherry construct was a gift from (AddGene Plasmid #55144) was a gift from Michael Davidson lab. The CALR-GFP constructs were cloned in the pMSCV vector.

[^{3}H] Thymidine incorporation assay

Ba/F3 TpoR Calrmut were seeded in a 96-well plate (5000 cells per well) and incubated for 72 h at 37°C treated daily with or without increasing doses of rhCALR del52. Four hours before analysis, 0.02 Ci of [methyl-3H]-thymidine (Perkin Elmer #NET027X005MC, Zaventem, Flandre, Belgium) was added. Cells were next harvested in GF/C glass fiber filter-bottom 96-well microplates (Perkin Elmer) and dried. Incorporated tritium was measured using a TopCount instrument (Perkin Elmer) after addition of Scintillation liquid (MicroScint-O, Perkin Elmer).

Western Blots and immunoprecipitation

Western blots were performed on NP40 extracts as described [2]. For Western blots on plasma, we first used immunoprecipitation to enrich mutant CALR and remove the large amounts of albumin and immunoglobulins present in plasma. Anti-CALR mutant antibody (CAL2, Dianova) was purified with rProtein G prior to biotinylation using a commercial antibody biotinylation kit (Thermo Scientific, Merelbeke, Belgium). 100-300µl of plasma was incubated with 1:25 biotinylated anti-CALR mutant antibody (CAL2, Dianova) overnight and pull-down with Streptavidin-coated magnetic beads (DynaBeads MyOne Streptavidin C1, Thermo Scientific, Merelbeke, Belgium). Samples were washed 5 times with PBS and eluted with the elution buffer (Glycine 0.15M – HCl, pH 2.5) before analysis by western blotting in denaturing and reducing conditions with an anti-CALR mutant antibody (SAT602). For quantification by western blotting in plasma, known amount of rh CALR del52 (described in **Supplementary Figure 2**) or rh sTFRC (R&D systems, Catalog #2474-TR) were loaded in parallel to immunoprecipitation product from plasma. Optical density (OD) was quantified for each lane with ImageJ and the amount of plasma CALR mut and plasma sTFRC in the immunoprecipitation product was estimated by comparing their OD with this of the ladder of known amount of rh sTFRC or rhCALR del52.

Mass spectrometry

Anti-mutant CALR antibody (Dianova, CAL), was biotinylated using Pierce™ antibody biotinylation (Catalog number:90407). The antibody was added to plasma from controls or patients at a 1:25 dilution and incubated overnight on a spinning wheel at 4°C. Pull-down was performed with Dynabeads™ MyOne™ Streptavidin C1 (Invitrogen). Samples were eluted in 0.15M Glycine*HCl pH 2.5 prior to analysis by non-targeted mass spectrometry.

For analysis of glycosylation profile of TFRC in UT-7/Tpo CALR WT or CALR del52, endogenous TFRC was immunoprecipitated with anti-human TFRC/CD71 antibody (D7G9X, Cell Signaling). Immunoprecipitation product were run on SDS-PAGE and stained with Coomassie Blue. The identity of the bands was

confirmed by western blotting with an anti-TFRC antibody (ab84036, abcam). The band corresponding to the cleaved and total form were cut from the gel and analyzed by mass spectrometry. Post-acquisition analysis was performed with MSFragger.

1. 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.
2. Chachoua I, Pecquet C, El-Khoury M, et al. Thrombopoietin receptor activation by myeloproliferative neoplasm associated calreticulin mutants. *Blood.* 2016;127(10):1325-1335.
3. Balligand T, Achouri Y, Pecquet C, et al. Pathologic activation of thrombopoietin receptor and JAK2-STAT5 pathway by frameshift mutants of mouse calreticulin. *Leukemia.* 2016;30(8):1775-1778.
4. Jia R, Balligand T, Atamanyuk V, et al. Hematoxylin binds to mutant calreticulin and disrupts its abnormal interaction with thrombopoietin receptor. *Blood.* 2020.
5. Balligand T, Achouri Y, Pecquet C, et al. Knock-in of murine Calr del52 induces essential thrombocythemia with slow-rising dominance in mice and reveals key role of Calr exon 9 in cardiac development. *Leukemia.* 2020;34(2):510-521.

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Supplemental Figures:

Supplemental Figure 1: Secretion of CALR del52 in cells that express or not TpoR

(A) CALR del52 secretion is decreased in cell co-expressing TpoR. Top:Western blot showing CALR del52 in whole cell lysates (left) and culture medium (right) of HEK293T transiently transfected with empty vectors, CALR del52 alone, or CALR del52 with TpoR. Medium was harvested 48h post-transfection. Detection of mutant CALR was achieved using a polyclonal anti-mutant CALR antibody obtained from MyeloPro (SAT602). Bottom: quantification of the supernatant/total lysate ratio of CALR del52 with or without TpoR. Statistics: unpaired student t-test (N = 2, n = 6).

(B) Flow cytometry analysis of cell surface CALR del52-FLAG in presence or absence of TpoR. HEK293T were transiently transfected with indicated constructs and cell surface CALR del52-FLAG was measured with an anti-FLAG primary antibody (Genscript) and an APC-coupled anti-mouse antibody 48h post-transfection. Geometric mean and median of compensated APC (Comp-APC) are indicated.

Supplemental Figure 2: Analysis of recombinant human CALR proteins

SDS-PAGE gel of recombinant human CALR del52 (A) and CALR WT (B). Recombinant proteins produced in *E. Coli* were purified using a Ni+ column and concentrated by ultrafiltration. (C) Analysis of protein quality (folding/structural integrity and purity) by Tycho system (thermal shift assay). (D) Table summarizing the stability study of rhCALR del52 in different kinds of medium or plasma. Indicated samples were maintained at 37°C for various time points prior to quantification by ELISA and analysis using a one-phase decay model (Prism6) to determine the averaged half-life (t1/2) and the Coefficient of determination (R^2). (E) Western blot showing rhCALR del52 stability in medium. Cells were

maintained for various time points (0 to 480 min) at 37°C and the samples were subjected to Western blotting analysis. Detection of mutant CALR was achieved using a polyclonal rabbit anti-mutant CALR antibody obtained from MyeloPro (SAT602).

Supplementary Figure 3: Quantification of the sTFRC-CALR mutant complex in plasma

(A) Western blotting showing the presence of plasma CALR mutant (up) and sTFRC (bottom) after immunoprecipitation of control or patient plasma with biotinylated anti-CALR mutant antibody (Dianova).

(B) Representative western blotting used for quantification of CALR mutant and sTFRC from patient plasma after immunoprecipitation with anti-CALR mutant antibody (Dianova). The immunoprecipitation products were loaded alongside different known amounts of rh CALR del52 and rh sTFRC proteins to approximate the amount of plasma CALR mutant and the amount of sTFRC bound to plasma CALR mutant.

(C) Approximation of the fraction of CALR mutant bound to sTFRC in the plasma of patients. The amount of plasma CALR mutant and sTFRC was quantified by western blotting like in (B) after immunoprecipitation of 300 µL of plasma with anti CALR mutant antibody (Dianova) to estimate (1) the amount of CALR in plasma and (2) the amount of sTFRC bound to plasma CALR. The estimated percentage of CALR bound to TFRC was estimated based on a 1:1 molar ratio using the following formula:

$$\% \text{ of CALR bound to TFRC} = \frac{[sTFRC]_{ng/ml}}{[plasma\ CALR]_{ng/ml)}} * \frac{MM(CALR\ mut)_{g/mol}}{MM(sTFRC)_{g/mol}}$$

Where [x] represents the concentration of sTFRC or plasma CALR in ng/ml and MM(x) represents the molar mass of each protein.

Supplementary Figure 4: Exogeneous CALR del52 induces higher maximum growth response than Tpo in CALR mutant expressing cells with EC50 of 99.42 ng/ml

(A) Dose response of Ba/F3 TpoR *Calr*^{WT} in presence of varying concentrations of Tpo (n = 6) measured by their proliferation using the Celltiter-Glow (CTG). EC50 and non-linear curve fit were calculated with GraphPad Prism version 9.0.

(B) Dose response of Ba/F3 TpoR *Calr*^{mut} in presence of varying concentrations of Tpo (n = 9) measured by their proliferation using the Celltiter-Glow (CTG). EC50 and non-linear curve fit were calculated with GraphPad Prism version 9.0.

(C) Dose response of Ba/F3 TpoR *Calr*^{mut} in presence of varying concentrations of rhCALR del52 (n = 12) measured by their proliferation using the Celltiter-Glow (CTG). EC50 and non-linear curve fit were calculated with GraphPad Prism version 9.0.

(D) Maximum growth of Ba/F3 TpoR *Calr*^{WT} or Ba/F3 TpoR *Calr*^{mut} in presence of saturating concentrations of Tpo (2.000ng/ml) or rhCALR del52 (20.000 ng/ml). Values represent mean (+ SD), n = 6-12. Statistic: Two-ways ANOVA followed by SIDAK multiple comparison test. **: p <0.01, ****: p < 0.000

Supplementary Figure 5: Exogenous CALR del52 is inducing cell growth preferentially in Ba/F3 expressing TpoR and mutant CALR.

(A) rhCALRdel52 induces cell growth of Ba/F3 TpoR *Calr*^{mut} in a doses dependent manner. Rate of [^{3}H]thymidine incorporation of Ba/F3 TpoR *Calr*^{mut} was assessed after daily exposition of the indicated doses of recombinant human CALR del52 during 72h. Values shown represent the average of 4 experiments with at least 29 biological replicates ± SEM. Statistical analysis (Jmp pro12) was performed by the non-parametric multiple comparisons Steel's test with a control group (vehicle). **** = p < 0.0001.

(B-C) Short term proliferation of parental Ba/F3 and stable Ba/F3 expressing the indicated constructs were analyzed after daily exposition of the indicated doses of rhCALR del52 during 72h with CTG assay (Promega). Values shown represent the average of 5 experiments with at least 29 biological replicates ±

SEM. Statistical analysis (Jmp pro14) was performed by the non-parametric multiple comparisons Steel's test with a control group (vehicle). *** = $p < 0.001$, ** = $p < 0.01$.

(D) Cell-Titer Glo assay measuring the proliferation of UT-7/Tpo and UT-7/Tpo CRISPR CALR del52 stably expressing an empty vector or over-expressing TFRC 3 days after removal of GM-CSF. Data represents mean +/- SD of 4 independent experiments in quadruplicate (N = 4, n = 16). Tpo stimulation corresponds to 10ng/ml.

Supplementary Figure 6: STAT5 signaling induced by rhCALRdel52 in cell line and CD41-enriched primary cells

(A) Western blots showing TpoR and STAT5 phosphorylation in Ba/F3 TpoR *Calr*^{mut}, Ba/F3 TpoR *Calr*^{WT} and Ba/F3 parental cells treated or not with the indicated condition for 30 min. P-Y626-TpoR denotes phosphorylation of tyrosine residue 112 of the intracellular chain of TpoR. HA denotes detection of total HA-tagged TpoR.

(B) Flow cytometry analysis of CD41-positive cell enrichment. Bone marrow cells from *Calr* $^{del52/WT}$ knock-in mouse were cultivated in Tpo for 5 days before CD41+ isolation with magnetic beads (MACS technology). Purity assessment was monitored by flow cytometry analysis using an anti-CD41/APC antibody (Biolegend, clone MWreg30) and Median Fluorescent Intensity (MFI) were indicated in the table. Cell debris and dead cells were excluded from the analysis based on forward and side light-scatter.

(C) Western blot showing STAT5 phosphorylation of CD41-positive cells obtained after *in vitro* megakaryopoiesis, with or without the addition of 10 µg/mL of rhCALR del52 in the culture medium 30 minutes before cell lysis. Primary cells were harvested from the bone marrow of a KI-*Calr* $^{del52/WT}$ mouse. Detection of mutant CALR was achieved using a polyclonal rabbit anti-mutant CALR antibody obtained from MyeloPro (SAT602).

Supplemental Figure 7: BRET analysis

(A) Map of modified NanoBRET vectors that were made to express a secreted CALR-HaloTag construct and N-terminal fused Nano-Luciferase linked TpoR or EpoR (original vectors supplied in the NanoBRET PPI starter kit of Promega, ref N1821). (B) Culture media of HEK293 cells were collected 24-hours post-transient transfection with either the CALR WT-HaloTag or CALR del52-HaloTag. Media were diluted in NP-40 lysis buffer 1:1 (v/v), then re-diluted 3:1 (v/v) with LDS sample buffer (containing 1/10 of sample reduction buffer 10X from Thermo Fisher Scientific, Merelbeke, Belgium), heated 5 minutes at 96°C and loaded onto an SDS-PAGE 4-12% Bis-Tris Plus gel (Thermo Fisher Scientific, Merelbeke, Belgium). After transfer, we used the anti-HaloTag (Promega) mouse monoclonal antibody to detect the secreted proteins. As shown, all fusion constructs were well expressed in the relevant media (expected size of a CALR-HaloTag fusion protein is ~85 kDa, and HaloTag alone is ~38 kDa). Some degradation products are seen with fusion constructs, where HaloTag appears to be separated from the original molecule (lower bands seen in fusion construct expressing conditions). (C) The nanoluciferase-TpoR and -EpoR fusion constructs are correctly exposed to the cell surface after transient transfection of HEK293 cells. HEK293 cells were washed 24 hours post transfection with vectors encoding either the N-terminal HA-tagged nanoluciferase-TpoR (Nano-TpoR) or the N-terminal HA-tagged nanoluciferase-EpoR (Nano-EpoR) construct (A) in PBS – 2% FBS – 2 mM EDTA. As a control, untransfected HEK293 were similarly processed. After washing, cells were marked using a 1/400 dilution of anti-HA-APC antibody (clone 16B12, BioLegend, San Diego, CA) and the Aqua Live/Dead fixable viability dye (Thermo Fisher Scientific, Merelbeke, Belgium) before analysis on a FACSVerse flow cytometer (BD, Franklin Lakes, NJ). Dead cells were discarded in a previous gate before quantifying APC fluorescence (not shown). (D) Western blot showing CALR protein in HEK293 cells expressing stably the hCALR WT-HaloTag or the hCALR del52-HaloTag by using an anti-HaloTag antibody and anti-actin antibody as control.

To test whether these fusion constructs retain the original signaling properties of the non-fused protein, we set-up two STAT5 reporter luciferase assays. (E) Ba/F3 expressing NL-TpoR were electroporated to take up the STAT5 firefly luciferase reporter Spi-Luc along with either an empty vector (pMSCV empty), a vector coding for unmodified human CALR WT or a vector coding for unmodified human CALR del52. Cells were then kept for 6 hours at 37°C with or without presence of Tpo (50 ng/mL) or eltrombopag (0.5 µg/mL) in the medium, after which they were lyzed to perform the luciferase reporter assay. The result shown are from 2 independently performed experiments, and confirm that NL-TpoR is able to be activated by Tpo, eltrombopag or hCALR del52. Statistics were performed by Jmp pro 14, using the Steel with control (no Tpo, pMSCV empty) multiple non-parametric comparison test, p-value shown above. (F) Ba/F3 expressing either CALR WT-HaloTag or CALR del52-HaloTag were electroporated to take up the to take up the STAT5 firefly luciferase reporter Spi-Luc along with either an empty pMegix vector or a human TpoR WT coding pMegix vector. Cells were then kept for 6 hours at 37°C with or without presence of Tpo (50 ng/mL) in the medium, after which they were lyzed to perform the luciferase reporter assay. The result shown are from 2 independently performed experiments, and confirm that CALR del52-HaloTag but not CALR WT-HaloTag is able to activate the wild-type human TpoR. Statistics were performed by Jmp pro 14, using Student's two-tailed t-test. E and F. electroporation was performed in OptiMeM medium, with 10 µg of total plasmid DNA and 500.000 cells per condition, using a BTX ECM 630 electroporator with 260 V, 750 ohm and 1500 µF as settings.

Supplementary Table 1: Relationship between single dose treatment and average concentration.

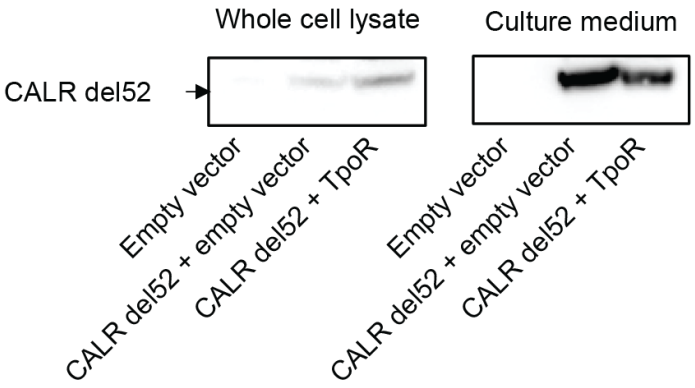
The relationship between single dose treatment (D) and average concentration (C_p) was calculated with the following formula:

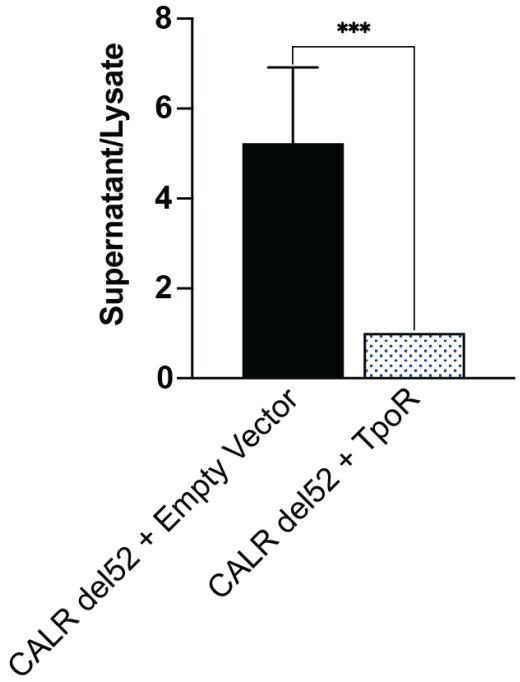
$$C_p = \frac{1}{0.693} * F * D * t_{\frac{1}{2}} * \frac{1}{\tau}$$

Where F (bioavailability) is set at 1, $\tau = 24\ \mathrm{h}$ (dose interval) and $t_{\frac{1}{2}}$ is the measured half-life.

Supplementary Figure 1

A





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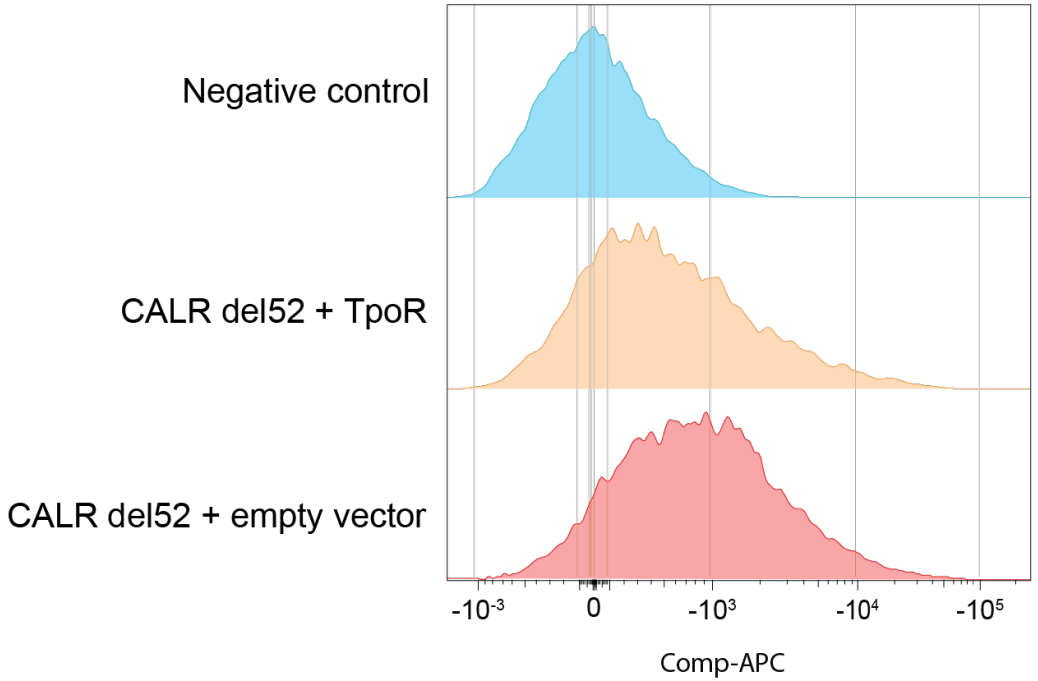
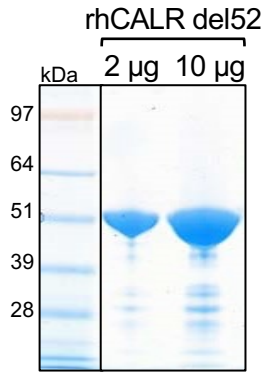


	TUBE NAME	Geometric Mean : Comp-APC	Median : Comp-APC
	Negative control	27.9	7.52
	CALR del52 + Empty vector	542	450
	CALR del52 + TpoR	854	823

Supplementary Figure 2

A



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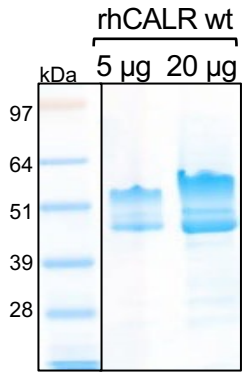

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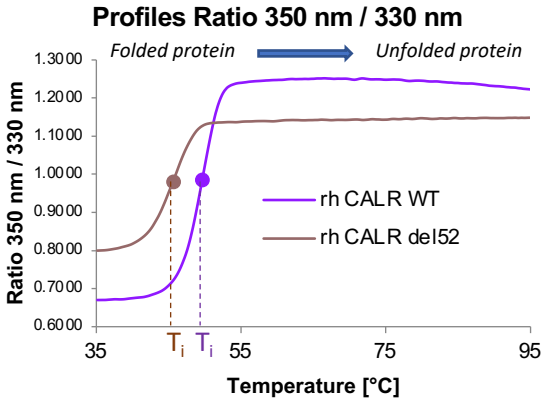

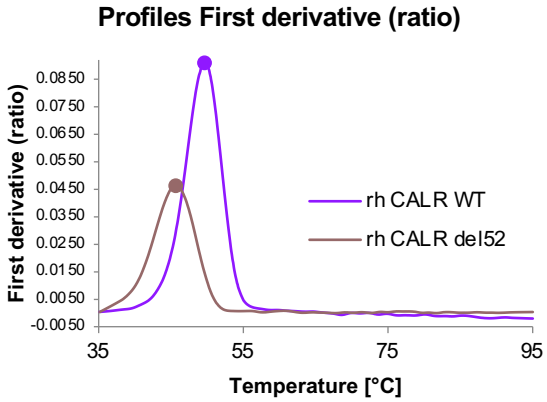

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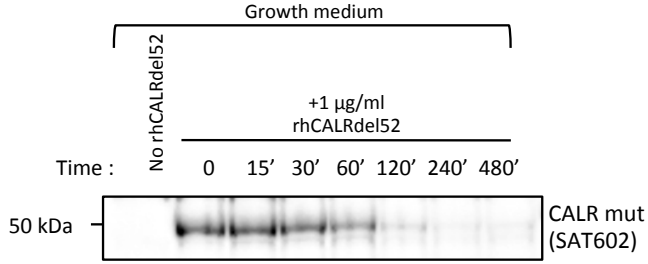
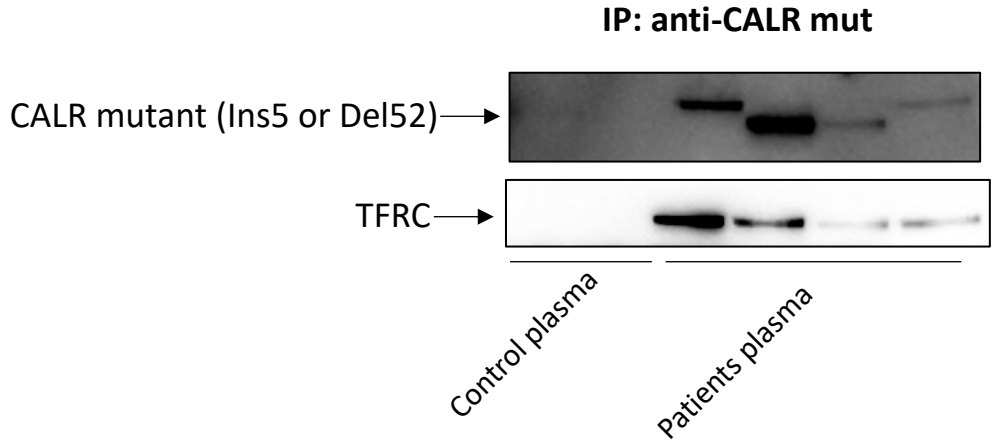


D

Stability at 37°C	rhCALR del52 in culture medium without FBS	rhCALR del52 in healthy plasma (n=6)	rhCALR del52 in culture medium with FBS	rhCALR del52 in culture medium with FBS + 4 µg/ml sTFRC	Endogenous CALR mutant in patient plasma
t(1/2) (min)	30.72	45.19	73.38	143.2	426.3
R^2	0.8560	0.8409	0.9375	0.9580	0.8824

Supplementary Figure 3

A



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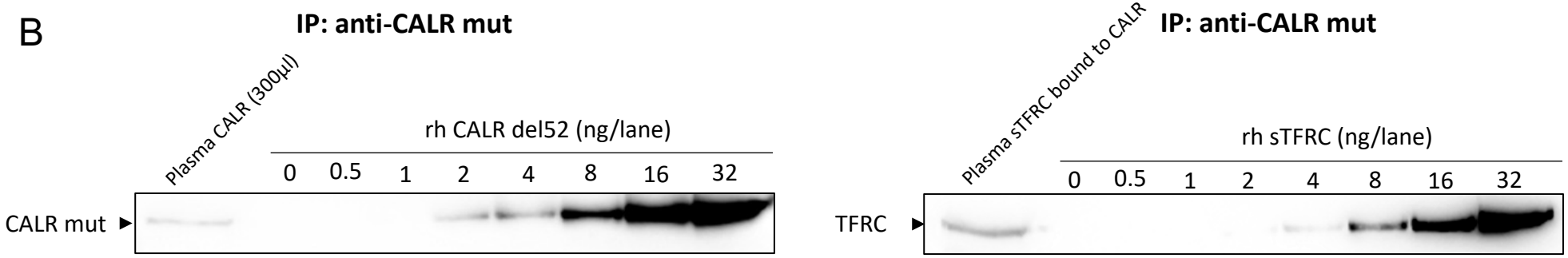
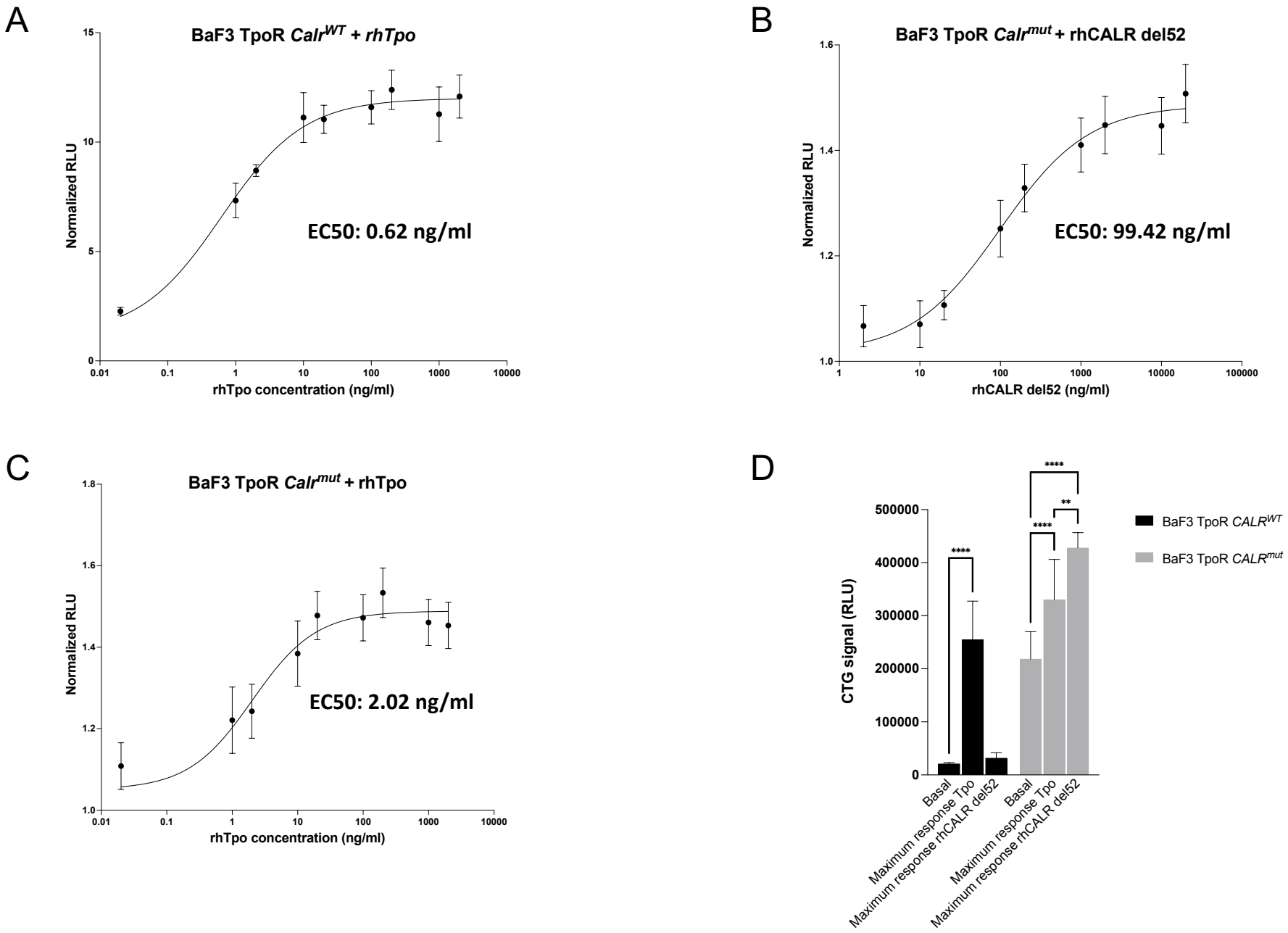


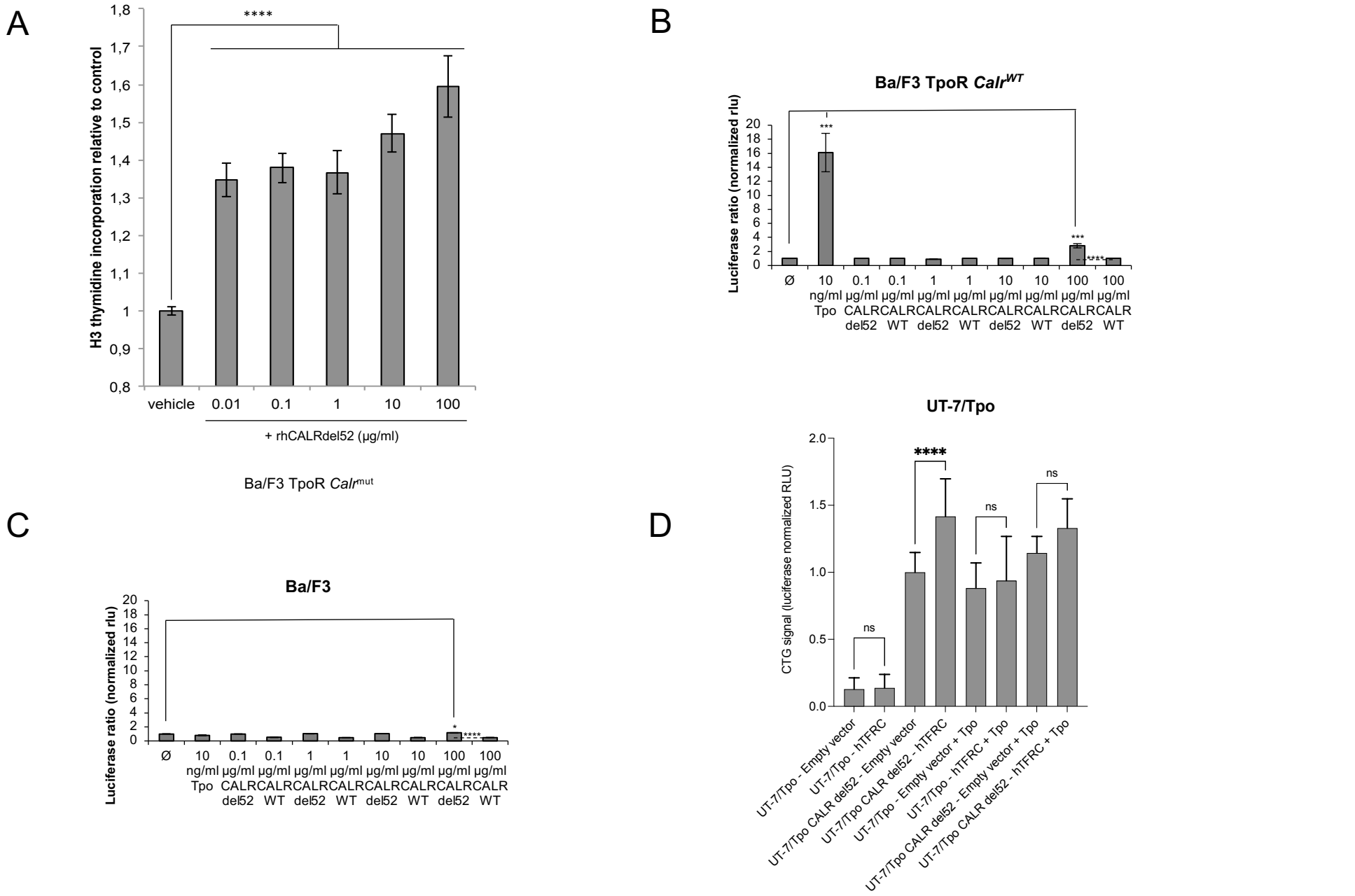
C

Sample	Allele burden	CALR mutation	Estimated CALR in plasma	Estimated sTFRC bound to CALR	Estimated % of CALR bound to (1:1 molar ratio)
Patient 1	24%	CALR del52	42.3 ng/ml	19.2 ng/ml	28.3%
Patient 2	51%	CALR del52	48.20 ng/ml	76.39 ng/ml	99%
Patient 3	25.30%	CALR del52	19.07 ng/ml	19.29 ng/ml	62%
Patient 4	30%	CALR Ins5	20.84 ng/ml	17.88 ng/ml	53.6%

Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6

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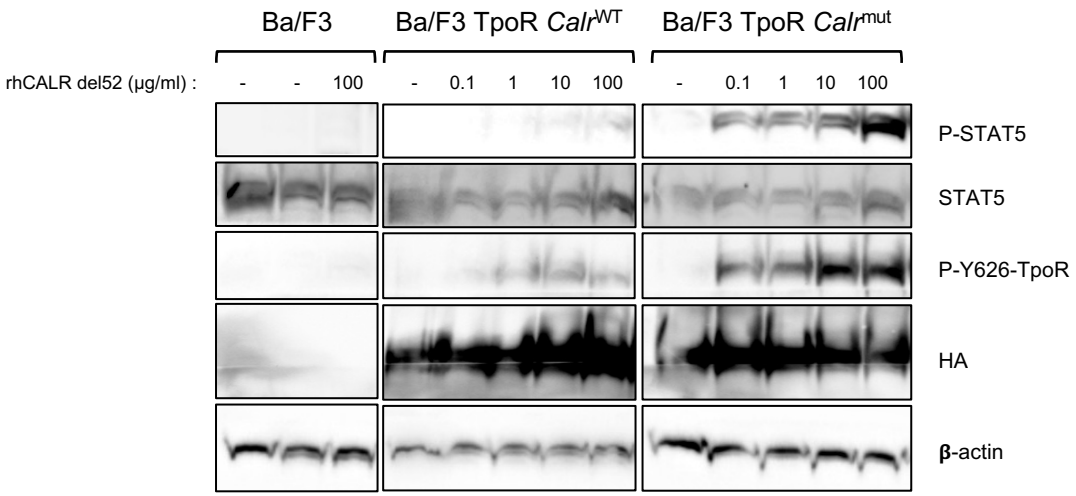
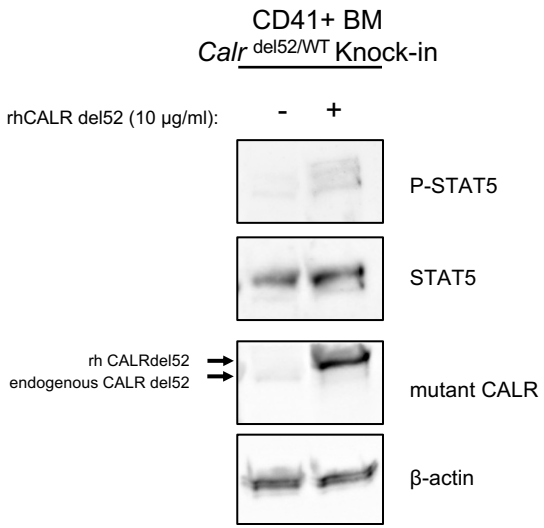


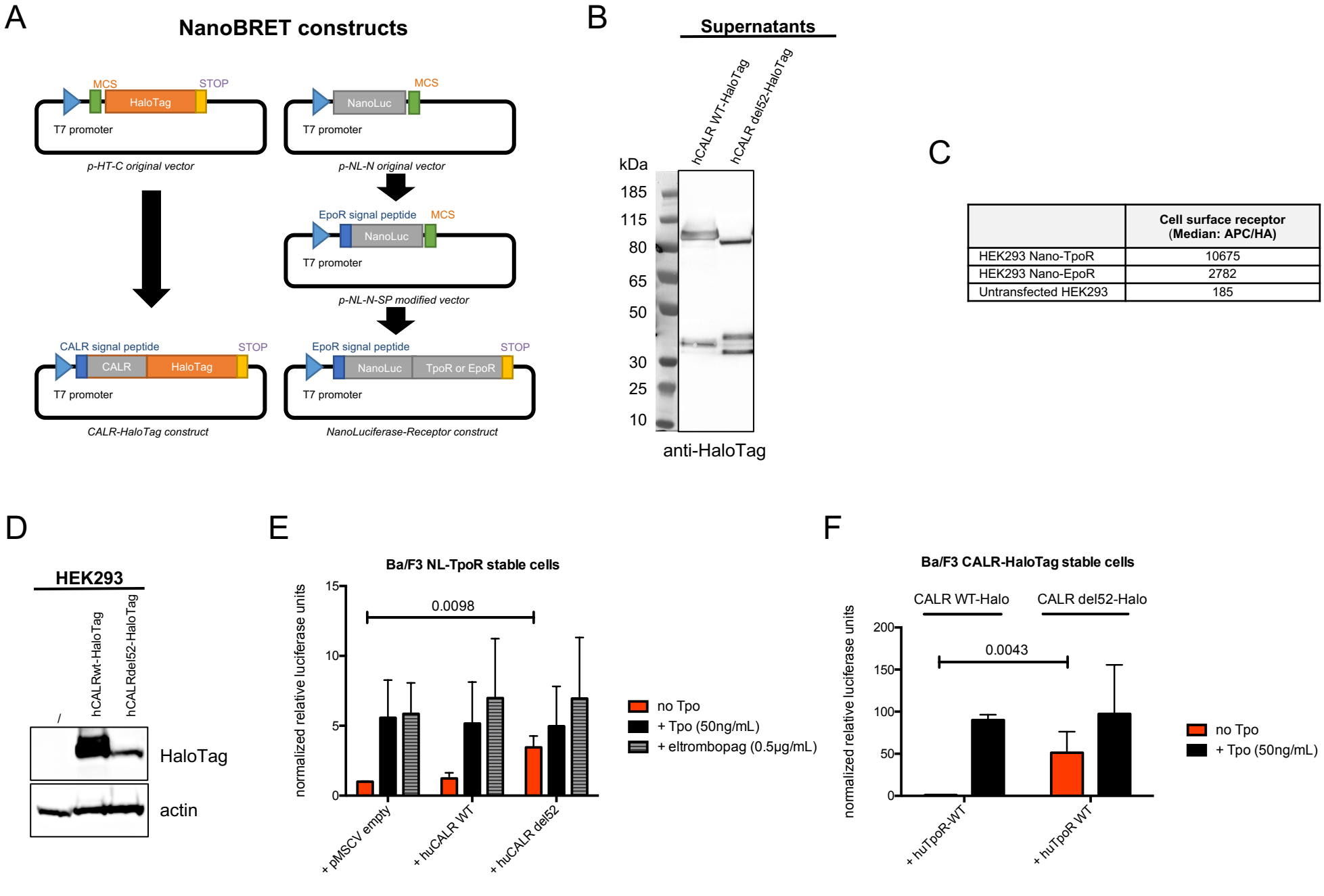
B

	Median: APC/CD41
CD41-positive population	27142
CD41-negative population	1048
Total BM population	4009

C



Supplementary Figure 7



Supplementary Table 1

Single dose (D) in ng/ml	Average concentration over 24h in medium without FBS (C_p)	Average concentration over 24h in medium with 10% FBS (C_p)
10	0.30	0.73
100	3	7.3
1000	30	73
10000	300	730
100000	3000	7300

Table 1: Correlation between single dose treatment every 24h and average concentration over experiment interval.