**Title: Defective interaction of mutant calreticulin and SOCE in megakaryocytes from patients with myeloproliferative neoplasms**

**Short Title:** CALR mutants drive SOCE activation in MPN

**Authors:**

Christian A. Di Buduo[1,2,*], Vittorio Abbonante[1,2,*], Caroline Marty[3], Francesco Moccia[4], Elisa Rumi[5], Daniela Pietra[5], Paolo M. Soprano[1-2], Dmitry Lim[6], Daniele Cattaneo[7], Alessandra Iurlo[7], Umberto Gianelli[7], Giovanni Barosi[8], Vittorio Rosti[8], Isabelle Plo[3], Mario Cazzola[4], Alessandra Balduini[1,2,9]

**Affiliations:**

[1]Department of Molecular Medicine, University of Pavia, Pavia, Italy; [2]Laboratory of Biochemistry, Biotechnology and Advanced Diagnosis, IRCCS Policlinico San Matteo Foundation, Pavia, Italy; [3]INSERM, UMR1170, Gustave Roussy, Université Paris-Sud, Villejuif, France; [4]Laboratory of General Physiology, Department of Biology and Biotechnology "Lazzaro Spallanzani", University of Pavia, Pavia, Italy; [5]Department of Hematology Oncology, IRCCS Policlinico San Matteo Foundation, Pavia, Italy; [6]Department of Pharmaceutical Sciences, Università del Piemonte Orientale, Novara, Italy; [7]Hematology Division, Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; [8]Center for the Study of Myelofibrosis, IRCCS Policlinico San Matteo Foundation, Pavia, Italy; [9]Department of Biomedical Engineering, Tufts University, Medford, MA, USA. *These authors equally contributed.

**Corresponding Author:** Alessandra Balduini, Department of Molecular Medicine, University of Pavia, via Forlanini 6, 27100, Pavia, Italy. Phone + 39 0382 502968. Fax +39 0382 502990. E-mail: alessandra.balduini@unipv.it.

**Supplemental Materials and Methods**

**Materials.**

2-Aminoethyl diphenylborinate (2-APB), 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM), cyclopiazonic acid from *Penicillium cyclopium* (CPA), and Protein A-Sepharose from *Staphylococcus aureus* were from Sigma-Aldrich (Milan, Italy). N-(4-[3,5-Bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide BTP-2) was from Calbiochem (Merck Millipore, Milan, Italy). Fura-2 acetoxymethyl ester (Fura-2 AM) was from Molecular Probes Europe BV (Leiden, the Netherlands). Precision Plus protein standard was from Bio-Rad (Milan, Italy). The following antibodies were used: anti-human CD42b (phycoerythrin (PE)- conjugated, clone HIP1); anti-human CD41a (fluorescein isothiocyanate (FITC)- conjugated, clone HIP8) (eBioscience, USA); mouse monoclonal anti-STIM1; rabbit polyclonal anti-STIM1; rabbit polyclonal anti-ERp57; mouse monoclonal anti-PCNA; rabbit monoclonal anti-calreticulin (clone EPR3821); mouse monoclonal anti-calreticulin (clone FMC 75); rabbit monoclonal anti-c-Mpl (clone EPR4194) (Abcam, Cambridge, UK); anti-mutated CALR (clone CAL2) (Dianova, Hamburg, Germany); rabbit monoclonal anti-Orai1 (clone H-46), mouse monoclonal anti-TRPC1 (clone E-6); goat polyclonal anti-CD61 (clone C-20); anti-goat HRP conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA); rabbit monoclonal anti-STAT5 (clone RH7); rabbit monoclonal anti-phospho-STAT5 (Tyr694) (clone C11C5); rabbit monoclonal anti-phospho-Akt (Ser473); rabbit polyclonal anti-Akt; rabbit monoclonal anti-ERK1/2 (clone 137F5) (Cell Signaling Technology, MA, USA); rabbit monoclonal anti-phospho-ERK1/2 (Thr185/Tyr187) (clone AW39) (Millopre, Milan, Italy); anti-mouse and anti-rabbit HRP conjugated secondary antibodies (Bio-Rad, Milan, Italy). In some immunoprecipitation experiments EasyBlot anti-mouse and anti-rabbit HRP conjugated secondary antibodies (GeneTex, USA) were used. EasyBlot specifically reacts with the native, non-reduced form of rabbit and mouse IgG but does not bind the reduced, denatured form, thus decreasing the interference caused by the heavy (~50 kDa) and light chains (~25 kDa) of the IgG used for immunoprecipitation.

Recombinant human thrombopoietin (rhTPO), recombinant human interleukin-11 (rhIL-11) and recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF) were from Peprotech (Peprotech, UK). Enhanced chemiluminescence reagents (ECL) were from Millipore (Milan, Italy).

**Solutions.**

Physiological salt solution (PSS) had the following composition: NaCl 150 mM, KCl 6 mM, $CaCl_2$ 1.5 mM, $MgCl_2$ 1 mM, glucose 10 mM, Hepes 10 mM. In $Ca^{2+}$-free solution (Ø $Ca^{2+}$), $Ca^{2+}$ was substituted with NaCl 2 mM and EGTA 0.5 mM was added. Solutions were titrated to pH 7.4 with NaOH.

**Patients.**

This study was approved by the institutional ethics committee of IRCCS Policlinico San Matteo, Pavia, Italy and Gustave Roussy hospital, Villejuif, France. The procedures followed were in accordance with the Helsinki Declaration of 1975 (revised 2000). Samples were obtained with written informed consent. Diagnosis of MPN was made according to the 2016 revision of the World Health Organization classification of myeloid neoplasms.[1] Genetic mutations were studied as previously described.[2] Patient and healthy subjects characteristics are described in **Supplemental Table 1**.

**UT-7 infection and culture.**

The human megakaryoblastic UT-7 cells expressing c-Mpl and CALR Type I mutant were maintained in Dulbecco's modified Eagle's medium (DMEM; Euroclone, Milan, Italy), supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine and 10 ng/mL GM-CSF. For down-modulation of endogenous levels of CALR wild type (WT) in UT-7 cells, lentivirus-mediated short hairpins RNAs (shRNAs) specifically targeting CALR WT were used. UT-7 transduced and sorted to express FLAG-tagged c-Mpl (huCD4) and HA-tagged CALR (muCD2) were infected with either shRNAs or a scramble sequence (scr) (GFP) and sorted. UT-7/GM cell line (UTRRID: CVCL_5203) was a generous gift from N. Komatsu (Japan). A Mycoplasma screening was routinely performed, according to the manufacturer instructions (Sigma, Saint-Quentin Fallavier, France). Cells were kept in culture for a limited amount of passages (p25).

For the analysis of cell proliferation, UT-7 were stained with 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE, BioLegend) and cultured for 4 days in cytokine-starved conditions. Labeled cells were analysed by a Beckman Coulter FacsDiva flow cytometer (Beckman Coulter Inc.) on day 1 and day 4 of culture.

**Immunofluorescence analysis.**

For immunofluorescence imaging samples were processed as previously described.[3] Briefly, samples were fixed in 4% paraformaldehyde (PFA) for 20 min, permeabilized with 0.1% Triton X-100 (Sigma) for 5 min and then blocked with 5% bovine serum albumin (BSA, Sigma) for 30 min, at room temperature. Samples were probed with anti-CALR (1:100) or anti-mutated CALR (1:20), 2 hours at room temperature and then immersed in Alexa Fluor secondary antibody (1:500) for 1 hour at room temperature. Nuclei were stained with Hoechst. Samples were imaged by confocal microscopy (FV10i, Olympus).

**Flow cytometry.**

In order to analyze Mks immunophenotype in the different tested treatments, all samples, were routinely characterized as $CD41a^+CD42b^+$. Briefly, Mks were cultured in PSS or in Ø $Ca^{2+}$

solutions, in the presence or absence of 10 μM CPA, and pre-treated (for 30 min) or not with 20 μM BAPTA-AM, 20 μM BTP-2 or 20 μM 2-APB. Then, samples were stained with a FITC-conjugated antibody against human CD41a and a PE-conjugated antibody against human CD42b, at room temperature, in the dark for 10 minutes. After incubation, samples were acquired with a Beckman Coulter Navios flow cytometer. Flow cytometry settings were established as previously described.[4] The relative isotype controls were used to set the correct analytical gating. For ploidy analysis, cells from the different tested conditions were fixed with 70% ethanol at -20°C overnight, centrifuged at 4000xg for 10 minutes at RT and then suspended in PBS containing 1μg/ml Propidium Iodide (Sigma), 1 μg/ml RNAse (Sigma) and FITC-conjugated antibody against CD41a, at room temperature, in the dark for 30 minutes. Off-line data analysis was performed using Beckman Coulter Navios Kaluza version software package.

**Human inositol trisphosphate dosage**

For inositol trisphosphate (IP3) dosage, Mks were starved and stimulated or not with 100 ng/mL rhTPO or 1U/mL thrombin, used as standard control, for 10 min before being lysed with Hepes-glycerol lysis buffer (Hepes 50 mM, NaCl 150 mM, 10% glycerol, 1% Triton X-100, $MgCl_2$ 1.5 mM, EGTA 1mM, NaF 10mM, $Na_3VO_4$ 1mM, 1 μg/mL leupeptin, 1 μg/mL aprotinin). Lysates were clarified by centrifugation at 15700xg and stored at -80°C until ELISA analysis. Human inositol 1,4,5,-trisphosphate IP3 Assay kit was from Tebu-Bio. ELISA assays were performed according to the manufacturer's instructions. Samples from 7 independent experiments were analyzed in triplicate. Results are reported as mean±Standard Deviation (SD) with respect to untreated controls.

**References**

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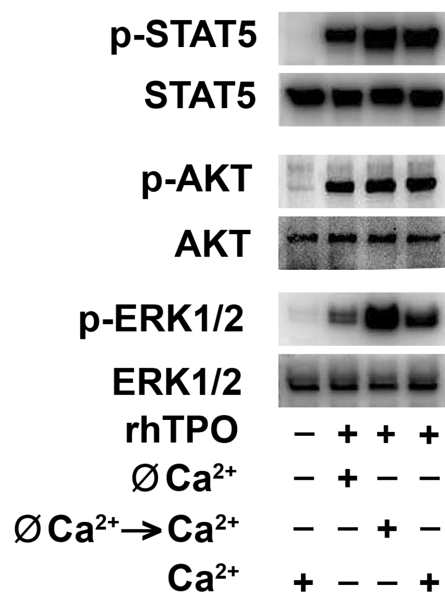
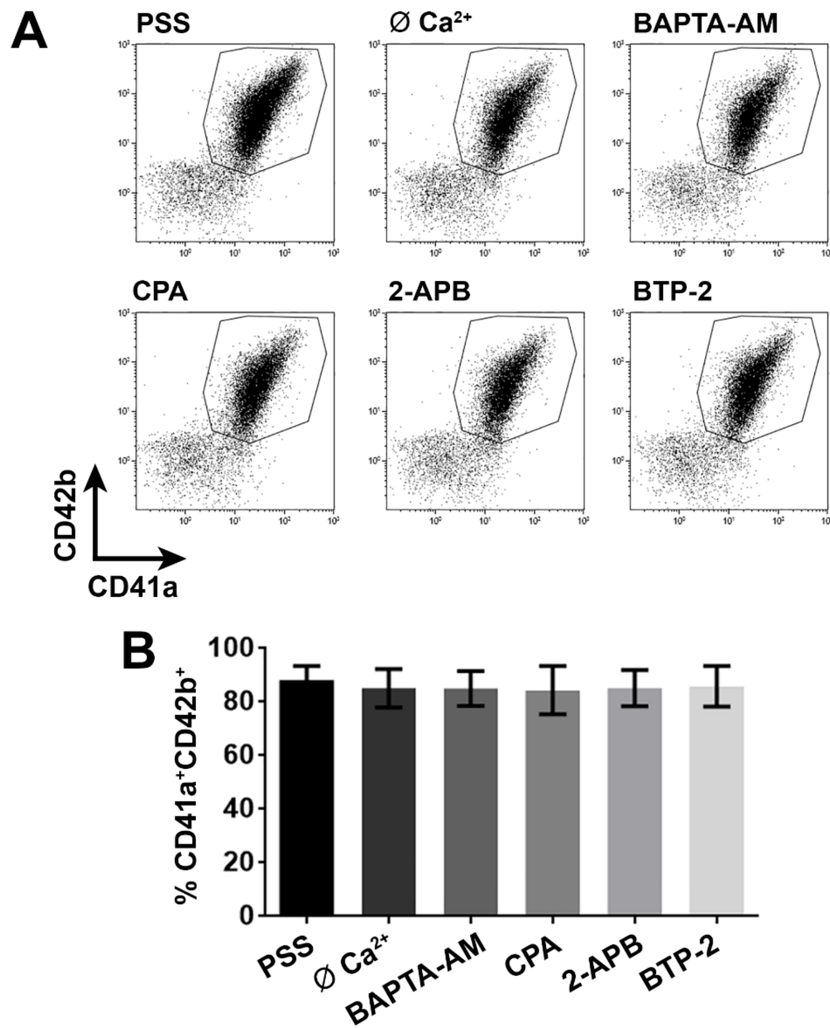
**Supplemental Figures and Figure Legends**

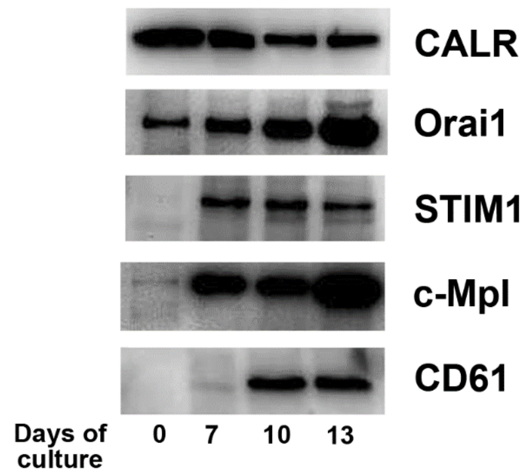



**Supplemental Figure 1. Thrombopoietin-dependent intracellular signaling is sustained by intracellular and extracellular calcium flows.** Western blot analysis of STAT5, AKT and ERK1/2 phosphorylation (p-STAT5, p-AKT and p-ERK1/2 respectively) after 10 min stimulation with recombinant human thrombopoietin (rhTPO) (100 ng/mL) in presence of extracellular calcium ($Ca^{2+}$) (1.5 mM), absence of extracellular $Ca^{2+}$ (Ø $Ca^{2+}$) or upon re-addition of physiological extracellular $Ca^{2+}$ after stimulation in absence of extracellular $Ca^{2+}$ (Ø $Ca^{2+}$ -> $Ca^{2+}$). Total STAT5, AKT and ERK1/2 were stained to ensure the equal loading.

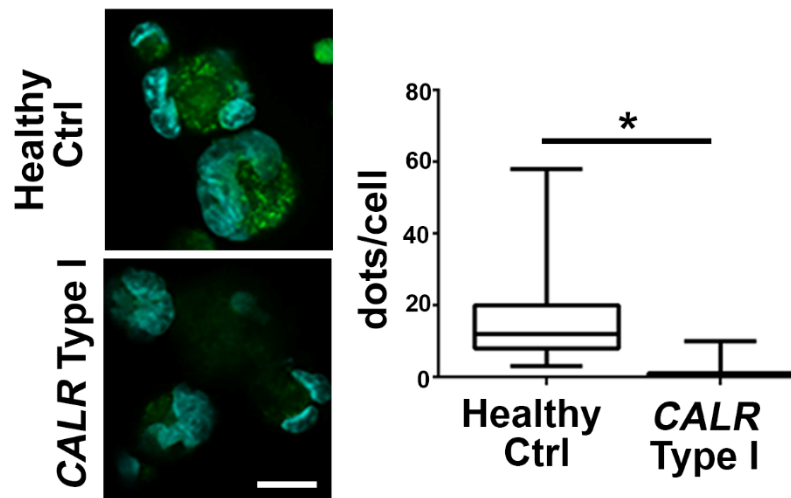



**Supplemental Figure 2. Analysis of megakaryocyte immunophenotype.** (A) Flow cytometry gates for CD41a$^{+}$CD42b$^{+}$ megakaryocytes in the different tested conditions. (B) Percentage of

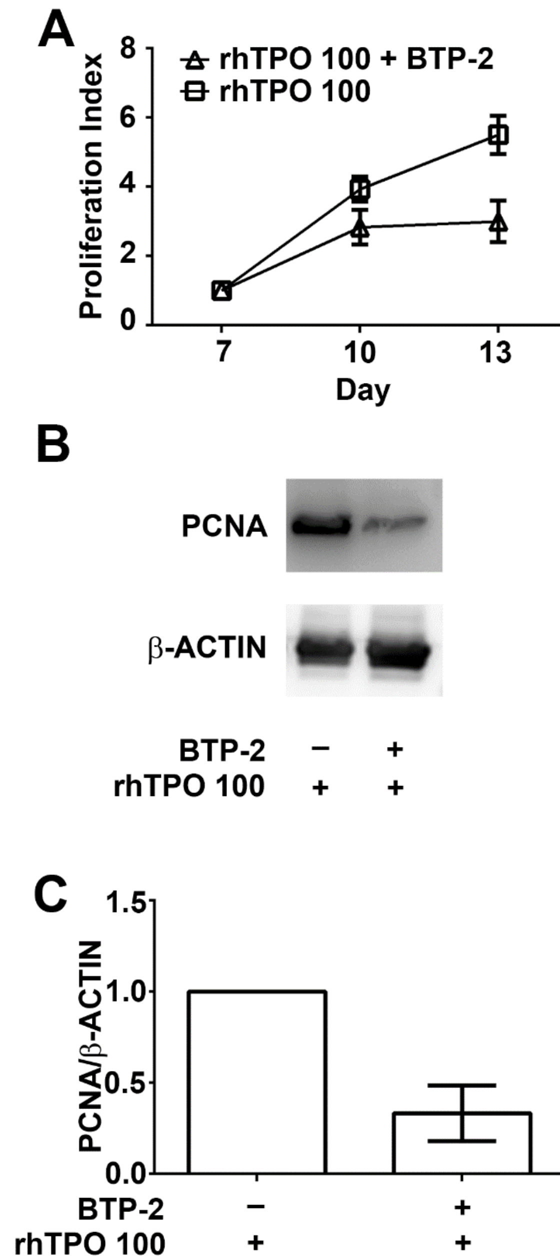
$CD41a^+CD42b^+$ events calculated with respect to the total number of acquired events. Data are presented as mean±SD of 3 independent experiments (p=NS).



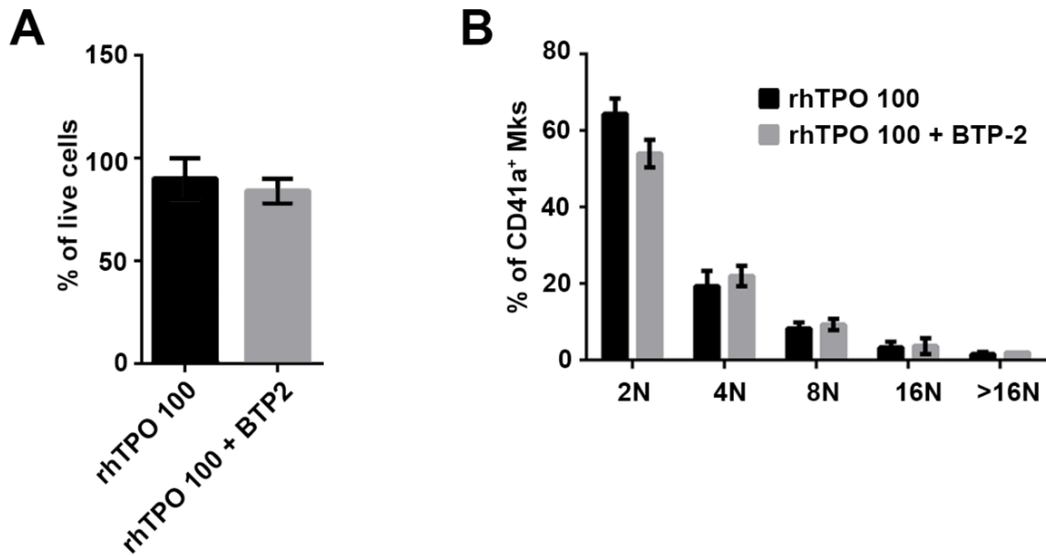

**Supplemental Figure 3. Increased expression of store-operated calcium entry (SOCE) effectors during megakaryocyte differentiation.** Representative western blot analysis of CALR, Orai1, STIM1, c-Mpl and CD61 expression by megakaryocytes (Mks) during differentiation form cord-blood derived hematopoietic stem cells (day 0).



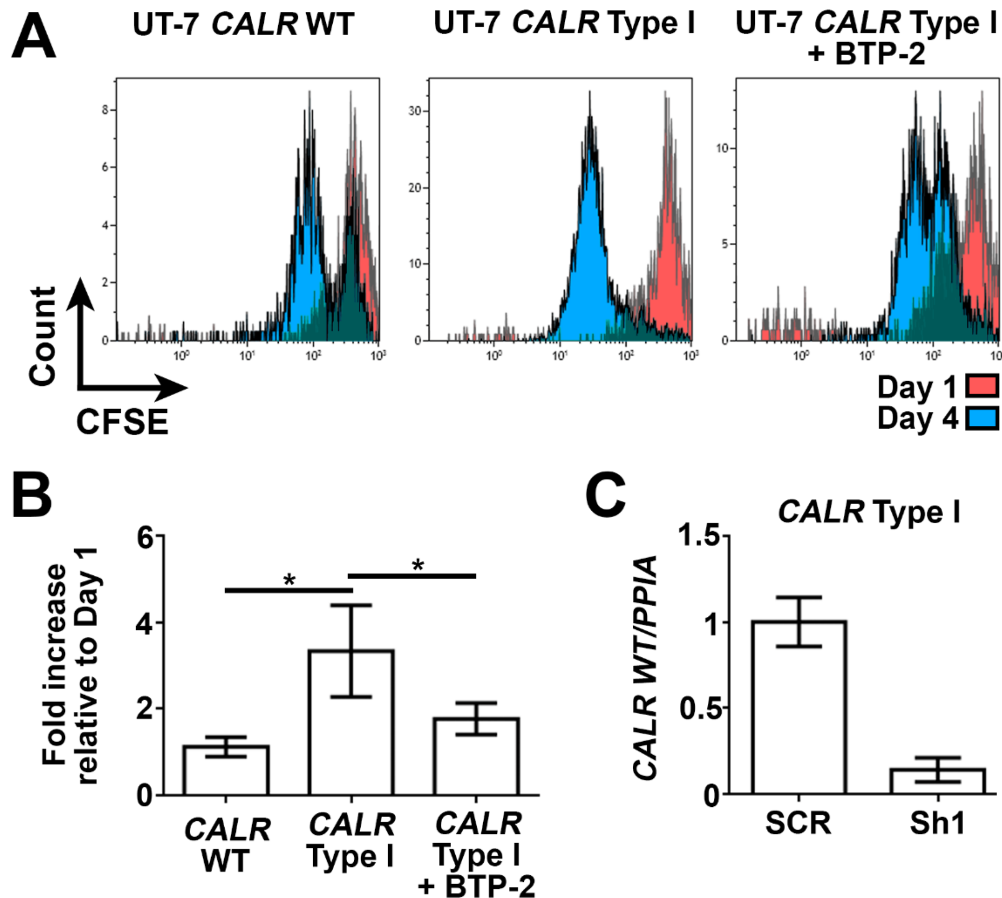

**Supplemental Figure 4. Altered CALR-STIM1 binding in the presence of homozygous *CALR* Type I mutation.** *In situ* proximity ligation assay (PLA) imaging of STIM1 and CALR WT or CALR Type I interaction (green dots) in starved mature Mks from one healthy control (Ctrl) and one PMF patient having homozygous *CALR* Type I mutation (blue = nuclei; scale bar = 25 µm; original magnification = 60x/NA1.2). Quantification of dot number per single Mk (overall, data derives from 107 measurements, $P<0.05$).




**Supplemental Figure 5. Megakaryocytes proliferation in response to increased thrombopoietin signaling is due to store-operated calcium entry activation. (A)** Cell proliferation assay of cord blood derived-megakaryocytes (Mks) in presence of recombinant human thrombopoietin (rhTPO) 100 ng/mL or rhTPO 100 ng/mL plus 20 μM of the store-operated calcium entry inhibitor BTP-2 (n=3). **(B)** Western blot analysis of proliferating cell nuclear antigen (PCNA) expression in mature Mks in presence of rhTPO 100 ng/mL or rhTPO 100 ng/mL plus 20 μM BTP-2. β-ACTIN serves as loading control. **(C)** The band densitometry analysis of 3 independent experiments is shown.




**Supplemental Figure 6. Analysis of the effects of tested compounds on megakaryocyte viability and ploidy.** Cord blood derived-megakaryocytes (Mks) were cultured in presence 100 ng/mL recombinant human thrombopoietin rhTPO in the presence or absence of the tested compounds. **(A)** Percentage of live cell. Data are expressed as mean±SD (n=3; *P*=NS). **(B)** Effects of tested treatments on Mk ploidy. Data are expressed as mean±SD (n=3; *P*=NS).




**Supplemental Figure 7. Proliferation of UT-7 cell lines harboring *CALR* Type I mutation. (A)** Representative histograms of carboxyfluorescein succinimidyl ester (CFSE) proliferation assay in wild type (WT) and *CALR* Type I mutated UT-7 cells cultured in cytokine-starving conditions. Where indicated, *CALR* Type I UT-7 were cultured in presence of 20 μM BTP-2. **(B)** Cell proliferation assay of megakaryocyte (Mk) culture from WT and *CALR* Type I UT-7 cells in cytokine starved conditions (n=3; $P$<0.05). **(C)** Efficacy and specificity of short hairpin RNAs (shRNAs) directed against *CALR* WT in *CALR* Type I UT-7 cells: UT-7 cells expressing *CALR* Type I were transduced with shRNA specifically targeting *CALR* WT or a scramble sequence (SCR) and sorted. The expression level of *CALR* WT was quantified in these cells by qRT-PCR related to peptidylprolyl isomerase A (PPIA). Results are the mean±SD of duplicate data.

**Supplemental Table 1.**

| | **HEATLHY SUBJECTS** | ***JAK2*** $^{V617F}$ | ***CALR* Type I** | ***CALR* Type II** |
|---|---|---|---|---|
| AGE (median and range) | 54 (34-60) | 61 (40-81) | 61 (50-75) | 61 (31-80) |
| FEMALE/MALE | 5/5 | 6/4 | 5/5 | 5/5 |
| PMF/ET | - | 8/2 | 8/2 | 8/2 |