Supplemental Material

Targeting the CALR Interactome in Myeloproliferative Neoplasms

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Supplemental Methods

Co-Immunoprecipitation assay. Cells were washed in PBS and resuspended in lysis buffer (NaCl 100mM, 0.1% Tween 20) containing 1X Protease Arrest (G-Biosciences) and 1X Phosphatase Inhibitor Cocktail II (EMD). Protein extracts were incubated with 5µg of MBP monoclonal antibody, CALR N-term antibody or corresponding IgG (Cell Stem Technologies) on a rotating wheel at 4°C overnight and subsequently mixed with A/G agarose beads (Santa Cruz) on a rotating wheel at 4°C for 1 hour. Extracts were then washed three time with PBS with 0.05% Tween 20. Finally, bound proteins were extracted by boiling at 65°C for 7 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and subsequently electrophoresed on SDS/PAGE followed by silver staining for mass spectrometry or blotted onto nitrocellulose membranes (Millipore) for immunoblot analysis.

Mass spectrometry. Unless otherwise specified, all chemicals were from Sigma-Aldrich at the highest available purity. Lanes containing SDS PAGE-resolved proteins were excised from the gel and de-stained using 30mM K3Fe(CN)6/100mM Na2S2O3, washed with 25mM NH4HCO3 pH8.4 / 25% (v/v) Optima LC/MS acetonitrile (Fisher Scientific) and de- hydrated using a vacuum centrifuge. Proteins were reduced (10mM DTT/100mM ammonium bicarbonate pH8.4, 56°C for 60 minutes) and alkylated (55mM C2H4INO/ 100mM NH4HCO3 pH8.4, 25°C for 30 minutes, dark), and excess iodoacetamide was removed by three cycles of dehydration (100 µl C_2H_3N) and rehydration (100 µl 100mM NH4HCO3 pH8.4) prior to final dehydration in a vacuum centrifuge. Gel slabs were re- hydrated using 50mM NH4HCO3 pH8.4 containing 0.04µg sequencing grade modified porcine trypsin (Promega) per reaction and proteolysis was allowed to proceed for 16 hours at 37°C. Tryptic peptides elution from the polyacrylamide slabs was achieved by two consecutive 30 minutes incubation in 1% formic acid/70% acetonitrile (v/v) under continuous shaking. Eluates from each

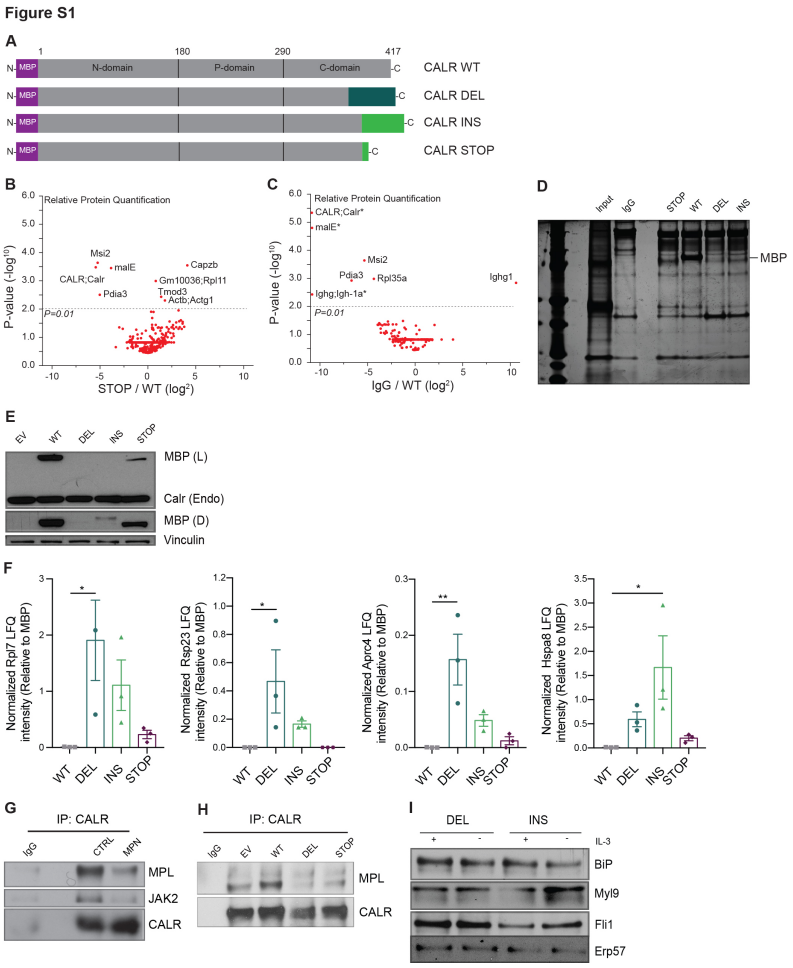
sample were pooled, lyophilized and stored at - 80°C until analysis. Peptide pellets were resuspended in 0.1% formic acid (Thermo Scientific) and 5% of the solution was analyzed by LC/MS. The LC system consisted in a vented trap-elute setup (Ekspert nanoLC 425, Eksigent) coupled to the Orbitrap Fusion mass spectrometer (Thermo) via a nano electro-spray DPV-565 PicoView ion source (New Objective). The trap column was fabricated capping a 5cm×150µm internal diameter silica capillary (Polymicro Technologies) with a 2mm silicate frit, and pressure loaded with Poros R2-C18 10µm particles (Life Technologies). The analytical column consisted of a 40cm×75µm internal diameter column with integrated electrospray emitter (New Objective), and was packed with ReproSil-Pur C18-AQ 1.9µm particles (Dr. Maisch). Samples were loaded on the trap column at 1µl/minute with analytical column excluded from the flow path. The analytical column was then put in-line with the trap, and peptides were resolved over 120 minutes using a 5-40% gradient acetonitrile/0.1% formic acid (buffer B) gradient in water/0.1% formic acid (buffer A) at 300nl/minute. Precursor ion scans were recorded from 400–1800m/z in the Orbitrap (240,000 resolution at m/z 200) with automatic gain control target set 10^5 ions and maximum injection time of 100ms. We used data-dependent mass spectral acquisition with monoisotopic precursor selection, range 800-1800Th for singly charged ions, and 400- 1800Th for ions with charge 2-6, dynamic exclusion (90 sec, 10 ppm tolerance), HCD fragmentation (normalized collision energy 31, isolation window 1.2Th) using the top speed algorithm with a duty cycle of 2 sec. Product ion spectra were recorded in the linear ion trap (“enhanced” scan rate, automatic gain control= 10^4 ions, maximum injection time = 100ms). Raw files were submitted to MaxQuant Version 1.5.4.1to be searched against the murine UniProt database (version 05/02-17, containing isoforms) supplemented with contaminant cRAP database, and the sequences of

human CALR and *E. coli* MBP (MalE gene). FDR was set at <0.01 for both peptide and protein level. Mass tolerance was set at 5ppm for orbitrap spectra and 0.5Da for linear trap spectra. C- carbamidomethylation was set as fixed modification, while acetylation of protein N- terminus, N-deamidation and M-oxidation were allowed as variable. The software was set to align chromatographic features (Matching time window= 1 min, Alignment time window= 20 min), and label-free quantification (LFQ) was enabled as quantification method. MaxQuant output and raw files are available via the ProteomeXchange repository with accession number PXD008476. Protein quantification was retrieved from the proteinGroups.txt table, after removing decoy identifications and common contaminants matching the cRAP database. For proteins quantified in at least one conditions, missing values were imputed by replacing them with the local minima within each experiment. For each mutant, protein intensity ratios (mutant/wild-type) were calculated and $\log_2$-transformed. To estimate statistical significance of differential protein precipitation, two-tailed t-test was performed testing the hypothesis that the means of LFQ intensities for each protein from cells harboring mutant and wild type CALR respectively were the same.

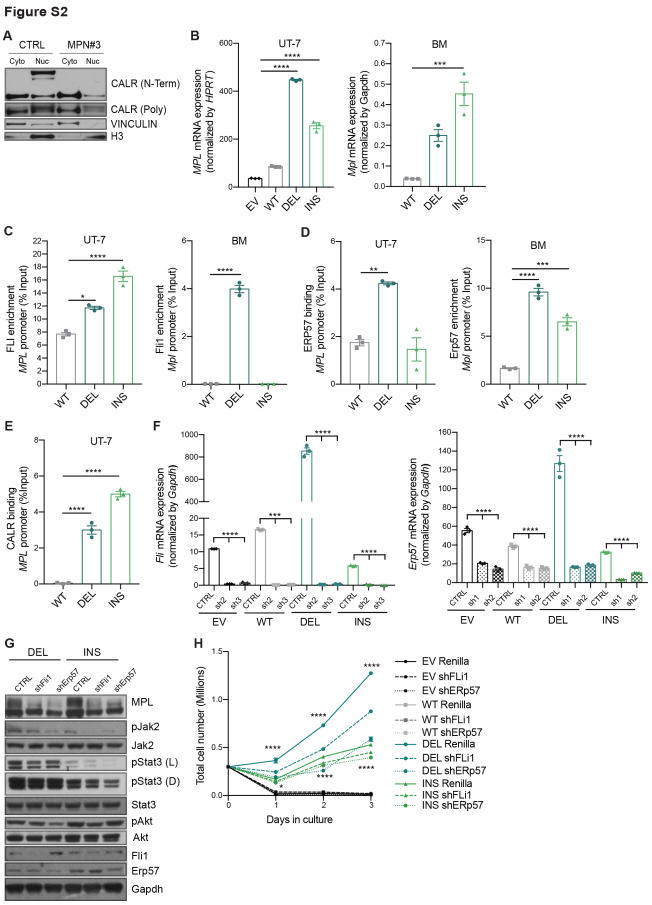
Western blotting. Cells were washed in PBS and resuspended in complemented RIPA lysis buffer. Total protein concentration was obtained by Bio-Rad Bradford assay, separated on 4%–12% Bis-Tris gels (Invitrogen) and blots probed for BIP, MYL9, GRP75, FLI1, ERP57, pJAK2, JAK2, pSTAT3, STAT3, pSTAT5, STAT5, pTYK2, pAKT, AKT, VINCULIN, GAPDH, HISTONE H3, CALR N-term (Cell Stem Technologies, #12238, clone D3E6), MPL/TPO-R (Millipore, #06-944), MBP (NEB, #E8032), CALR Polyclonal (Poly) (abcam, #ab4109) and TYK2 (Santa Cruz). Membrane, cytoplasmic, nuclear and chromatin fractions were prepared using Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific) according to manufacturer's instruction. NIH Image J Software was used to calculate densitometry.

In vitro and in vivo Jak2 inhibitor assays. To assess in *vitro* anti-proliferative effects of inhibitors, all cell lines were cultured at 10.000 cells/200µl with increasing inhibitor concentrations in triplicate. Proliferation was assessed at 48 hours using the Cell viability luminescent assay (Promega) and normalized to cell growth in media with an equivalent volume of DMSO. The concentration inhibiting proliferation by 50% (IC_{50}) was determined with Graph Pad Prism 5.0. Apoptosis induction was quantified by Caspase-3 MAB apoptosis kit (BD) detected by flow cytometry on LSRFortessa (BD). *In vivo*, 6 to 8 weeks old BALB/c female mice were sub lethally irradiated (5.5 Gy) and injected with 10.000 MPL-WT- Ba/F3 cells expressing both *CALR* mutations (DEL and INS). Mice were randomized 7 days after injection according to blood counts to receive vehicle or 30 mg/kg of CHZ868 by gavage for 10-14 days (25). Animal care was in strict compliance with institutional guidelines established by Memorial Sloan Kettering Cancer Center, the Guide for Care and Use of Laboratory animals, and the Association for Assessment and Accreditation of Laboratory Animal Care International and were approved by the Institutional Animal Care and Use Committees at Memorial Sloan Kettering Cancer Center.

Flow cytometry. The following antibodies from BD biosciences were used MPL (APC-conjugated), pSTAT3 (pY705, Alexa Fluor 647-conjugated), pSTAT5 (pY694, phycoerythrin-conjugated) and cleaved caspase-3 (phycoerythrin-conjugated). Cell surfaces were stained for 15–20 min with the appropriate 'cocktail' of fluorescence-labeled antibodies. For intracellular staining, cells were resuspended in Fixation-Permeabilization solution (BD Cytofix/Cytoperm kit; BD pharmingen) and intracellular cytokine staining was done according to the manufacturer's protocol.

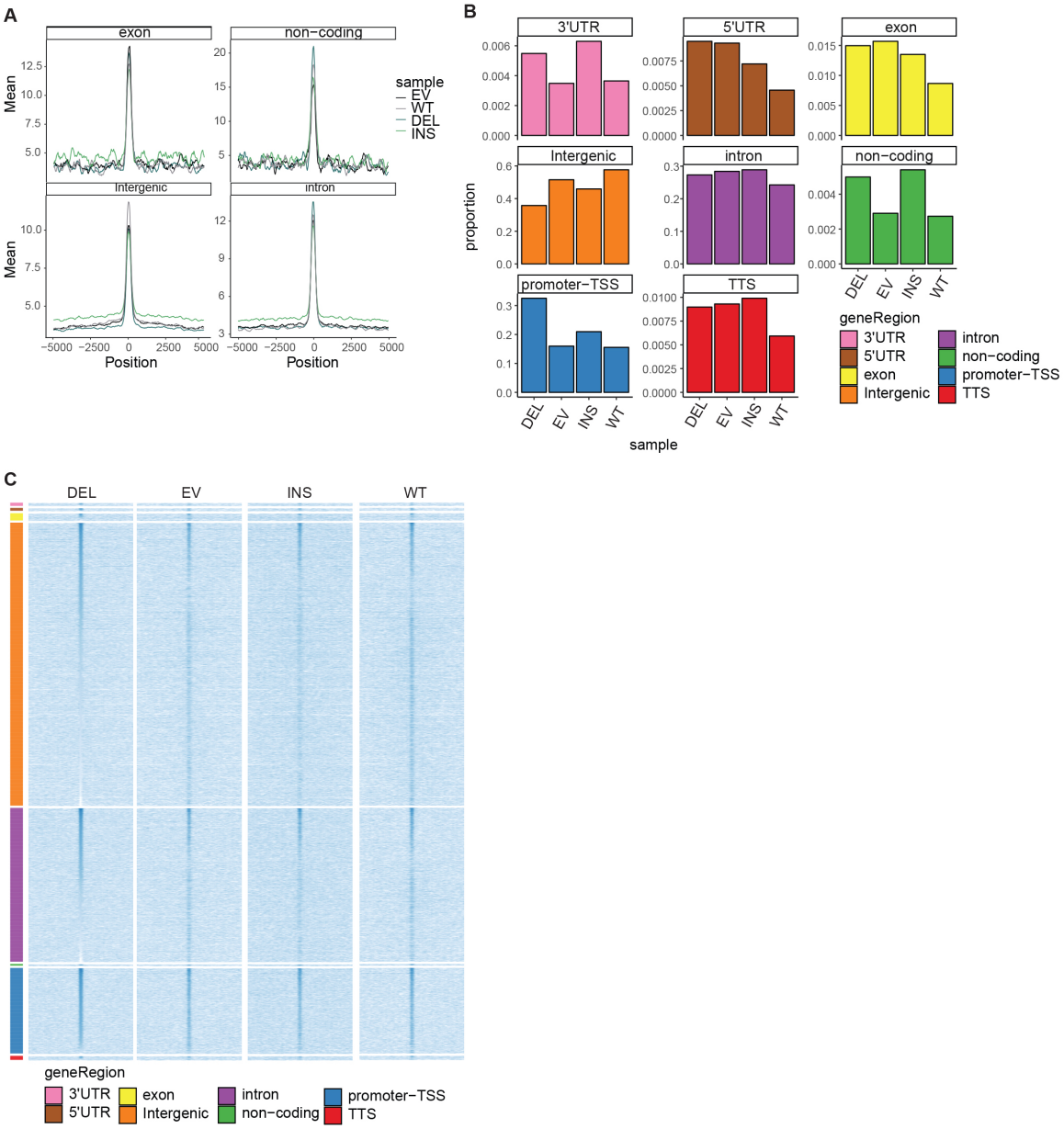


Supplemental Figure 1 Mass spectrometry-based identification of CALR mutant interactomes. (**A**) Schema of CALR WT and mutant retrovirus constructs designed for the study. Volcano plots showing the degrees of enrichment (ratio of label-free quantitation (LFQ) protein intensities) in MPL-WT-Ba/F3 cells expressing STOP (**B**) and IgG control (**C**) compared to WT CALR-expressing cells. The x-axis depicting the fold change in protein levels and the y-axis the -log^{10} p-value. Three independent experiments were performed for each condition and the data were analyzed with MaxQuant. Changes in partner's abundance are shown for each condition. (**D**) Representative silver-stained SDS-PAGE gel used for mass spectometry analysis. (**E**) Endogen CALR (Endo) and Tagged-CALR (MBP) expression level analysis by Western Blot on total protein extracts from MPL-WT-Ba/F3 cells expressing different CALR constructs. (L) and (D) correspond to different exposure times (L= Light, D= Dark). Vinculin was used as a loading control. (**F**) Normalized LFQ intensities of a subset of candidate proteins (Rpl7, Rsp23, Aprc4 and Hspa8) to the MBP LFQ of each condition. (**G**) Immunoprecipitation of CALR were followed by blotting with MPL and JAK2 using total protein extracts from CD34^{+} cells of healthy donors (CTRL) and MPN patients (MPN). (**H**) CALR was immunoprecipitated using a N-terminal antibody, followed by Western Blot analysis of MPL using protein extracts from MPL-WT-Ba/F3 cells. (**I**) Immunoblotting of immunoprecipitated CALR-mutant targets: BiP, Myl9, Fli1 and Erp57 proteins in MPL-WT-Ba/F3 cells stably expressing DEL or INS CALR cultured in media with (+) or without (-) IL-3. In (**F**), mean values ± s.e.m. are represented. Statistical significance was assessed using one-way ANOVA, $^{*}p < 0.05$ and $^{**}p < 0.01$. Blots are representative of three independent experiments.

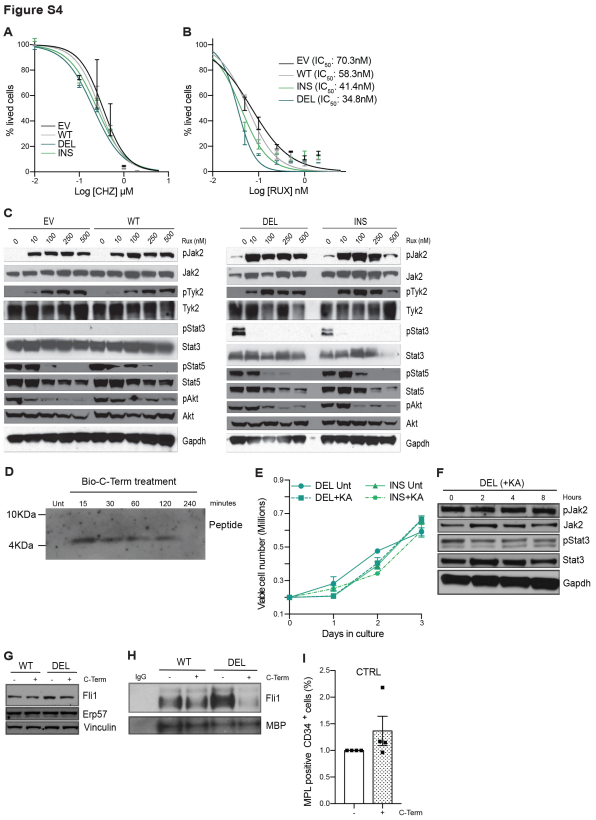


Supplemental Figure 2 CALR mutants binding to a subset of partners affect their cellular localization and influence *MPL* transcription. (**A**) Cytoplasm (Cyto) and nuclear (Nuc) extracts from mononuclear cells isolated from a healthy donor (CTRL) or a *CALR*-mutated MPN patient were used to detect, by Western Blot, the cellular localization of CALR. Two CALR antibodies were used; N-terminal (N-term) and Polyclonal (Poly). (**B**) Quantitative PCR (qPCR) analysis of *MPL* in MPL-WT-UT-7 cells and in c-Kit$^+$ cells isolated from WT mice bone marrow (BM) stably expressing WT, DEL or INS CALR. qPCR analysis of ChIP assays showing FLI1 (**C**) and ERP57 (**D**) binding to the *MPL* promoter in MPL-WT-UT-7 cells and in c-Kit$^+$ cells (BM). (**E**) qPCR analysis of ChIP assays showing CALR binding to the *MPL* promoter in MPL-WT-UT-7 cells. Data are expressed as the percent of pre-IP input for each sample and are representative of at least 3 independent IPs. (**F**) qPCR analysis of *Fli1* (left) and *Erp57* (right) expression in EV, WT, DEL or INS CALR MPL-WT-Ba/F3 cells expressing *Fli1* and *Erp57* shRNA compared to Renilla controls (CTRL). (**G**) Jak/Stat axis analysis by Western Blot of MPL-WT-BA/F3 cells expressing DEL or INS CALR transduced by *Fli1* or *Erp57* shRNA compared to Renilla controls (CTRL). (**H**) Proliferation assays, in absence of IL-3, of MPL-WT-BA/F3 cells transduced by *Fli1* or *Erp57* shRNA compared to Renilla controls (CTRL). Growth curves are means (in total number of viable cells) ± s.e.m (n=6 in triplicate). In (**B**-**F**,**H**), mean values ± s.e.m. are represented. Statistical significance was assessed using one-way ANOVA. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$. Blots are representative of three independent experiments. *HPRT* or *Gapdh* were used as housekeeping genes. Gapdh and HISTONE H3 were used as loading controls.

Figure S3



Supplemental Figure 3 DEL CALR mutant enhance genome-wide Fli1 recruitment to chromatin. (**A**) Fli1 Peaks in exon, intron, non-coding and intergenic regions. (**B**) Plots of Fli1 peaks proportion in different genomic regions: 3'UTR, 5'UTR, exon, intergenic, intron, non-coding, promoter-TSS and TSS regions. (**C**) Heatmap displaying Fli1 enrichment at several genomic regions: 3'UTR, 5'UTR, econ, intergenic, intron, non-coding, promoter-TSS and TSS in EV, WT, DEL and INS-expressing MPL-WT-BaF3 cells. TSS: Transcription start sites.



Supplemental Figure 4 *CALR* mutated cells are sensitive to JAK1/2 inhibitor and to a peptide corresponding to the C-term sequence of the WT CALR. Representative proliferation curves of MPL-WT-Ba/F3 cells stably expressing CALR mutants cultured for 48 hours with increasing concentrations of CHZ868 (CHZ, µM) (**A**) or ruxolitinib (Rux, nM) (**B**). (**C**) Jak-Stat axis analysis, by Western Blot, of MPL-WT-Ba/F3 cells expressing different CALR constructs cultured with increasing concentrations of ruxolitinib (Rux, nM) as compared to negative controls (EV and WT). (**D**) Peptide uptake analysis, by Western Blot, of MPL-WT-Ba/F3 cells expressing DEL CALR treated with the Bio-C-Term peptide for different time points (0 to 240 minutes) followed by streptavidin immunoprecipitation. (**E**) Growth curves of MPL-WT-Ba/F3 cells expressing different CALR mutant constructs treated with the KA mutant peptide in absence of IL-3. Unt: Untreated; +KA: KA mutant peptide. Growth curves are means (in total number of viable cells) ± s.e.m. (n=6 in triplicate). (**F**) Jak-Stat axis analysis, by Western Blot, of MPL-WT-Ba/F3 cells expressing DEL CALR and treated with the KA mutant peptide (+KA) for different incubation times (0-8 hours). (**G**) Western Blot analysis of Fli1 and Erp57 expression in MPL-WT-Ba/F3 cells expressing WT or DEL CALR after peptide treatment for one hour. (**H**) Immunoblotting of immunoprecipitated MBP-tagged CALR and Fli1 in MPL-WT-Ba/F3 cells expressing WT or DEL CALR cultured in media with (+) or without (-) C-Term peptide for one hour. MBP was used as an immunopreciptation efficacy control. (**I**) Quantification of change in MPL positive cells (%) among $CD34^+$ cells isolated from bone marrow of healthy donors (CTRL) treated with the C-Term peptide (+) for one hour compared to untreated cells (-). In (**A**, **B**), percentages (%) of lived cells relative to DMSO treated control cells are represented as mean ± s.e.m (n=3, in triplicate). In (**I**) mean ± s.e.m values are represented. Blots are representative of three independent experiments. Gapdh or vinculin were used as loading controls.

Supplemental Table 1. MPN Patient samples description.

Patient #	Mutation	Mutation type	Prognostic
3	L367fs*46	DEL	MF
4	L367fs*46	DEL	ET
17	L367fs*46	DEL	ET
29	L367fs*46	DEL	ET
30	K385Nfs*47	INS	ET
35	K385Nfs*47	INS	ET vs. prefibrotic MF
54	ins/del exon 9	-	MF
95	L367fs*46	DEL	ET

Supplemental Table 2. List of primers used for ChIP and qRT-PCR experiments.

qPCR	Forward	Reverse
Actin	5'-ggctgtattcccctccatcg-3'	5'- ccagttggtaacaatgccatgt-3'
Gapdh	5'-accacagtccatgccatcac-3'	5'-tccaccaccctgttgctgta-3'
Mpl	5'-taaaccagactcggactcagc-3'	5'-cggagttcg tacctcaggaaa-3'
Jak2	5'-ccttcaggtgaggagatttttgc-3'	5'-tgtacgtcctgttctgtcagtg-3'
Stat3	5'-aggacatcagtggcaagacc-3'	5'-ttggtcttcaggtacggggc-3'
Fli1	5'-cgctacaacaacaaacgtgc-3'	5'-cagagcctccttaatagtcccg-3'
Erp57	5'-atgcctcagtggtgggtttt-3'	5'-gttggtgtgtgcaaatcggt-3'
HPRT	5'-agatggtcaaggtcgcaag-3'	5'-gtattcattatagtcaagggcatatc-3'
MPL	5'-taaaccagactcggactcagc-3'	5'-cggagttcgtacctcaggaaa-3'
FLI1	5'-cccaggaagtggaattgaggc-3'	5'-gtgggaggggttgatcttg-3'
ERP57	5'-gagttctcgcgtgatgggaa-3'	5'-gaccacaccaaggggcataa-3'

ChIP	Forward	Reverse
Mpl	5'-ctgccaacagaaggctcatg-3'	5'-ctgtcagatacagccccacgt-3'
MPL	5'-catcctcccttcaggaagctg-3'	5'-tagcacagatacagaggctgagtt-3'