Effects of Calreticulin Mutations on HLA Class I Expression in Myeloproliferative Neoplasms

Supplementary Methods, Tables, Figures, Figure legends and References

Supplementary Methods

Materials

All laboratory chemicals were purchased from Sigma. Vacutainer® ACD solution A tubes (364606), 7-AAD dye, BD GolgiStop™ (554724) and BD GolgiPlug™ (555029) were obtained from BD. Ficoll® Paque-Plus density gradient media, apyrase, saponin, ACD solution, and protease inhibitor cocktail (P8340) were obtained from Sigma. FlowPRA single antigen HLA beads, anti-Bw6 FITC, and anti-Bw4 FITC were purchased from One Lambda. All cell culture media, FBS, antibiotic-antimycotic, puromycin, geneticin, Puromycin, Blasticidin, Opti-MEM™ I reduced serum media, Recovery™ Cell Culture Freezing Medium, Live-dead fixable Aqua dead cell stain and Sodium Pyruvate were obtained from Gibco (Invitrogen). BCA assay protein estimation kit and Pierce™ ECL western blotting substrate (32106) were obtained from Pierce. QuikChange II XL Site-Directed Mutagenesis Kit was procured from Agilent. The BsmBI enzyme was purchased from Thermo Fisher Scientific. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. Wizard® Plus SV Miniprep kit, GoTaq® Green Master Mix, and FuGENE® HD transfection reagent were purchased from Promega. Lipofectamine™ LTX with Plus™ reagent was procured from Invitrogen. The gel extraction kit, PCR purification kit, DNeasy Blood and Tissue kit, and RNeasy Plus Mini kit were procured from Qiagen. Nucleofection kits were purchased from Lonza. The normal mouse serum (Cat. No. 015-000-001) and normal goat serum (Cat. No. 005-000-121) were purchased from Jackson ImmunoResearch.

sgRNA and gene constructs

The single guide RNA (sgRNA) targeting a sequence 5'-GGCCACAGATGTCGGGACCT-3' located at the boundary of intron 3 and exon 4 of the *CALR* gene was used for knockdown of CRT expression (1). The oligos were procured from IDT after adding the specific adapter sequences (Supplementary Table 4) for cloning at the BsmBI site in pLentiCRISPRv2-blasticidin (pLB) vector.

The sgRNA used for CRISPR knock-in experiments (Supplementary Table 4) targeting intron 7 of the *CALR* gene (2) was purchased from Synthego with chemical modifications (2'-O-Methyl group at 3 first and last bases and 3' phosphorothioate bonds between the first 3 and last 2 bases).

The pMSCV constructs of wild-type human calreticulin, as well as the CRT_{Del52} and CRT_{Ins5} mutants have been described earlier (3). Site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit, Agilent) was used to introduce silent mutations within the sgRNA-binding region of wild-type and mutant *CALR* constructs using primers given in the supplementary table 4. Six silent mutations were introduced in two rounds of mutagenesis using primers C1QF and C1QR in the first round and primers C2QF and C2QR and mutated products from round 1 as templates in the second round of mutagenesis. The PCR was set up following the manufacturer's protocol, followed by treatment with the DpnI enzyme for 1h at 37°C. XL10-Gold Ultracompetent bacterial cells (Agilent) were transformed with the DpnI-digested PCR product following the manufacturer's recommendations.

For reconstitution of CRT expression in *CALR*-KO cells, the sgRNA-resistant versions of human wild-type ($CALR_{WT}$) and mutant ($CALR_{Del52}$ *and* $CALR_{Ins5}$) CRT cDNA, generated as mentioned above, were cloned in the pSIP-*ZsGreen* (pLVX-SFFV-IRES-Puro) (4, 5) vector that was a gift from Dr. Mary Carrington at the Frederick National Laboratory for Cancer Research (Frederick, Maryland). The *CALR* genes were amplified by PCR using primers given in supplementary table 4 and GoTaq® Green Master Mix (Promega). The amplicons and the pSIP-*ZsGreen* (pLVX-SFFV-IRES-Puro) vector were digested with EcoRI and NotI enzymes. The digested vector was subjected to agarose gel electrophoresis and purification using the Qiagen gel extraction kit. The digested *CALR* inserts were purified using Qiagen PCR purification kit. The insert and vector were combined in a molar ratio of 3:1 for ligation using the T4DNA ligase enzyme. The STBL3 competent *E. coli* cells were transformed with the ligation mix, and colonies were screened for the presence of the insert and verified by Sanger Sequencing.

The pMSCV-*neo* constructs of *HLA-B*07:02, HLA-B*08:01, HLA-B*15:01, HLA-B*18:01, HLA-B*35:01, HLA-B*40:01, HLA-B*44:02, HLA-B*44:03, HLA-B*45:01, HLA-B*53:01 and HLA-B*57:01* alleles have been described earlier (6). The pMSCV-*neo* construct used for expressing HA-tagged HLA-A*02:01 was a gift from Dr. Kathleen L. Collins at the University of Michigan and has been described previously (7). For cloning of specific HLA class I allotypes in the pSIP vector for expression in knock-in cell lines, the *HLA-B*07:02*, *HLA-B*08:01*, and *HLA-B*15:01* were amplified using primers given in the supplementary table 4 and subcloned at the EcoRI and BamHI sites in the pSIP-*ZsGreen* (pLVX-SFFV-IRES-Puro) vector. Digestion of the pSIP-*ZsGreen* vector with EcoRI and NotI/BamHI enzymes during the cloning of different *CALR* or *HLA class I* constructs allowed the removal of the *ZsGreen* fragment. The empty pSIP vector without *ZsGreen* was generated by replacing the *ZsGreen* sequence with a PmeI restriction enzyme site using primers given in supplementary table 4. The pSIP-*ZsGreen* vector was digested with EcoRI and BamHI enzymes and ligated to annealed and phosphorylated oligos, followed by transformation of *E. coli* STBL3 cells with the ligation mix.

Construction of homology-dependent repair (HDR) templates for CRISPR knock-in

The HDR templates used for CRISPR knock-in were designed to insert the exon 8 sequence of wild-type *CALR* fused to either wild-type exon 9 or $CALR_{Del52}$ exon 9 sequence at the cut site, as described in a recent study (2). The left homology arm (LHA) consisted of 242 bases of *CALR* sequence upstream of the cut site for Cas9 enzyme within the sgRNA target sequence, and the right homology arm (RHA) extended 400 bases downstream from the cut site. The splice acceptor arm included a sequence identical to the 150 bases upstream of the exon 8 in the wild-type *CALR* gene and was placed after the LHA within the HDR templates. Exon 8 and 9 sequences in the HDR templates were modified compared to the endogenous *CALR* exon 8 and 9 sequences using codon optimization. The wild-type *CALR*-specific knock-in template included a GFP reporter gene,

and the *CALR*$_{Del52}$-specific knock-in template included a BFP reporter gene. The specific reporter gene was added downstream of the *CALR* knock-in fragment sequence and the spleen focus-forming virus (SFFV) promoter. An SV40 polyadenylation sequence was added after the wild-type or mutant exon 9 sequence, and a bovine growth hormone (bGH) polyadenylation sequence was added after the reporter gene sequence. The HDR templates were synthesized by GenScript and subcloned at the BamHI and NotI sites of pAAV-MCS2 vector (Addgene, Plasmid #46954).

AAV generation

Recombinant adeno-associated virus (AAV) packaged with the HDR templates carrying wild-type *CALR* or *CALR*$_{Del52}$-specific knock-in fragments was produced at the University of Michigan Vector Core. Briefly, AAV helper vector pAd-Delta6 (200 µg), serotype vector pAAV2/8 (150 µg), and transfer pAAV-MCS2 vector carrying wild-type or Del52 knock-in HDR template (100 µg) were co-precipitated using standard PEI precipitation methods. PEI precipitation was performed by incubating plasmids with 270 µg PEI (molecular weight 2500, Polysciences, Inc.) in 60 mL Opti-MEM™ I reduced serum media (Life Technologies) at room temp for 15 min, before adding to fresh 60 mL DMEM, 10% FBS media. This DNA/PEI-containing media was then distributed equally to three T300 flasks (Falcon) containing HEK293T cells. Supernatant was collected at 72 h and frozen. Fresh DMEM with 5% FBS was then added, and both supernatant and cells were collected 48 h later. Cells were pelleted out and resuspended in 10 mL of Buffer A (150 mM NaCl2, 1 mM MgCl2, 50 mM Tris pH 8.5), frozen at -80°C for 20 min, thawed, and sonicated to release AAV particles. Following sonication, cell debris was pelleted out, and the supernatant was combined with the previously collected supernatant. AAV was then precipitated and pelleted out using PEG 8000 (Fisher). The pellet was resuspended in 10

mL Buffer A. The resuspended AAV was run through an iodixanol gradient (60%, 40%, 25%, and 15%). The 40% fraction was collected and applied to a 4 mL, 100kDa MWCO Amicon centrifugal filter and washed with PBS to remove iodixanol and concentrate the virus. Titers were assessed by qPCR using the protocol published by Addgene (https://www.addgene.org/protocols/aav-titration-qpcr-using-sybr-green-technology/).

Variant allele frequency analysis

The wild-type or mutant *CALR*-specific exon 9 fragments were amplified using the primers given in the supplementary table 4, and genomic DNA isolated from MPN patient PBMC as template. The amplified fragments were purified by Qiagen PCR purification kit (Catalog #28106). The purified amplicons were submitted to Genewiz (Azenta Life Sciences) for variant allele frequency analysis using the Amplicon-EZ next-generation sequencing service.

FlowPRA screening of BB7.2 binding specificities

The HLA class I-binding specificities of the BB7.2 antibody were checked by using the FlowPRA single antigen HLA class I antibody detection test (One Lambda). We checked for binding to beads coated with antigens derived from common HLA-A, B, and C alleles, including FlowPRA single antigen beads Groups 1,2,3,4,6,9, and 10. The beads were vortexed, and 5 µL of beads were incubated with 20 µL of the BB7.2-PE/Cy7 antibody at a 1:200 dilution for 30 minutes at room temperature, followed by two washes with the 1X dilution of wash buffer (included in the kit) in water. The beads were resuspended in 1X PBS and analyzed by flow cytometry. Each group contains 9 bead populations that exhibit different levels of fluorescence in the PE channel. Additionally, each group includes negative control beads without antigens that can be used to set the threshold for negative binding.

qRT-PCR

RNA was isolated from cells using the Qiagen RNeasy® Plus Mini kit (Catalog #74134) following the manufacturer's protocol. RNA was quantified by nanodrop, and the first strand of cDNA was synthesized from equal amounts of each RNA sample (~1 µg) using the SuperScript™III First-Strand synthesis kit (Invitrogen; Catalog #18080051) with Oligo-dT primers, followed by digestion with RNase H enzyme for 20 minutes at 37°C. The cDNA was either used immediately or stored at -80°C. For qPCR, 1 µL of cDNA was mixed with the SYBR™ Green PCR master mix (1X) (Applied Biosystems, catalog #4309155) and 500 nM each of forward and reverse primers targeted against the wild-type *CALR* exon 9 (Supplementary Table 4) or the housekeeping genes. The qPCR was performed in an Applied Biosystems 7500 Fast Real-Time PCR machine. The primers for human GAPDH (catalog #VHPS-3541), human beta actin (catalog #VHPS-4263), and human hypoxanthine phosphoribosyltransferase 1 (HPRT1) (catalog #VHPS-3541) housekeeping genes were procured from Real Time Primers, LLC.

S.No	Healthy donor/ Patient ID	Sex	Mutation/ Healthy	Clinical Treatment	Disease, lab VAF or clinical VAF	Race/Ethnicity	W6/32 ABC platelets	W6/32 ABC classical monocytes	BB7.2 ABC platelets	BB7.2 ABC classical Monocytes	Bw4 ABC CD14^{+} HLA-DR^{+} Monocytes
1	***CALR*7438**	F	*CALR* (Ins5)	Pegylated IFN	ET 28.56	Caucasian		2374926.554			
2	***CALR*8178(A) *CALR*8178(B)**	F	*CALR* (Ins5)	Hydroxyurea & Pegylated IFN	ET 9.02 (B)	Caucasian	40958.079 (B)	1915640.437 (A) 2995756.220 (B)			
3	***CALR*4401**	M	*CALR* (Ins5)	Pegylated IFN	ET 4.69	Caucasian	92288.011	3547358.41			
4	***CALR*4725**	F	*CALR* (Del52)	Pegylated IFN & Aspirin	ET 1.13	African American	34677.659	1463075.204			
5	***CALR*9845**	M	*CALR* (Ins5)	Hydroxyurea & Xarelto	ET 29.13	Caucasian		1846640.970			
6	***CALR*7557**	M	*CALR* (Del52)	Hydroxyurea	ET 0.72	Caucasian		2150330.301		496071.733	
7	***CALR*2288**	F	*CALR* (Del52)	Hydroxyurea & Aspirin	ET 31.2#	Caucasian	26777.506		15425.147		
8	***CALR*7471**	F	*CALR* (Ins5)	Hydroxyurea & Plavix	Pre-PMF 76.2#	Caucasian	15121.281	872521.439			10917.582
9	***CALR*8251**	F	*CALR* (Ins5)	Ruxolitinib & Hydroxyurea	Post ET-MF 91.3	Caucasian	17539.712	896655.371			
10	***CALR*7610**	F	*CALR* (Ins5)	Ruxolitinib	Post ET-MF 21.45	Caucasian		886639.456			
11	***CALR*9342**	M	*CALR* (Del52)	Ruxolitinib	Post ET-MF 33.9#	Caucasian	22624.090	1450642.144			
12	***CALR*1983**	M	*CALR* (Del34)	Ruxolitinib	Post ET-MF 31.3	Caucasian	22312.841	628808.804			
13	***CALR*0266**	F	*CALR* (Del52)	LSD1inhibitor	Post ET-MF 1.22	Caucasian	51689.279	1545874.332	6402.392	200528.520	42976.301
14	***CALR*0387**	M	*CALR* (Del52)	Danazol	PMF 1.62	Caucasian	61213.889	1169475.406			33158.296
15	***CALR*2077**	F	*CALR* (Del52)	PRBC transfusion	PMF 0.82	Caucasian	27455.839	1439724.413	7137.012	211752.949	
16	***CALR*5628**	M	*CALR* (Del52)	Aspirin	PMF 13.5#	Caucasian	23087.115				
17	***JAK*6440**	F	*JAK2* V617F	Aspirin & Ruxolitinib	PV	Caucasian	28881.544	692761.752			20020.856
18	***JAK*0621**	M	*JAK2* V617F	Hydroxyurea & Aspirin	PV	Other	17759.310	***482450.924***¶			
19	***JAK*0196**	M	*JAK2* V617F	Hydroxyurea & Aspirin	PV	Caucasian	43247.766	1713080.441			
20	***JAK*1761**	F	*JAK2* V617F	Aspirin	PV	Caucasian	16035.165	2045781.240	11709.179	444087.406	
21	***JAK*5092**	F	*JAK2* V617F	Fedratinib	Post-PV MF	Caucasian	23010.722	***2032950.519***¶			
22	***JAK*0922**	M	*JAK2* V617F	Ruxolitinib	PMF	Caucasian	11963.067	1143270.398	14454.868	615816.897	
23	***MPL*8524**	M	*MPL*	BET inhibitor	PMF	Caucasian	63881.640	1032644.214			

			W515K	2018-090							
24	**HD1001**	F	Healthy			Not known		1555580.447			
25	**HD1002**	M	Healthy			Not known		1336275.326			
26	**HD255**	F	Healthy			Caucasian		1052015.857			
27	**HD1003**	M	Healthy			Not known		1478268.031	9975.116	182813.552	
28	**HD1004**	M	Healthy			Not known	63485.073	1186217.548			
29	**HD1005**	F	Healthy			Not known	83400.905	1876420.004			
30	**HD1006**	F	Healthy			Not known	56722.238	1854469.143			
31	**HD237**	M	Healthy			Caucasian	30797.939 (A) 73674.249 (B)	1576273.352 (A) 1568532.596 (B)			19237.292 (A) 11043.741 (B)
32	**HD259**	F	Healthy			Asian	34666.741 (A) 23119.198 (B) 40634.265 (C) 38199.731 (D)	***1293488.098***¶ (A) 1517286.667 (B) 863553.733 (C) 1399483.013 (D)			
33	**HD258**	F	Healthy			Asian	52911.981 (A)	1527245.391 (A) 2943547.538 (B)			
34	**HD171**	F	Healthy			Caucasian	46650.580	1154325.872	18289.526	238900.436	
35	**HD118**	F	Healthy			Caucasian	38754.923	1173267.481	9258.154	354588.409	
36	**HD24**	F	Healthy			Caucasian	24285.677	1116187.333	4759.248	118612.320	
37	**HD14**	F	Healthy			Caucasian	22905.313	2164293.696	7199.235	645976.953	
38	**HD64**	F	Healthy			Caucasian					17216
39	**HD94**	F	Healthy			Caucasian					15338
40	**HD141**	M	Healthy			Caucasian					12642
41	**HD187**	F	Healthy			Caucasian					9367

Supplementary Table 1: HLA class I ABC values obtained with the indicated antibodies for MPN patients and healthy donors (HD) along with details of their sex, ethnicity, and for MPN patients, the disease, mutation type, mutational burden (or variant allele frequency (VAF)), and treatment. The *CALR* VAF values were measured using genomic DNA isolated from PBMC or obtained for some patient samples as part of the clinical information (indicated by **#**). VAF values were measured in the lab using the same PBMC samples as those used for surface HLA class I measurements except for *CALR*8251, for which the VAF was measured with a blood sample collected six years and 11 months prior to the sample collection for surface HLA class I measurements. Clinical VAF values were measured using peripheral blood 1 year 8 months before surface HLA measurements in *CALR*2288 PBMC, 3 months prior to surface HLA measurements in *CALR*7471 PBMC, 4 years 5 months before surface HLA measurements in *CALR*5628 PBMC, and 1 year 8 months after surface HLA class I measurements in *CALR*9342 PBMC. ABC values derived from surface staining of platelets and monocytes with W6/32, BB7.2 or anti-Bw4 antibodies are shown. Multiple collections from same patient or healthy donor are indicated by an alphabet within parentheses added to the VAF and ABC values. The Bw4 ABC values for $CD14^+$ $HLA\text{-}DR^+$ monocytes for healthy donors HD64, HD94, HD141 and HD187 were obtained from an earlier study (8). The ABC values italicized and bold-faced (indicated by **¶**) were excluded because the number of events within the relevant gate was <100. Healthy donor sample 253 (female, Asian) and *CALR*1596 patient sample (female, Caucasian, PMF patient with Del52 mutation, prescribed Ruxolitinib treatment) were included in immunoblots of platelet lysates, but not for surface HLA class I measurements. *CALR*8251 and *CALR*7471 with VAF>50 are highlighted in green. F, Female; M, Male; BET, Bromodomain and extraterminal protein; IFN, Interferon; PRBC, Packed Red Blood Cell; LSD1, Lysine-specific demethylase 1

S.No	Healthy donor/ Patient ID	HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-C1	HLA-C2
1	***CALR*7438**	A*24:02:01:01	A*03:01:01:01	B*07:02:01:01	B*07:02:01:66	C*07:02:01:03	C*07:02:01:03
2	***CALR*8178**	A*01:01:01:01	A*02:02:01:01	B*55:01:01:01	B*41:01:01:01	C*03:03:01:01	C*17:01:01:05
3	***CALR*4401**	A*02:01:01:01	A*03:02:01:01	B*18:01:01:02	B*49:01:01:01	C*07:01:01:16	C*07:01:01:12
4	***CALR*4725**	A*24:02:01:01	A*68:02:01:01	B*44:03:01:19	B*40:01:02:01	C*04:01:01:92	C*03:04:01:02
5	***CALR*9845**	A*01:01:01:01	A*01:01:01:01	B*07:02:01:01	B*35:02:01:02	C*07:02:01:03	C*06:02:01:01
6	***CALR*7557**	A*01:01	A*02:01	B*40:01	B*57:01	C*03:04	C*06:02
7	***CALR*2288**	A*01:01	A*02:01	B*14:01	B*57:01	C*06:02	C*08:02
8	***CALR*7471**	A*11:01:01:01	A*29:02:01:01	B*14:02:01:01	B*44:02:01:01	C*08:02:01:01	C*05:01:01:02
9	***CALR*8251**	A*24:02:01:01	A*25:01:01:01	B*08:01:01:01	B*18:01:01:05	C*07:01:01:01	C*12:03:01:01
10	***CALR*7610**	A*02:01:01:01	A*25:01:01:01	B*44:02:01:01	B*18:01:01:05	C*05:01:01:02	C*12:03:01:01
11	***CALR*9342**	A*03:01:01:01	A*23:01:01:01	B*07:02:01:01	B*44:03:01:19	C*07:02:01:03	C*04:01:01:14
12	***CALR*1983**	A*26:01:01:01	A*01:01:01:01	B*07:02:01:01	B*08:01:01:01	C*07:02:01:03	C*07:01:01:16
13	***CALR*0266**	A*02:01:01:01	A*03:01:01:01	B*44:02:01:01	B*44:03:01:19	C*07:04:01:03	C*02:02:02:01
14	***CALR*0387**	A*01:01:01:01	A*29:02:01:01	B*08:01:01:01	B*44:03:01:01	C*07:01:01:01	C*16:01:01:01
15	***CALR*2077**	A*02:01:01:01	A*03:01:01:01	B*27:05:02:05	B*07:02:01:01	C*02:02:02:01	C*07:02:01:03
16	***CALR*5628**	A*02:01	A*02:35	B*07:02	B*44:27	C*07:02	C*07:04
17	***CALR*1596**	A*26:08:01:01	A*03:01:01:01	B*40:01:02:04	B*07:02:01:01	C*03:04:01:01	C*07:02:01:03
18	***JAK*6440**	A*31:01:02:01	A*02:01:01:01	B*44:03:01:19	B*44:02:01:01	C*04:01:01:14	C*05:01:01:25
19	***JAK*0621**	A*02:06:01:01	A*23:01:01:01	B*27:05:02:11	B*44:03:01:19	C*02:02:02:01	C*04:01:01:14
20	***JAK*0196**	A*34:02:01:01	A*02:01:01:01	B*14:01:01:03	B*15:01:01:01	C*08:02:01:02	C*03:04:01:01
21	***JAK*1761**	A*02:01:01:01	A*11:01:01:01	B*51:01:01:65	B*41:02:01:05	C*02:02:02:01	C*17:03:01:01
22	***JAK*5092**	A*01:01:01:01	A*01:01:01:01	B*08:01:01:01	B*57:01:01:01	C*07:01:01:01	C*07:01:01:01
23	***JAK*0922**	A*02:01:01:01	A*02:01:01:01	B*44:03:01:01	B*13:02:01:01	C*16:01:01:01	C*06:02:01:01
24	***MPL*8524**	A*03:01:01:01	A*01:01:01:01	B*07:02:01:01	B*15:01:01:01	C*07:02:01:03	C*03:03:01:01
25	**HD1001**	Not genotyped					
26	**HD1002**	A*02:01:01:01	A*11:01:01:01	B*15:01:01:06	B*35:01:01:05	C*01:02:01:01	C*04:01:01:05
27	**HD253**	A*02:06:01:01	A*02:01:01:01	B*35:01:01:51	B*07:02:01:01	C*03:03:01:01	C*07:02:01:03
27	**HD255**	A*02:01:01:01	A*02:01:01:01	B*40:01:02:01	B*13:02:01:01	C*03:04:01:01	C*06:02:01:01
28	**HD1003**	A*02:01:01:01	A*24:02:01:01	B*44:02:01:01	B*57:02:01:01	C*05:01:01:02	C*18:02:01:01
29	**HD1004**	Not genotyped					

30	**HD1005**	Not genotyped					
31	**HD1006**	Not genotyped					
32	**HD237**	A*68:01:01:02	A*02:01:01:01	B*08:01:01	B*44:02:01:01	C*07:01:01:01	C*05:01:01:02
33	**HD259**	A*02:06:01:01	A*01:01:01:01	B*58:01:01:03	B*27:05:02:05	C*03:02:02:05	C*02:02:02:01
34	**HD258**	A*11:01:01:01	A*24:02:01:01	B*15:02:01:01	B*27:07:01	C*08:01:01:01	C*15:02:01:01
35	**HD171**	A*02:01:01:01	A*32:01:01	B*53:01:01	B*27:05:02	C*04:01:01:01	C*01:02:01e1
36	**HD118**	A*02:01:01:01	A*01:01:01:01	B*15:01:01:01	B*08:01:01	C*03:03:01e1	C*07:01:01:01
37	**HD24**	A*02:01:01:01	A*24:02:01:01	B*51:01:01:01	B*35:01:01:02	C*15:02:01:01	C*04:04:01
38	**HD14**	A*02:01:01:01	A*03:01:01:01	B*51:01:01:01	B*07:02:01	C*15:02:01:01	C*07:02:01:03
39	**HD64**	A*03:01:01:01	A*02:01:01:01	B*07:02:01	B*44:02:01:01	C*07:02:01:03	C*05:01:01:02
40	**HD94**	A*68:01:02:01	A*01:01:01:01	B*44:02:01:01	B*08:01:01e1	C*05:01:01:02	C*07:01:01:01
41	**HD141**	A*02:01:01:01	A*03:01:01:01	B*44:02:01:01	B*35:01:01:02	C*05:01:01:02	C*04:01:01:01
42	**HD187**	A*02:01:01:01	A*01:01:01:01	B*44:02:01:01	B*35:01:01:02	C*05:01:01:02	C*04:01:01:05

Supplementary Table 2: HLA class I genotypes of MPN patient and healthy donor samples used for surface HLA class I measurements: The table provides full HLA class I genotypes of MPN patients with *CALR, JAK2* and *MPL* mutations as indicated and healthy donors (HD). HLA-B*44:02^{+} or HLA-B*44:03^{+} samples used for anti-Bw4-based ABC measurements are highlighted in green and HLA-A*02:01^{+} samples included in the BB7.2-based ABC measurements are highlighted in cyan. Samples for which HLA genotype was not known are indicated as not genotyped.

HLA class I allele	NMDP_European (1,242,890)	UofM Healthy (223)	UofM-*CALR*-MPN, this study (16)	Gigoux *et al*. *CALR*-MPN NEUS (25)	Gigoux *et al.* *CALR*-MPN Danish (29)	Gigoux *et al*. *JAK2*-MPN NEUS (105)	Gigoux *et al.* *JAK2*-MPN Danish (149)
A*01:01	0.1646	0.1734	0.2188	0.1000	0.1724	0.1435	0.1678
A*02:01	0.2755	0.2838	0.2188	0.2340	0.2759	0.2098	0.2852
A*02:02	0.0009	0.0000	0.0313	0.0213	0.0000	0.0049	0.0000
A*02:35	0.0000	0.0000	0.0313	0.0000	0.0000	0.0000	0.0000
A*03:01	0.1399	0.1329	0.1563	0.1600	0.1897	0.1053	0.1846
A*03:02	0.0024	0.0045	0.0313	0.0000	0.0000	0.0048	0.0000
A*11:01	0.0609	0.0495	0.0313	0.0400	0.0172	0.0670	0.0638
A*23:01	0.0197	0.0180	0.0313	0.0800	0.0172	0.0239	0.0134
A*24:02	0.0846	0.0833	0.0625	0.1000	0.1207	0.0718	0.0671
A*25:01	0.0210	0.0248	0.0625	0.0000	0.0345	0.0239	0.0268
A*26:01	0.0309	0.0383	0.0313	0.0200	0.0517	0.0478	0.0101
A*26:08	0.0010	0.0023	0.0313	0.0000	0.0000	0.0000	0.0000
A*29:02	0.0353	0.0293	0.0625	0.0200	0.0000	0.0574	0.0470
B*07:02	0.1306	0.1592	0.2500	0.0800	0.1207	0.0857	0.1678
B*08:01	0.1144	0.1300	0.0938	0.0600	0.1034	0.1238	0.1242
B*14:01	0.0102	0.0112	0.0313	0.0000	0.0172	0.0143	0.0067
B*14:02	0.0286	0.0179	0.0313	0.0200	0.0000	0.0238	0.0168
B*18:01	0.0443	0.0426	0.0938	0.0000	0.0172	0.0333	0.0470
B*27:05	0.0373	0.0448	0.0313	0.0400	0.0517	0.0286	0.0436
B*35:02	0.0102	0.0090	0.0313	0.0400	0.0000	0.0144	0.0034
B*40:01	0.0528	0.0583	0.0625	0.0200	0.0862	0.0238	0.0403
B*41:01	0.0043	0.0067	0.0313	0.0000	0.0172	0.0095	0.0034
B*44:02	0.0952	0.0695	0.0938	0.0625	0.0517	0.0909	0.0872
B*44:03	0.0467	0.0381	0.0938	0.0417	0.0345	0.0574	0.0403
B*44:27	0.0000	0.0045	0.0313	0.0000	0.0000	0.0000	0.0000
B*49:01	0.0158	0.0112	0.0313	0.0000	0.0172	0.0048	0.0067
B*55:01	0.0186	0.0224	0.0313	0.0400	0.0172	0.0095	0.0101
B*57:01	0.0365	0.0336	0.0625	0.0200	0.0172	0.0143	0.0336
C*02:02	0.0435	0.0432	0.0625	0.0851	0.0690	0.0340	0.0570

C*03:03	0.0534	0.0455	0.0313	0.0000	0.0690	0.0146	0.0872
C*03:04	0.0749	0.0818	0.0625	0.0652	0.1379	0.0341	0.0906
C*04:01	0.1059	0.0841	0.0313	0.0851	0.1552	0.1456	0.0839
C*05:01	0.0939	0.0636	0.0625	0.0426	0.0690	0.0825	0.0570
C*06:02	0.0932	0.0932	0.0938	0.0638	0.0690	0.0922	0.0906
C*07:01	0.1600	0.1614	0.1563	0.0870	0.1207	0.1634	0.1409
C*07:02	0.1413	0.1614	0.2500	0.0435	0.1552	0.0990	0.1913
C*07:04	0.0154	0.0182	0.0625	0.0435	0.0000	0.0149	0.0302
C*08:02	0.0385	0.0273	0.0625	0.0213	0.0172	0.0340	0.0235
C*12:03	0.0486	0.0568	0.0625	0.0426	0.0690	0.0825	0.0369
C*16:01	0.0338	0.0205	0.0313	0.0426	0.0000	0.0437	0.0336
C*17:01	0.0088	0.0045	0.0313	0.0000	0.0000	0.0194	0.0034

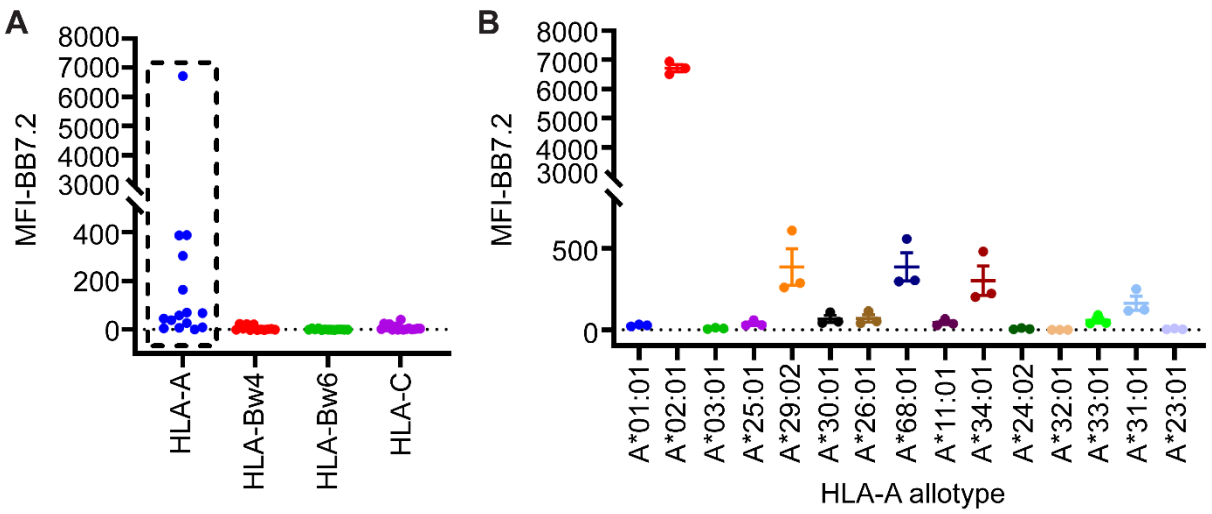
Supplementary table 3: Comparison of HLA class I allele frequencies in healthy donors and MPN patients. Frequencies of HLA class I alleles present in Caucasian donors from a University of Michigan healthy donor cohort (UofM) (n=223) (8), *CALR*-MPN patients from this study (n=16) and *CALR*-MPN and *JAK2*-MPN patients from the previously decribed Northeastern United States (NEUS) (*CALR*, n=25; *JAK2*, n=105) and Danish (*CALR*, n=29; *JAK2*, n=49) cohorts (9). Frequencies of HLA class I alleles in the USA National Marrow Donor Program (NMDP) European Caucasian population (Allele frequency net database) (n=1,242,890) (10) are also shown.

S.No	Name	Sequence (5' to 3')	Used for	References
1	*CALR* sgRNA	GGCCACAGATGTCGGGACCT	CRISPR/Cas9 based *CALR*-knockout	Previously published (1)
2	pLB-*CALR* sgRNA	FP: 5'-***CACCG***GGCCACAGATGTCGGGACCT-3'	Cloning of *CALR*-sgRNA in pLentiCRISPRv2-BLAST (pLB) vector; bases shown in bold italics correspond to the adapter sequences added for cloning	Previously published (1)
		RP: 5-***AAAC***AGGTCCCGACATCTGTGGCC***C***-3'		
3	pSIP Vector minus *ZsGreen*	FP (**PmeI**)- AATTCccg**GTTTAAAC**gccG	Replacement of the *ZsGreen* fragment of the pSIP-*ZsGreen* vector with the PmeI sequence	This study
		RP (**PmeI**)- Gggc**CAAATTTG**cggCCTAG		
4	pSIP-$CALR_{WT}$	FP (**EcoRI**)- CGAA**GAATTC**GCCGCCACCATGCTGCTATCCG	Cloning of $CALR_{WT}$ cDNA in pSIP vector	This study
		RP (**NotI**)- GCATTATT**GCGGCCGC**CTACAGCTCGTCCTTGGC		
5	pSIP-$CALR_{Del52}$ or $CALR_{Ins5}$	FP (**EcoRI**)- CGAA**GAATTC**GCCGCCACCATGCTGCTATCCG	Cloning of $CALR_{Del52}$ or $CALR_{Ins5}$ cDNA in pSIP vector	This study
		RP (**NotI**)- GCATTATT**GCGGCCGC**TCAGGCCTCAGTCCAGCC		
6	pSIP-*HLA-B*15:01*	FP (**EcoRI**)- CGAA**GAATTC**GCCGCCACCATGCGGGTCACGG	Cloning of *HLA-B*15:01* into pSIP vector	This study
		RP (**BamHI**)- CCAGCCG**GGATCC**GATCAAGCTGTGAGAGAC		
7	pSIP-*HLA-B*07:02*; pSIP-*HLA-B*08:01*	FP (**EcoRI**)- CGAA**GAATTC**GCCGCCACCATGCTGGTCATGG	Cloning of *HLA-B*08:01* and *HLA-B*07:02* into pSIP vector	This study
		RP (**BamHI**)- CCAGCCG**GGATCC**GATCAAGCTGTGAGAGAC		
9	Site-directed mutagenesis (SDM) of pSIP-$CALR_{WT}$, $CALR_{Del52}$ or $CALR_{Ins5}$	C1QF (FP)- ATACAACATCATGTTTGGGCCTGATATCTGTGGCCCTGGCACC	SDM to make pSIP-$CALR_{WT}$, $CALR_{Del52}$ or $CALR_{Ins5}$ constructs sgRNA resistant	This study
		C1QR (RP)- GGTGCCAGGGCCACAGATATCAGGCCCAAACATGATGTTGTAT		
		C2QF (FP)- CAACATCATGTTTGGGCCTGATATATGCGGACCTGGCACCAAGA		
		C2QR (RP)- TCTTGGTGCCAGGTCCGCATATATCAGGCCCAAACATGATGTTG		
10	sgRNA for CRISPR knock-in	CGCCTGTAATCCTCGCCCAG	CRISPR Knock-in after complexing with Cas9 to make Cas9-RNP	Previously published (2)

11	Primers for PCR-based confirmation of knock-in	Common FP- AAGTGATCCGTTCGCCATGACCTCCC	Verification of the knock-in of wild-type *CALR* or $CALR_{Del52}$-specific exon 8,9 sequence at the endogenous *CALR* locus	This study
		Endo RP- CTACAGCTCGTCCTTGGCCTGGCCG		
		WT-KI RP- CTAGAGTTCGTCTTTTGCTTGCCCGGGTAC		
		Del52-KI RP- CGACAGCTAGTTCTGGGCCTCGCCGG		
12	Primers for RT-qPCR	FP- GAAGACAAGAAACGCAAAGAGG RP- GGACATCTTCCTCCTCATCTTC	RT-qPCR-based measurement of wild-type *CALR* mRNA expression	This study
13	Primers for mutant *CALR* fragment amplification	FP- GGCAAGGCCCTGAGGTGT RP- GGCCTCAGTCCAGCCCTG	Amplification of mutant *CALR* fragments using genomic DNA from patient PBMC for VAF analysis	Previously published (11)

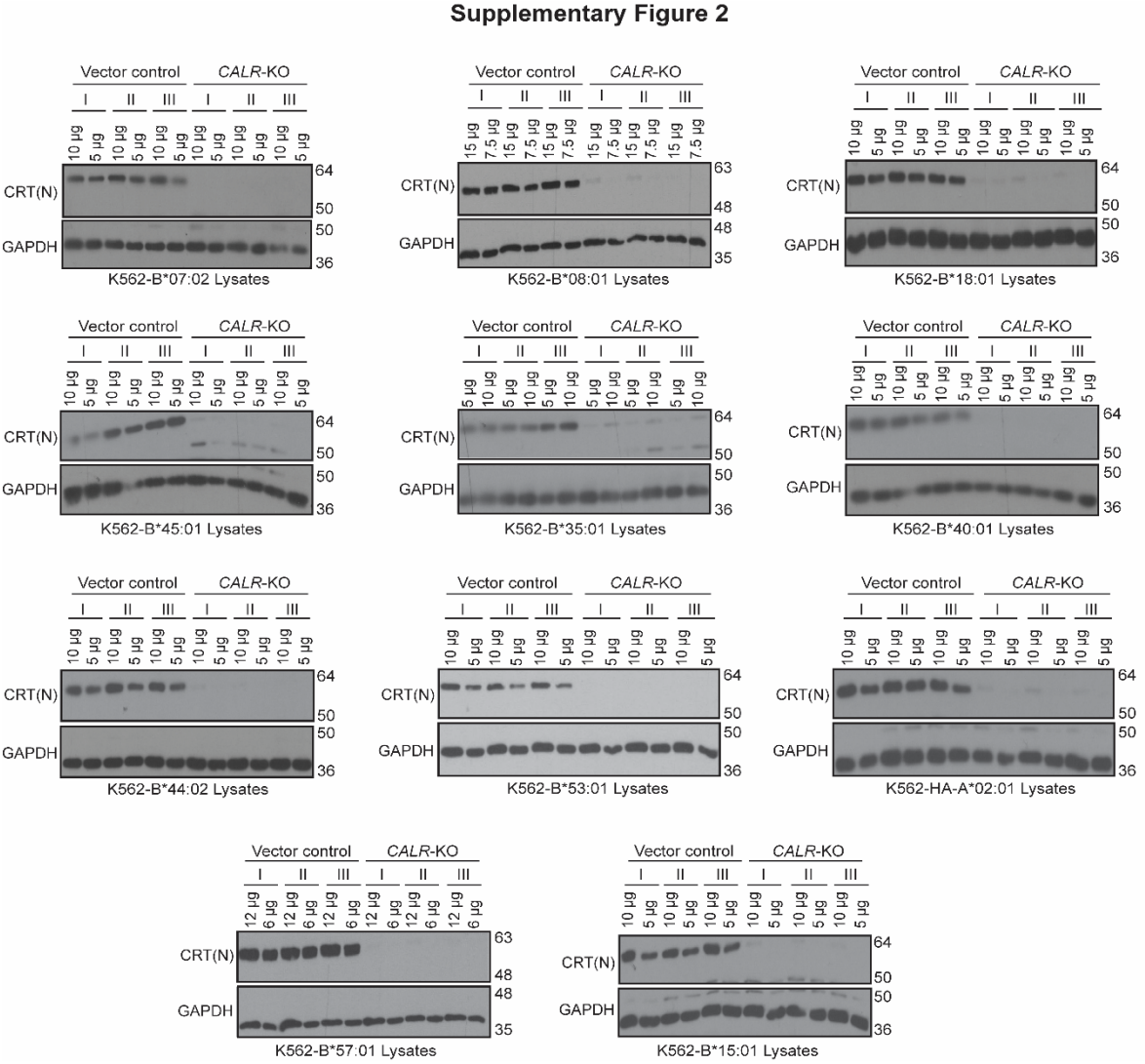
Supplementary Table 4: Sequences of primers, oligos and sgRNAs: Restriction enzyme recognition sites and adapters in sgRNA cloning oligos are shown in bold, and Kozak sequences are underlined. FP, Forward Primer; RP, Reverse Primer

Supplementary Figure 1

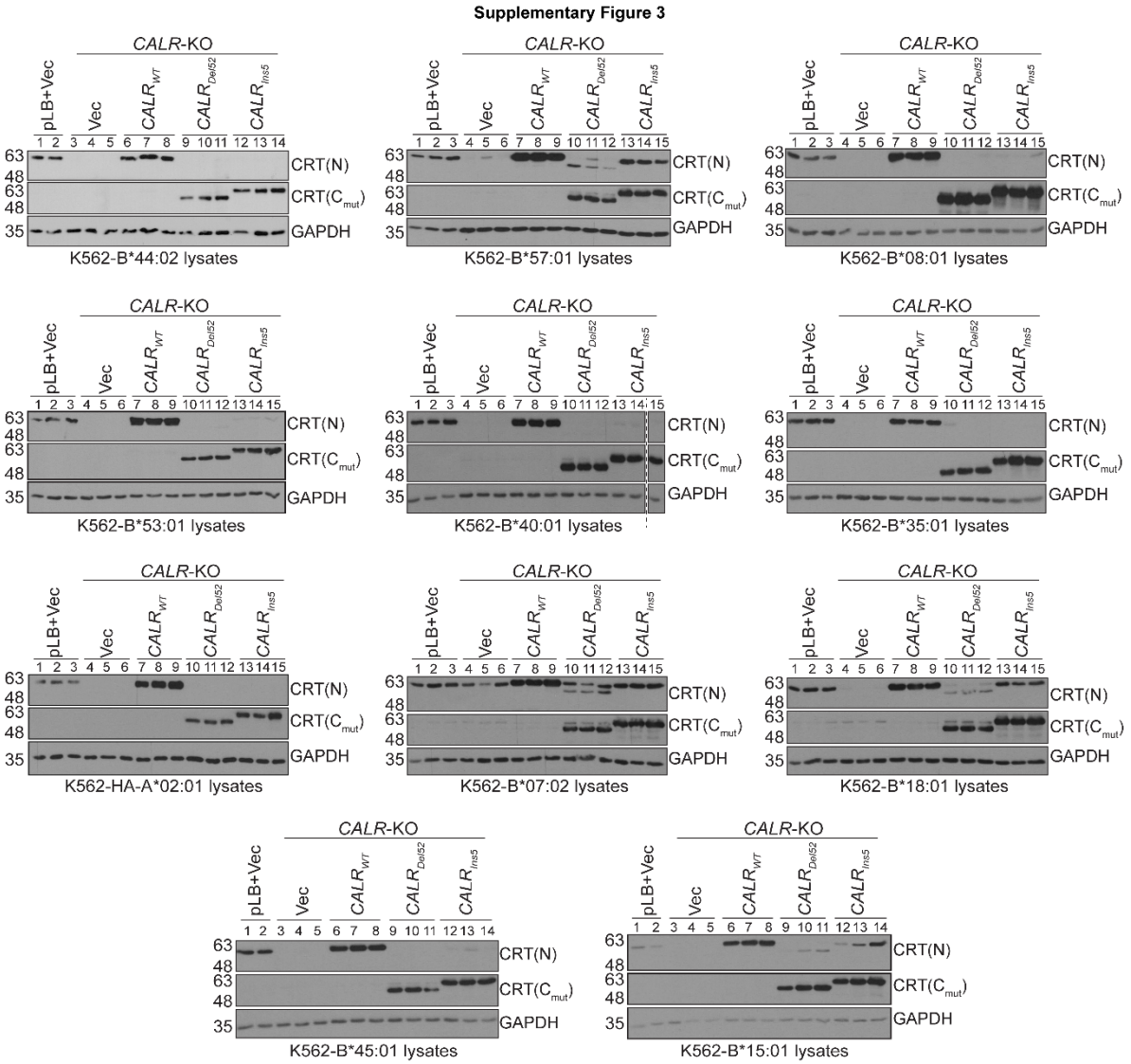


Supplementary Figure 1: Specificity screening of BB7.2 antibody using FlowPRA™ single antigen beads. (A) FlowPRA™ single antigen HLA class I antibody detection tests (One lambda) were used to test the binding specificities of BB7.2 antibody to groups of beads coated with purified HLA class I single or fractionated antigens as indicated. **(B)** Screening of BB7.2 antibody binding to beads coated with individual HLA-A antigens. Besides strong binding specificity for HLA-A*02:01, the BB7.2 antibody was also observed to show weaker non-specific binding to HLA-A*29:02, HLA-A*31:01, HLA-A*34:01 and HLA-A*68:01.

Supplementary Figure 2



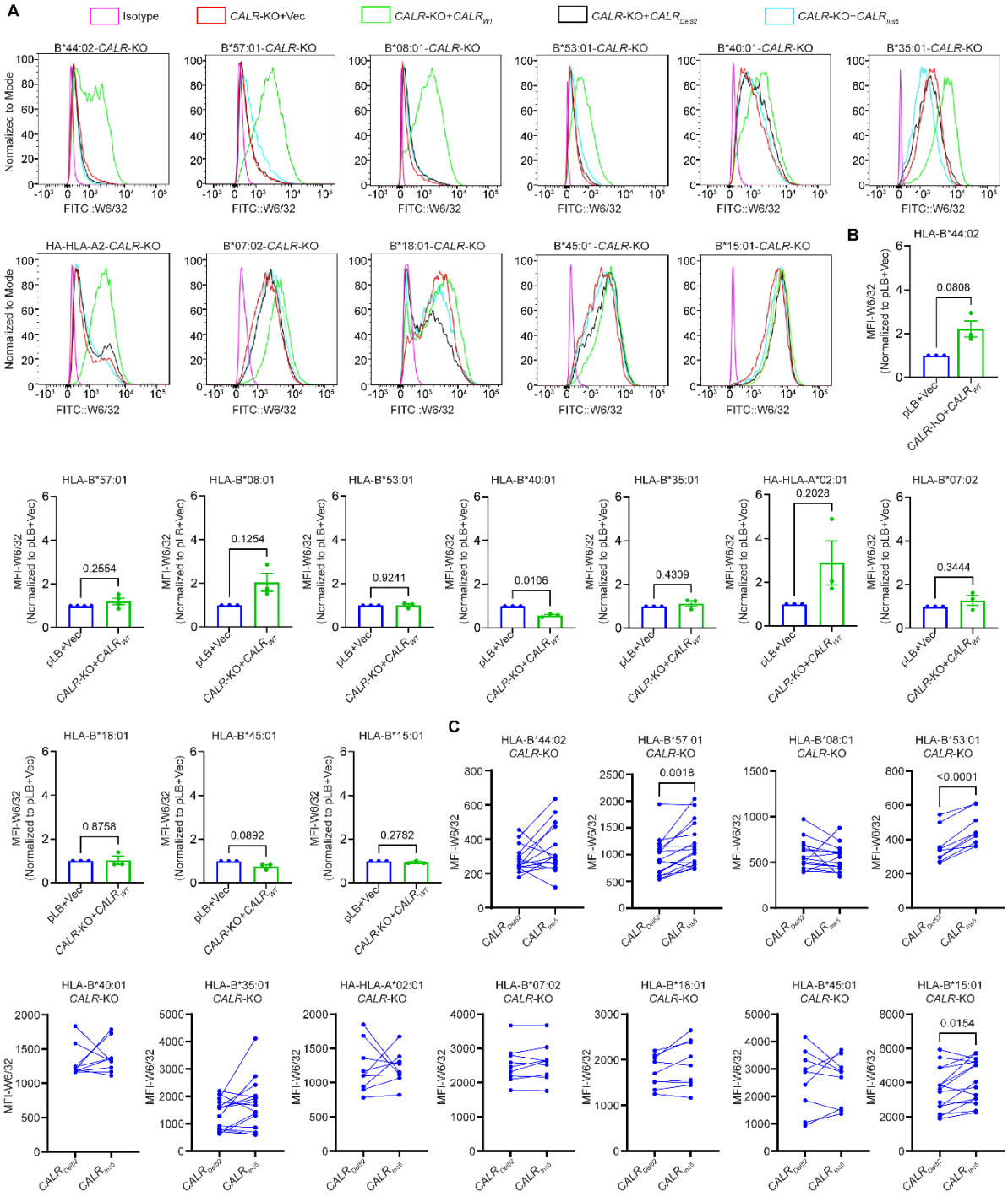
Supplementary Figure 2: CRISPR/Cas9-based *CALR* knockout in K562 cells: HLA class I null K562 cells were transduced with retroviruses to express individual HLA class I allotypes (monoallelic) and were then subjected to CRISPR/Cas9-based knockdown of CRT expression (*CALR*-KO). Immunoblots show CRT protein levels in K562 *CALR*-KO cells-expressing individual HLA class I allotypes compared to cells transduced with empty vector (Vector Control). Two different amounts of lysates from each of the three biological replicates (I, II and III) of *CALR*-KO and vector control cells were loaded as indicated. CRT was probed using anti-CRT(N) antibody and GAPDH is the loading control.



Supplementary Figure 3: Reconstitution of expression of wild-type CRT or MPN-linked CRT mutants in K562 *CALR*-KO cells expressing individual HLA class I allotypes: K562 cells expressing indicated HLA class I allotype were first transduced with lentivirus carrying either pLentiCRISPRv2-Blast empty vector (pLB) or pLB with *CALR*-targeting sgRNA (*CALR*-KO) to achieve *CALR* knockdown. The cells were subsequently transduced with virus carrying either the empty pSIP vector (pLB+Vec) or pSIP vector encoding wild-type CRT (*CALR*-KO+$CALR_{WT}$) or MPN-linked mutant CRT (*CALR*-KO+$CALR_{Del52}$ or *CALR*-KO+$CALR_{Ins5}$) proteins from the sgRNA-resistant cDNA constructs. Immunoblots show wild-type or mutant CRT protein levels, detected using anti-CRT(N) or anti-CRT(C_{mut}) antibody, respectively, in K562 cells,

following reconstitution of expression of the indicated CRT protein. Lysates from three biological replicates of viral transduction were loaded. GAPDH is a loading control.

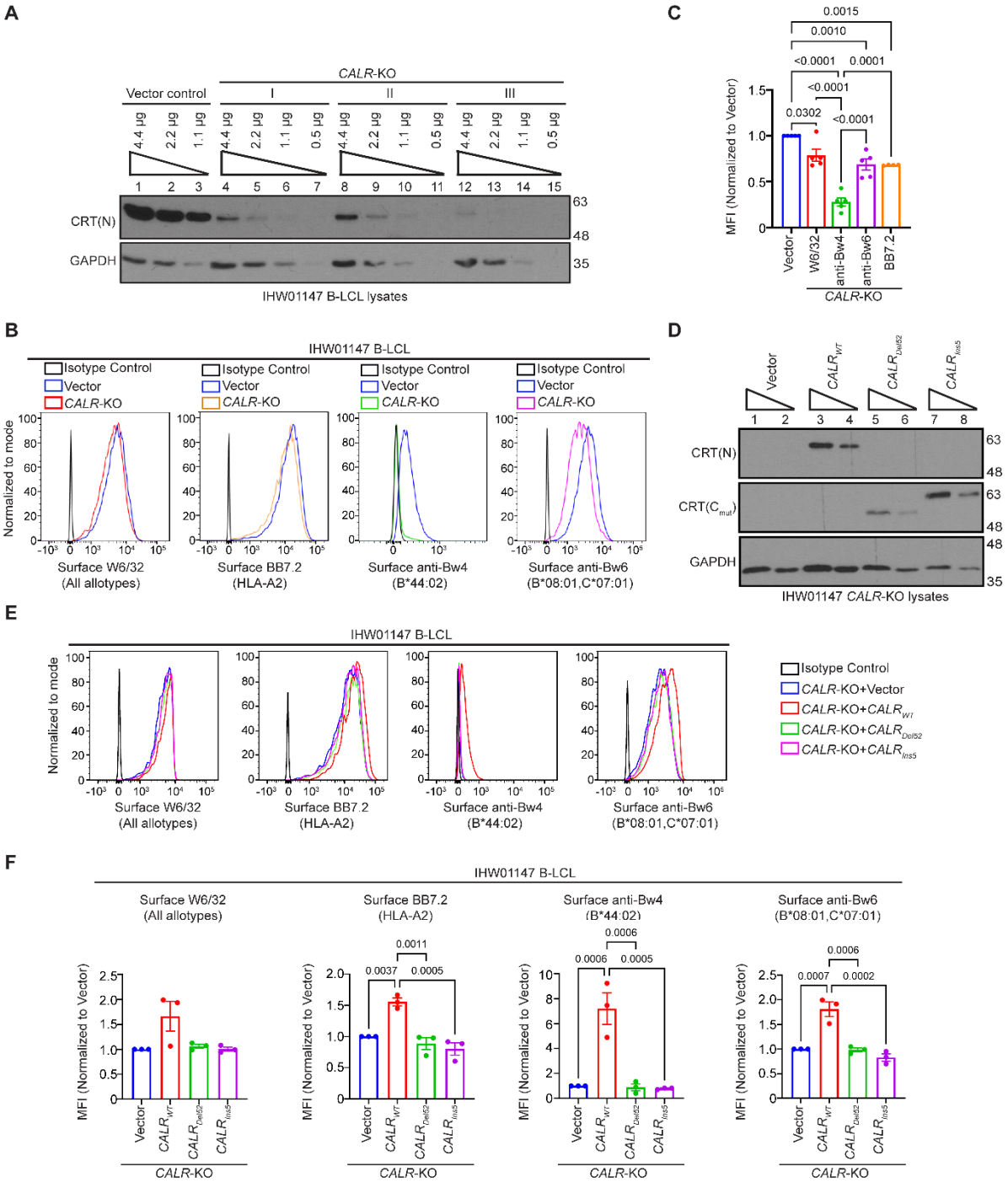
Supplementary Figure 4



Supplementary Figure 4: Reconstitution of wild-type CRT expression, but not the MPN-linked CRT mutants, rescues surface HLA class I levels in *CALR*-KO cells. K562 *CALR*-KO cells or cells transduced with empty pLentiCRISPRv2-Blast vector (pLB) expressing indicated HLA class I allotypes were transduced with virus carrying the empty pSIP vector (pLB+Vec) or (*CALR*-KO+Vec) or pSIP vector

encoding wild-type (*CALR*-KO+$CALR_{WT}$) or mutant (*CALR*-KO+$CALR_{Del52}$ or *CALR*-KO+$CALR_{Ins5}$) CRT proteins. Surface HLA class I expression was detected by flow cytometry using W6/32 antibody. **A)** Representative histograms show surface staining with W6/32 or isotype control antibody in K562 cells expressing indicated HLA class I allotypes following *CALR* knockdown and reconstitution of wild-type or mutant CRT expression. **(B)** Graphs show average ratios of MFI values of W6/32 staining, detected by flow cytometry, in *CALR*-KO cells reconstituted with wild-type CRT (*CALR*-KO+$CALR_{WT}$) relative to the vector control (pLB+Vec) cells-expressing endogenous wild-type CRT. Each dot represents an independent biological replicate of reconstitution (n=3, except HLA-B*57:01, for which n=4). **(C)** Paired line plots show comparison of MFI values of surface staining with W6/32 antibody of K562 *CALR*-KO+$CALR_{Del52}$ and *CALR*-KO+$CALR_{Ins5}$ cells expressing indicated HLA class I allotypes. Measurements from individual experiments (n=9-16) are connected by lines. Statistical significance in panels B and C was calculated using paired t-tests and graphs were plotted in GraphPad Prism.

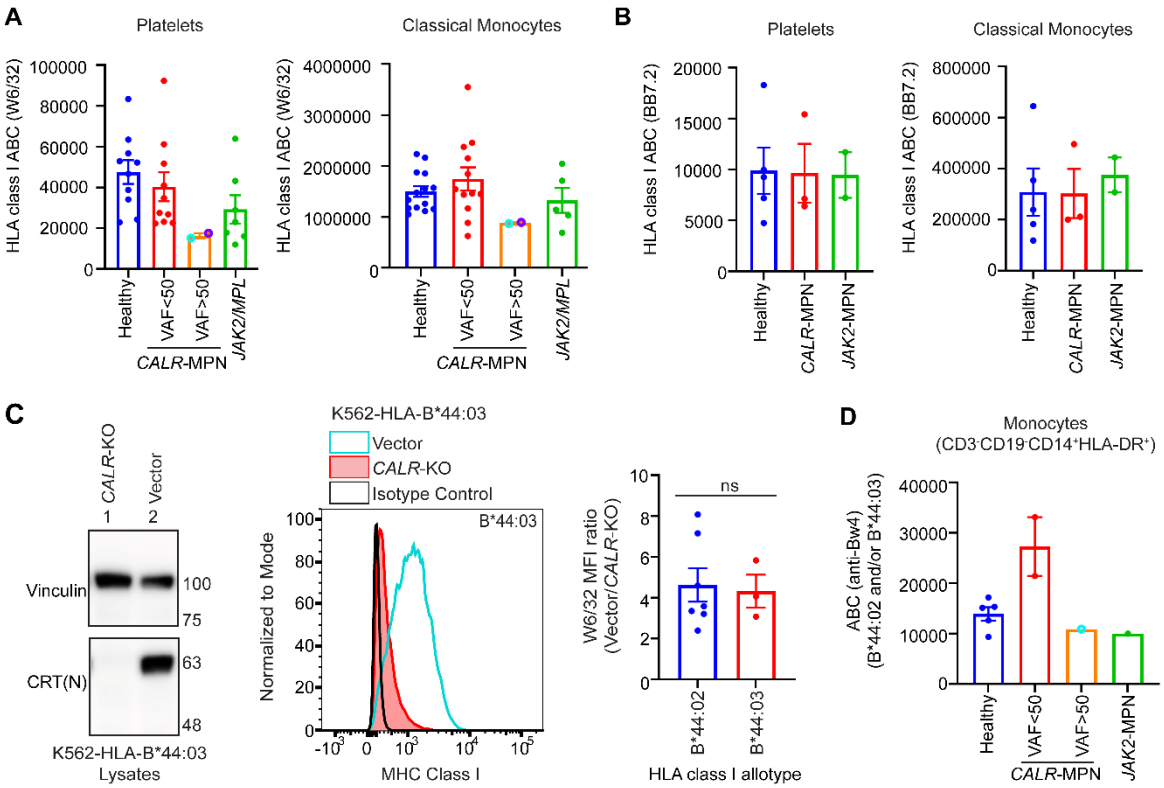
Supplementary Figure 5



Supplementary Figure 5: CRISPR/Cas9-based *CALR*-KO and reconstitution of wild-type or mutant CRT expression in IHW01147 B-LCL. (A) Immunoblots show CRT protein levels in lysates IHW01147 B-LCL (A*01:01/02:01, B*08:01/44:02, C*07:01/05:01) transduced with lentivirus packaged with either empty vector (Vector control) or the vector carrying *CALR*-targeting sgRNA for CRISPR/Cas9-based *CALR*

knockdown (*CALR*-KO). Three different cell lysate loads from vector control cells and each of the three biological replicates (I, II and III) of *CALR*-KO cells were loaded as indicated. **(B and C)** Surface HLA class I expression was measured by flow cytometry using the W6/32, anti-Bw4, anti-Bw6, or BB7.2 antibodies in the IHW01147 *CALR*-KO and vector control cells. Representative histograms show surface staining with the indicated anti-HLA class I antibody or the isotype control antibody (B), and the bar graph shows averaged data from flow cytometry experiments (C). Each dot shows the average MFI calculated from three biological replicates of *CALR*-KO cells, stained in parallel across multiple independent experiments (n=5 for W6/32, anti-Bw4, anti-Bw6, and n=4 for BB7.2). **(D)** Immunoblots show wild-type and mutant CRT expression in lysates of IHW01147 *CALR*-KO cells reconstituted with either wild-type (*CALR*-KO+$CALR_{WT}$) or mutant (*CALR*-KO+$CALR_{Del52}$ or *CALR*-KO+$CALR_{Ins5}$) CRT proteins. IHW01147 *CALR*-KO cells transduced with empty vector (*CALR*-KO+Vector) were also included. Wild-type and mutant CRT proteins were detected using anti-CRT(N) and anti-CRT(C_{mut}) antibodies, respectively, and GAPDH is a loading control. **(E and F)** Surface HLA class I expression was measured by flow cytometry in IHW01147 *CALR*-KO cells reconstituted with wild-type (*CALR*-KO+$CALR_{WT}$) or mutant (*CALR*-KO+$CALR_{Del52}$ or *CALR*-KO+$CALR_{Ins5}$) CRT or the vector control cells (*CALR*-KO+Vector). Cells were stained with the isotype control or the indicated anti-HLA class I antibody. Representative histograms (E), and averaged data (F) from independent flow cytometry experiments (n=3) are shown. Statistical significance in panels C and F was calculated using ordinary one-way ANOVA.

Supplementary Figure 6



Supplementary Figure 6: Quantitative measurements of total HLA class I, HLA-A*02:01 and HLA-B*44:02/HLA-B*44:03 levels in MPN patients and healthy donors and demonstration of similar CRT dependencies of HLA-B*44:02 and HLA-B*44:03. (A) Data from Figures 4E (Platelets) and 5D (Classical Monocytes) are re-plotted after grouping the *CALR*-MPN patients by variant allele frequency as VAF>50% (n=2) or VAF<50% (platelets, n=10; monocytes, n=12). *CALR*8251 and *CALR*7471 with VAF>50 are highlighted in purple and cyan, respectively. **(B)** Bar graphs show surface HLA-A*02:01 ABC values, measured using the BB7.2 antibody, on platelets (left) and classical monocytes (right) from MPN patients with *CALR* mutations (n=3), *JAK2* mutations (n=2) and healthy donors (n=5). ABC/2 is plotted for a donor with homozygous HLA-A2 expression (*JAK*0922). Donors carrying HLA-A allotypes with known cross-reactivity to BB7.2 or those that were not screened for BB7.2 binding were excluded from this analysis. **(C)** CRISPR/Cas9 editing was used in K562 cells to generate *CALR*-KO clones and control cells transduced with a vector lacking *CALR*-targeting sgRNA (Vector). The cells were further infected with viruses encoding

HLA-B*44:03. Immunoblots show wild-type CRT expression in K562-HLA-B*44:03 cells using the anti-CRT(N) antibody. Representative histograms show surface staining with W6/32 or isotype control antibody in HLA-B*44:02 and HLA-B*44:03-expressing K562 *CALR*-KO and control cells (vector). Graph shows ratios of surface expression of HLA-B*44:02 (data from Figure 2B) and HLA-B*44:03 in vector control relative to *CALR*-KO cells. Each dot represents an independent experiment (HLA-B*44:02, n=7; HLA-B*44:03, n=3). For each experiment, average MFI ratios from three biological replicates of vector control cells, K562 *CALR*-KO cells expressing-B*44:02 or three K562 *CALR*-KO single-cell clones expressing HLA-B*44:03 are shown. **(D)** Surface expression of HLA-B*44:02 and/or B*44:03 allotypes were measured on $CD3^{-}CD19^{-}CD14^{+}HLA\text{-}DR^{+}$ monocytes in MPN patients and healthy donors using the anti-Bw4 antibody and quantitative flow cytometry. Graph shows anti-Bw4 ABC values of monocytes from HLA-B*44:02/B*44:03-positive *CALR*-MPN patients with VAF<50 (n=2), VAF>50 (n=1, *CALR*7471 shown in cyan), a *JAK2*-MPN patient (n=1), and healthy donors (n=5; one $HLA\text{-}B*44:02^{+}$ healthy donor from this study and four $HLA\text{-}B*44:02^{+}$ donors included in an earlier study from our lab (8)). For donors expressing both B*44:02 and B*44:03 (*CALR*0266 and *JAK*6440), the measured ABC/2 is plotted in the graph. Patient and healthy control details are given in Supplementary Tables 1 and 2. Statistical significance in panel C (right) was calculated using an unpaired t-test with Welch's correction.

References

1. Mohan HM, Yang B, Dean NA, and Raghavan M. Calreticulin enhances the secretory trafficking of a misfolded alpha-1-antitrypsin. *J Biol Chem*. 2020;295(49):16754-72.
2. Foßelteder J, Pabst G, Sconocchia T, Schlacher A, Auinger L, Kashofer K, et al. Human gene-engineered calreticulin mutant stem cells recapitulate MPN hallmarks and identify targetable vulnerabilities. *Leukemia*. 2023;37(4):843-53.
3. Venkatesan A, Geng J, Kandarpa M, Wijeyesakere SJ, Bhide A, Talpaz M, et al. Mechanism of mutant calreticulin-mediated activation of the thrombopoietin receptor in cancers. *J Cell Biol*. 2021;220(7):e202009179.
4. Bashirova AA, Viard M, Naranbhai V, Grifoni A, Garcia-Beltran W, Akdag M, et al. HLA tapasin independence: broader peptide repertoire and HIV control. *Proc Natl Acad Sci U S A*. 2020;117(45):28232-8.
5. Garcia-Beltran WF, Holzemer A, Martrus G, Chung AW, Pacheco Y, Simoneau CR, et al. Open conformers of HLA-F are high-affinity ligands of the activating NK-cell receptor KIR3DS1. *Nat Immunol*. 2016;17(9):1067-74.
6. Rizvi SM, Salam N, Geng J, Qi Y, Bream JH, Duggal P, et al. Distinct Assembly Profiles of HLA-B Molecules. *The Journal of Immunology*. 2014;192(11):4967-76.
7. Roeth JF, Williams M, Kasper MR, Filzen TM, and Collins KL. HIV-1 Nef disrupts MHC-I trafficking by recruiting AP-1 to the MHC-I cytoplasmic tail. *J Cell Biol*. 2004;167(5):903-13.
8. Yarzabek B, Zaitouna AJ, Olson E, Silva GN, Geng J, Geretz A, et al. Variations in HLA-B cell surface expression, half-life and extracellular antigen receptivity. *Elife*. 2018;7:e34961.
9. Gigoux M, Holmstrom MO, Zappasodi R, Park JJ, Pourpe S, Bozkus CC, et al. Calreticulin mutant myeloproliferative neoplasms induce MHC-I skewing, which can be overcome by an optimized peptide cancer vaccine. *Sci Transl Med*. 2022;14(649):eaba4380.
10. Gonzalez-Galarza FF, McCabe A, Santos E, Jones J, Takeshita L, Ortega-Rivera ND, et al. Allele frequency net database (AFND) 2020 update: gold-standard data classification, open access genotype data and new query tools. *Nucleic Acids Res*. 2020;48(D1):D783-D8.
11. Klampfl T, Gisslinger H, Harutyunyan AS, Nivarthi H, Rumi E, Milosevic JD, et al. Somatic Mutations of Calreticulin in Myeloproliferative Neoplasms. *N Engl J Med*. 2013;369(25):2379-90.