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LETTER TO THE EDITOR

Pathologic activation of thrombopoietin receptor and JAK2-STAT5 pathway by frameshift mutants of mouse calreticulin

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Frameshifting (−1/+2) mutations in the exon 9 of the calreticulin gene (*CALR*) lead to the development of BCR-ABL-negative myeloproliferative neoplasms (MPNs) in humans under the forms of essential thrombocythemia (ET) and myelofibrosis.[1,2] We have shown that mutant human CALR proteins require interaction with the thrombopoietin receptor (TpoR) to exert an oncogenic activation of the JAK/STAT pathway.[3,4] Here, we report that homologous mutations in the murine *Calr* gene result in a highly similar, but not identical mutant CALR protein as found in human patients that also requires TpoR interaction to be able to activate the JAK/STAT pathway.

CALR is a highly conserved protein throughout the species:[5] both human and mouse *CALR* transcript sequences share 88% of base-pair identity (1098/1254 bp, human sequence as reference). Similarly, mouse and human wild-type CALR protein amino acid sequences are highly similar, but not identical: 94.25% identity; 377/400 exact same AA with human sequence as reference (Supplementary Figure 1A). Modeling the charge and structure of the mutant CALR proteins, we observed high levels of similarity in charge and predicted properties (Supplementary Figures 1C and D). By comparing type-1 and type-2 human mutant CALR amino acid sequences with the mouse wild-type (WT) CALR sequence, we created three murine CALR mutants homologous to their human counterparts for our study: del52 (type 1), ins5 (type 2) and del61 (type 1) (Supplementary Figures 1B–D), with del61 being highly similar to del52, but more easily engineered in the murine genome using the CRISPR/Cas9 system (Supplementary Figure 2). Interestingly, a very similar 61 bp deletion in the human *CALR* gene (c.1099_1159del) has already been reported in the MARIMO myeloid cell line[6] issued from a patient treated for acute myeloblastic leukemia with maturation with Busulfan who had a history of ET.[7]

The *mCalr* del52, ins5 and del61 complementary DNAs were obtained using the QuickChange mutagenesis kit from Agilent, Santa Clara, CA, USA.[8] STAT5 activation by the murine CALR mutants was subsequently confirmed by dual-luciferase reporter assay by transiently transfecting *mCalr* constructs in γ2A cells (a JAK2-deficient human fibrosarcoma derived cell line)[9] and using the Dual-Glo Kit from Promega, Madison, WI, USA as previously described.[3] For CRISPR/Cas9 engineering of *mCalr* del61 Ba/F3 cells, oligos encoding designed single guide RNAs were cloned into the CRISPR/Cas9 px330 vector (gift from Feng Zhang:[10] Addgene #42230). Efficiency of the obtained px330-sg1 and px330-sg2 constructs to target and cleave *Calr* exon 9 was verified by cloning the *Calr* exon 9 sequence in the pCAG-EGxxFP vector (gift from Ikawa Mashiko:[11] Addgene # 50716) and transiently transfecting both constructs in HEK-293T cells to quantify enhanced green fluorescent protein expression after 48 h of culture. After confirmation, either parental or TpoR-expressing Ba/F3 cells were electroporated with either both px330-sg1, –sg2 constructs or empty pMX-IRES-GFP. Cells were immediately kept in Interleukin-3 containing medium for 48 h before subsequent assays. We confirmed the presence of the 61 bp deletion in the *Calr* gene in TpoR/CRISPR cells by genomic DNA extraction and polymerase chain reaction amplification of the *Calr* exon 9 locus. All Ba/F3 populations were then used in proliferation assays by automatic cell counting or CellTiter-Glo assay (Promega, by following manufacturer's protocol) at different timepoints, and were lysed for western blotting. Lastly, fluorescence activated cell sorting (FACS) analysis of these Ba/F3 populations was performed using PE-linked anti-CD110 (TpoR) antibody from Miltenyi Biotec (Leiden, Netherlands) using a FacsVerse cytometer from BD technologies (Durham, NC, USA). Detailed procedures are described in Supplementary Material.

As it is the case for their human CALR mutant counterparts,[3,4] mCALR del52, mCALR ins5 and mCALR del61 were able to activate the JAK-STAT pathway when compared with mCALR WT in presence of human TpoR (Figure 1a and Supplementary Figure 10). This was also the case for mCALR mutants with murine TpoR (Supplementary Figure 3). In the case of granulocyte colony-stimulating factor receptor (GCSFR), mCALR mutants were able to activate the JAK-STAT pathway to a lower extent than with TpoR. No differences between mCALR WT and mCALR mutants were noticeable when cotransfected with EpoR.

Next, we show that Ba/F3 endogenously expressing mCALR del61 after CRISPR/Cas9 modification are able to proliferate in the absence of IL-3, but only if also expressing TpoR. All other cell populations, including parental Ba/F3 cells subjected to CRISPR/Cas9, stopped proliferating and underwent apoptosis in the absence of IL-3 (Figure 1b, Supplementary Figure 4). Ba/F3-expressing GSCFR instead of TpoR were not able to proliferate in long-term conditions (>72 h) after CRISPR modification (data not shown). Sequential dilutions allowed isolation of a TpoR-expressing Ba/F3 clone homozygous for a 4 bp deletion of the *Calr* gene, resulting in a close to type-2 mCALR mutant allowing the cells to proliferate in the absence of cytokines (Supplementary Figure 5). We subjected cytokine-independent proliferating TpoR/CRISPR Ba/F3 cells to 1 μM Ruxolitinib and observed that treated cells died after 48 h whereas control group proliferated normally (Figure 1b) demonstrating that JAK-STAT is therefore the key pathway for pathological proliferation of mCALR mutant cells and confirms that type-1 JAK2 inhibitors would be a viable treatment regimen in CALR-driven MPNs.[12,13] We next asked whether excess mCALR WT would act as a hypothetical competitive inhibitor of the mutant mCALR del61 by interaction with TpoR. Cytokine-independent proliferating TpoR/CRISPR Ba/F3 cells were transduced to overexpress mCALR WT (Supplementary Figure 6), they rapidly acquired autonomous growth and we could not detect any proliferation difference compared with control cells by automatic cell counting (Figure 1b).

All populations were next subjected to western blot for detecting constitutive and ligand-induced signaling in the presence of endogenously-expressed mCALR mutants

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(Figure 2a). A separate population of Ba/F3 TpoR/CRISPR that was preselected for a week in IL-3 free medium was also included to ensure selection for mCALR mutant cells. These last cells exhibited the presence of phosphorylated JAK2, phosphorylated TYK2 and phosphorylated STAT5/3/1 in the absence of ligand in contrast to all the other populations. Importantly, phosphorylated JAK2, TYK2, STAT5/3/1 were undetectable in the absence of ligand in Ba/F3 TpoR/CRISPR that were not preselected because mCALR mutant cells did not have time to achieve clonal superiority during the short selection (4 h) before lysis. Interestingly, in presence of Tpo, preselected TpoR/CRISPR cells show a weaker JAK2, TYK2, STAT5-/3/1 phosphorylation response than other TpoR-expressing populations. This could be due to a decrease of the surface levels of the TpoR in presence of mCALR mutants. We also searched for differences in Ras/MAPK/Erk and PI3'K pathway activation but could not detect any constitutive AKT phosphorylation and very weak MAP-kinase phosphorylation for the preselected CRISPR/TpoR cells. We also show that cells subjected to CRISPR show a second band < 48 kDa after anti-CALR staining, confirming the presence of type-1 mutant mCALR.

Based on our Western blot results showing that cytokine-independent TpoR/CRISPR Ba/F3 cells show lower levels of Tpo-dependent activation of the JAK-STAT pathway, we assessed cell surface levels of TpoR by FACS analysis. We show that, in the presence of the mutant mCALR, the surface levels of TpoR are down-regulated (Figure 2b). This may result from persistent signaling with internalization from the surface and from traffic defects induced by mutant mCALR within the secretory pathway or other cellular compartments. We have already shown that del52 and ins5 human CALR mutants induce a block in TpoR maturation and presence of endoglycosidase H-sensitive TpoR at the cell surface.[3] Although the surface levels of TpoR are decreased in our preselected TpoR/CRISPR Ba/F3 cells, the total TpoR levels remain the same on Western Blot (Figure 2a). Similarly, as TpoR was transduced using a pMX-IRES-GFP construct in these cells, GFP levels are correlated with the total level of expression of TpoR. By FACS, we show that the preselected TpoR/CRISPR Ba/F3 cells have the same or slightly higher expression of GFP than the other unselected TpoR/CRISPR or TpoR/empty vector cells, meaning that the TpoR expression is certainly not reduced in these cells (Figure 2c).

With this report, we show that the homologous mCALR del52, ins5 and del61 mutants also activate TpoR signaling via JAK-STAT pathway and behave similarly to the human counterparts, and

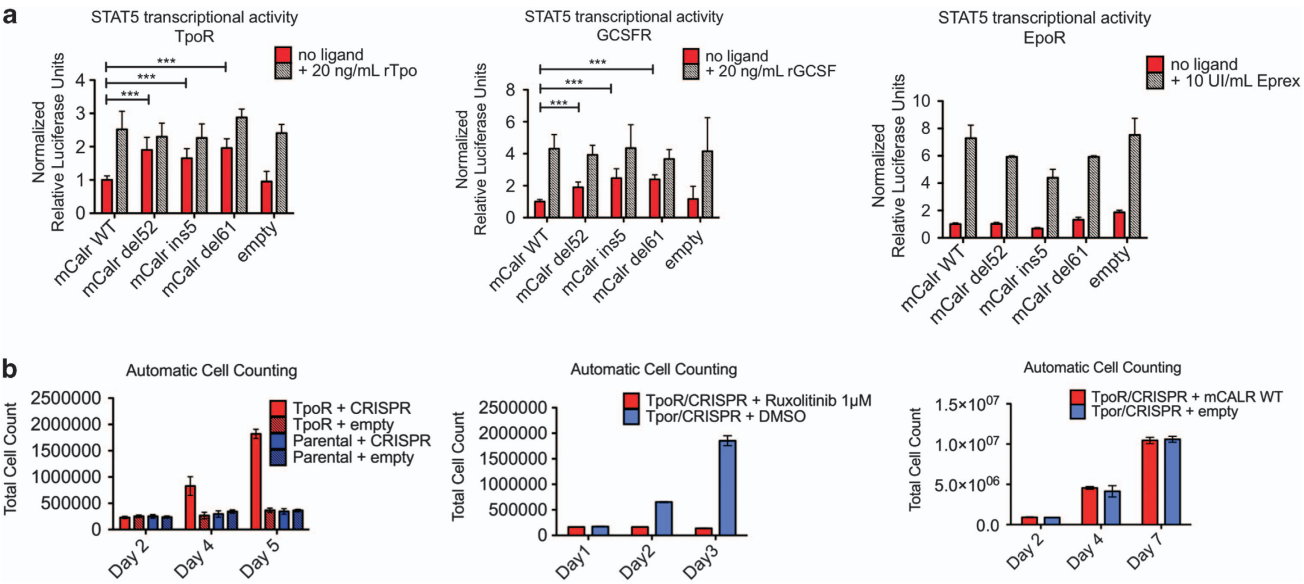

Figure 1. mCALR mutants activate the JAK-STAT pathway and enable cytokine-independence of Ba/F3 – TpoR cells. (**a**) mCALR mutants activate STAT5 when co-expressed with TpoR and GCSFR, but not EpoR *in vitro* when compared with mCALR WT. γ2A cells were transiently transfected with SpiLuc, a firefly luciferase reporter under a STAT5-specific promoter and a renilla luciferase reporter under a TK promoter as transfection control and complementary DNAs coding for either mCALR-WT, del52, ins5, del61 or empty vector (pMX-IRES-GFP), along with cytokine receptor (TpoR, GCSFR or EpoR), JAK2 and STAT5. Ligands or control medium were added 4 h after transfection. Cells were cultivated for 24 h before lysis and readings by luminometer. Shown are averages of separate experiments+s.d. ($N = 7$ for TpoR, $N = 4$ for GCSFR, $N = 2$ for EpoR); each experiment being performed with three biological repeats for each condition (triplicates). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Nonparametric multiple comparisons using Steel's test with a control group (CALR WT no ligand). (**b**) (Left) Only TpoR/CRISPR Ba/F3 cells are able to proliferate in absence of IL-3. Ba/F3 cells, either parental or transduced to express TpoR, were electroporated with either both px330-sg1 and px330-sg2 constructs or an empty vector. Cells were grown 48 h in the presence of IL-3 and then subsequently washed and put into IL-3 free growth medium on a 6-well plate. Initial total count at day 0 was 175 000 cells for all populations. Measurements were done using an automatic Coulter cell counter. Results are from one experiment (average of three biological repeats+s.d.) as representative of three separate experiments showing the same results. (Middle) Ruxolitinib inhibits proliferation of selected TpoR/CRISPR Ba/F3 cells signifying the critical importance of the JAK2/STAT5/3/1 pathway in the case of CALR mutant activation of TpoR. TpoR/CRISPR Ba/F3 cells that were able to grow independently of cytokines were washed three times in PBS and put at a starting concentration of 100 000 cells/ml on a 6-well plate in RPMI-Hepes+10% FBS free of any cytokines. 1 μl Ruxolitinib, diluted in DMSO was added at a final concentration of 1 μM to three wells. 1 μL DMSO was added to the other three wells. Measurements were done using an automatic Coulter cell counter. Results are from one experiment (average of three biological repeats+s.d.) as representative of three separate experiments showing the same results. (Right) mCALR WT overexpression does not interfere with mCALR mutant oncogenic activity. Selected TpoR/CRISPR cells that were able to grow independently from IL-3 stimulation were transduced with either a *mCalr*-WT/mCherry or empty/mCherry retrovirus construct and sorted by FACS using the mCherry marker. After sorting, cells were washed and put into IL-3 free growth medium on a 6-well plate, three wells per condition. Initial total cell count at day 0 was 200 000 cells for all conditions. Measurements were done using an automatic Coulter cell counter. Results are from one experiment (average of three biological repeats+s.d.) as representative of three separate experiments showing the same results. DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

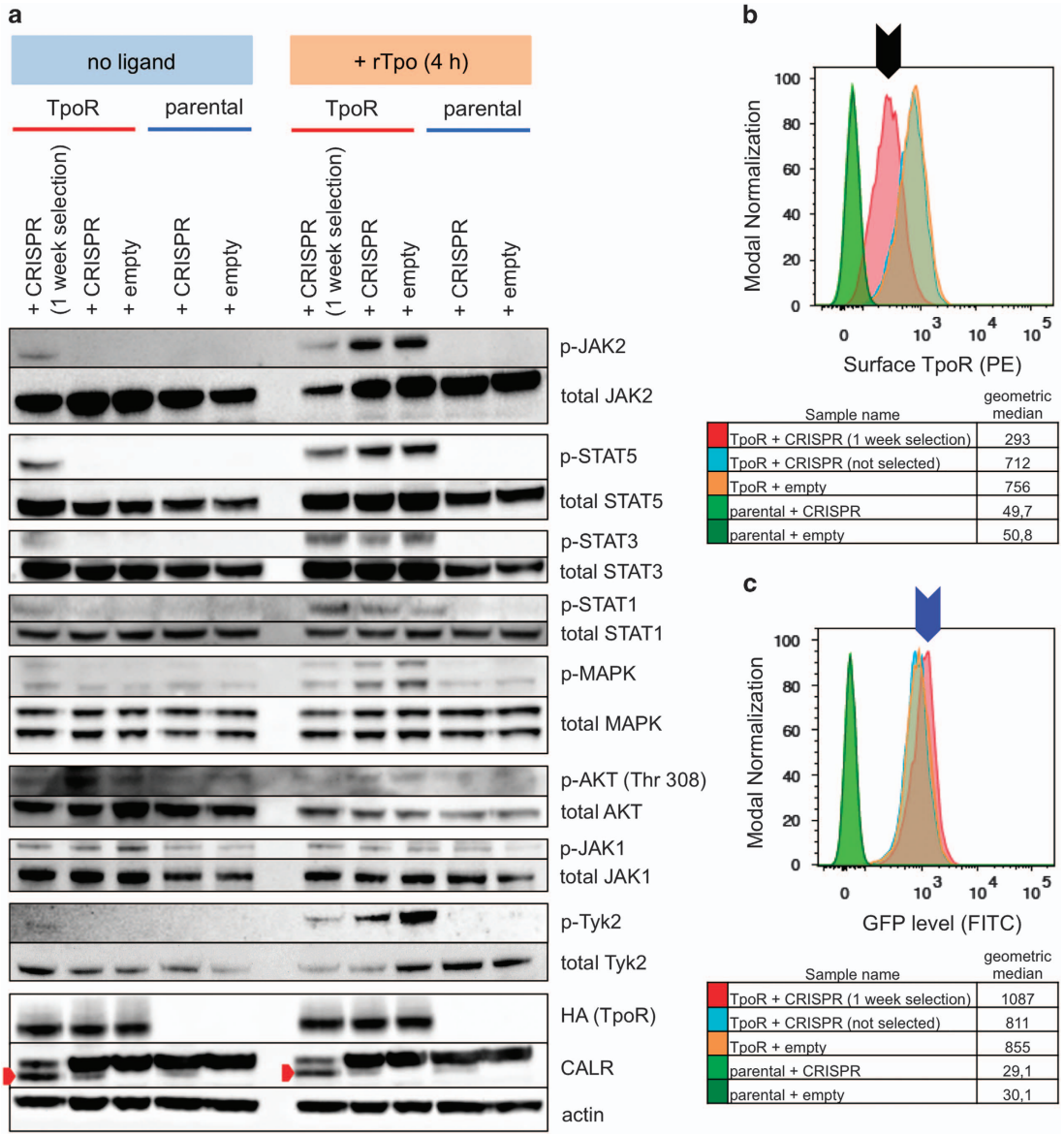

Figure 2. Signaling patterns and surface TpoR changes induced by mCALR mutants. (**a**) Parental or TpoR-expressing Ba/F3 cell populations previously electroporated with either CRISPR/Cas9 constructs targeting *Calr* exon 9 to create the del61 mutation (+CRISPR) or an empty vector (+empty) were washed three times to remove any IL-3 and put to grow in IL-3 free medium for at least 4 h to abolish all IL-3 signaling, in the presence or absence of recombinant Tpo at 20 ng/ml. A subset of TpoR+CRISPR Ba/F3 were washed free of IL-3, 1 week before the experiment, and selected in IL-3 and Tpo free medium, to ensure clonal dominance of mutant mCALR-expressing cells (+CRISPR 1 week selection). Only cells that were preselected a week before showed more phosphorylated JAK2, STAT5, STAT3, STAT1, MAPK and TYK2 in the absence of ligand when compared with the other populations, demonstrating an activation of these pathways by the mutant mCALR. TpoR +CRISPR Ba/F3 that were not preselected have undetectable levels of phosphorylated JAK2, STAT5, STAT3, STAT1, MAPK and TYK2 as the mCALR mutant cells have not had time to achieve clonal dominance. No differences were observable for phosphorylated AKT (threonine 308) or JAK1. Preselection 1 week before western blotting was not possible on other than TpoR+CRISPR Ba/F3 populations because those cells die, as shown in Figure 1b and Supplementary Figure 4. Total levels of JAK2, STAT5, STAT3, STAT1, MAPK, AKT, JAK1, TYK2 and β-actin are shown as references. hemagglutinin-tagged TpoR was only detected in TpoR-expressing cells as intended. A second CALR band is visible in CRISPR cells, corresponding to the mCALR mutants (red arrow tip). Preselected TpoR+CRISPR (1 week selection) Ba/F3 have a stronger presence of mCALR mutant than TpoR+CRISPR (not preselected) Ba/F3 as the preselection allowed the mCALR mutant cells to achieve clonal superiority. These results are from one experiment as representative of three separate experiments showing similar results. (**b**) FACS analysis of electoporated Ba/F3 populations. Surface TpoR level of all Ba/F3 populations was assessed by FACS using a PE-linked anti-CD110 (TpoR) antibody. Only the TpoR+CRISPR (1 week selection) Ba/F3 preselected in absence of IL-3 show a decrease of surface TpoR levels (black arrow tip). This is representative of three separate experiments. Again, TpoR+CRISPR Ba/F3 that were not preselected do not show a decrease in surface TpoR as the mCALR mutant cells have not achieved clonal superiority. (**c**) Total GFP levels of cells analyzed in (**b**) are also shown: as TpoR was expressed using a pMX-IRES-GFP vector, expression of GFP is correlated to TpoR expression. Only populations expressing TpoR show a significant level of GFP, with the TpoR+CRISPR (1 week selection) Ba/F3 preselected in IL-3 free medium showing a slightly increased GFP expression (blue arrow tip). These results are from one experiment as representative of three separate experiments showing same results. Below are the calculated geometric medians for each population, determined from 10 000 events per gate.

that creation of a del61 endogenous *Calr* mutation rapidly leads to cytokine-independent transformation of Ba/F3 TpoR, but not Ba/F3 parental or Ba/F3 GCSFR. The previously reported ability of human CALR del52 to transform parental Ba/F3 cells[1] was explained by induction of expression of the endogenous mouse MPL/TpoR of Ba/F3 cells.[14] In that study, a global CRISPR/Cas9 approach was also used to target *Calr* exon 9. Frameshift mutations in all three frames were obtained in Ba/F3 cells, but only −1/+2 frameshift mutations transformed Ba/F3 TpoR cells; those were different than our *Calr* del52, ins5 and del61 mutations.[14] Our experiments allowed us to study signaling in more physiologic expression conditions, and to observe that, due to cell surface TpoR down-modulation, response to ligand was blunted and also that mCALR WT does not interfere with the oncogenic properties of mutant CALR, which is relevant to the heterozygous state of *CALR* mutation in MPN patients.[15] These results also pave the way for the creation of new mouse models of mutant CALR-driven MPNs using the CRISPR/Cas9 tool to easily engineer a type-1 (or type-2) mutation in mouse zygotes, which would be different from the human CALR dependent mouse model generated by retroviral bone marrow transduction and transplantation.[4]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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