

# Calreticulin mutation does not contribute to disease progression in essential thrombocythemia by inhibiting phagocytosis

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**Calreticulin mutation does not contribute to disease progression in  
essential thrombocythemia by inhibiting phagocytosis**

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## Introduction

Myeloproliferative neoplasms (MPNs) are chronic hematopoietic stem cell (HSC) disorders characterised by an excess of mature myeloid lineage cells (i.e. granulocytes, erythrocytes and/or platelets)<sup>1</sup>. *Janus kinase 2 (JAK2)* gain-of-function mutation is the gene mutation most frequently involved in the pathogenesis of *breakpoint cluster region (BCR) – Abelson leukemia virus (ABL)* negative MPNs such as polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF)<sup>2-6</sup>. Previous reports suggest that *JAK2 V617F* definitely induces MPNs and that phosphorylated signal transducers and activators of transcription 5 (STAT5) play a crucial role in MPN pathogenesis. However, although a *JAK2* mutation is detected in most PV cases, approximately 50% of all ET and PMF cases harbour the wild-type *JAK2*; other mutations associated with these cases still remain unclear.

Using whole exome sequencing, a somatic mutation in *calreticulin (CALR)* exon 9 was recently detected in a considerable number of ET or PMF cases harbouring a non-mutated *JAK2* or non-mutated *myeloproliferative leukemia virus oncogene (MPL)*<sup>7,8</sup>. *CALR* mainly localizes within the endoplasmic reticulum (ER) lumen and is involved in lectin-like chaperoning of proteins and glycoproteins<sup>9,10</sup>. The vast majority of *CALR* exon 9 mutations found in MPN cases can be divided into two types, namely a 52-base pair (bp) deletion or type 1 mutation, which is the most common type, and a 5-bp insertion or type 2 mutation. Regardless of the type, all *CALR* mutations cause a common frame shift and result in an abnormal peptide sequence in the C-domain of *CALR*. This mutant *CALR* loses its calcium ion-binding capacity and the sequence necessary for retention and retrieval into the ER lumen and is known to cause a change in the intracellular distribution<sup>7,8</sup>.

Klampfl et al. showed that type I *CALR* mutations contribute to hypersensitivity to interleukin-3 (IL-3), IL-3-independent proliferation and constitutive STAT5 phosphorylation when transduced into an IL-3-dependent cell line<sup>7</sup>, a situation that closely resembles *JAK2* V617F mutation<sup>3,6</sup>. Moreover, an earlier gene expression study of peripheral blood (PB) granulocytes from patients with MPN showed universally activated JAK2 signaling, regardless of the *JAK2* or *CALR* mutational status, and a significantly enriched gene expression signature in patients with *CALR*-mutated MPN as compared with those with *JAK2*-mutated MPN<sup>11</sup>. These observations indicated that the extent of JAK-STAT pathway activation by *CALR* mutations was similar to that by *JAK2* mutations in the pathogenesis of MPNs. Recently, several groups revealed the direct interaction between CALR and MPL by using cell lines transfected with mutant CALR<sup>12-15</sup>. Mutant CALR binds to the extracellular domain of MPL, and also induced the dimerization of MPL and the phosphorylation of JAK2. These interaction activate JAK-STAT pathway and result in enhanced megakaryopoiesis. These findings indicate both of *CALR* and *JAK2* mutation cause JAK-STAT pathway activation and contribute to the pathogenesis of MPNs, however, several studies have described differences between the clinical features of MPNs harbouring mutant *CALR* and those harbouring mutant *JAK2*; patients with *CALR* mutation (frequently observed in males) tended to be younger at the time of diagnosis as well as have lower white blood cell counts, a lower hemoglobin concentration, higher platelet counts and a lower thrombosis occurrence rate<sup>16-30</sup>. These findings indicate that mutant *JAK2* and *CALR* may induce myeloproliferation via different mechanisms.

A small fraction of the total CALR pool is distributed over the cell surface<sup>31</sup>. These molecules have a function distinct from that of ER-residing CALR and are known to transduce pro-phagocytic 'eat me' signals to phagocytes by binding to low-density lipoprotein receptor-

related protein 1 (LRP1) on phagocytes<sup>31-39</sup>. On apoptotic cells, surface CALR acts as a receptor when conjugated with LRP1 and stimulates phagocytes to promote efficient apoptotic cell removal. Thus, we hypothesized that *CALR* mutations changes the cell surface expression level of CALR and affect the intracellular signaling, which mediates the phagocytic signals, resulting in the inhibition of phagocytic removal of myeloid cells and in turn facilitating excess myeloproliferation.

Herein, we have attempted to elucidate the pathogenesis of ET harbouring *CALR* mutations relative to those harbouring *JAK2* mutations from a standpoint of phagocytic regulation.

## **Materials and methods**

### **Patients and samples**

We obtained bone marrow (BM) or PB samples from patients diagnosed with *BCR-ABL* negative MPNs, including PV, ET and PMF, at institutions affiliated to Fukuoka Blood and Marrow Transplantation Group (FBMTG) and from healthy volunteers after obtaining their written informed consent. This study was approved by the ethics committee of each institution. Diagnoses of MPNs were made in accordance with the categories specified in 2008 by the World Health Organization. For DNA extraction, granulocytes were isolated from PB by dextran sedimentation, hypotonic lysis of contaminating erythrocytes and centrifugation with lymphocyte separation medium (LSM) (MP Biomedicals, Irvine, CA, USA), as described previously<sup>40</sup>.

### **Mutation analysis and DNA sequencing of JAK2, CALR and MPL**

Genomic DNA was obtained from PB cells with a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Genomic regions of interest were amplified by polymerase chain reaction (PCR) using pfu turbo DNA polymerase (Agilent, Santa Clara, CA, USA). The primers that were used are listed in Table S1. For the *JAK2* V617F mutation, PCR was performed as follows: 95°C for 2 min; 35 cycles of 95°C for 30 s, 52°C for 30 s and 72°C for 1 min; 72°C for 10 min and then maintained at 4°C. To detect *JAK2* exon 12 mutations, PCR was performed as follows: 95°C for 2 min; 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min; 72°C for 7 min and then maintained at 4°C. To detect *CALR* exon 9 mutations, PCR was performed as follows: 95°C for 2 min; 35 cycles of 95°C for 30 s, 61°C for 30 s and 72°C for 1 min; 72°C for 2 min and then maintained at 4°C.

To detect *MPL* exon 10 mutations, PCR was performed as follows: 95°C for 2 min; 35 cycles of 95°C for 30 s, 68°C for 30 s and 72°C for 1 min; 72°C for 2 min then and then maintained at 4°C. The PCR products were purified and subjected to sequencing with a BigDye Terminator Cycle Sequencing Kit, version 1.1 (Life Technologies, Waltham, MA, USA). Sequencing products were analyzed using an ABI3130XL DNA sequencer (Applied Biosystems, Foster City, CA, USA).

### **Antibodies, cell staining and sorting**

To sort hematopoietic progenitor cells (HSPCs), BM mononuclear cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD34 (581), a PerCP-Cy5.5-conjugated lineage cocktail including anti-CD3 (SK7), anti-CD4 (OKT4), anti-CD8 (HIT8a), anti-CD19 (HIB19), anti-CD20 (2H7), anti-CD235a/b (HIR2), APC-Cy7-conjugated anti-CD38 (HIT2), brilliant violet 421-conjugated anti-CD45RA (HI100) and biotinylated anti-CD123 (6H6) monoclonal antibodies as well as phycoerythrin (PE)-Cy7-conjugated streptavidin (antibodies were purchased from Biolegend, San Diego, CA, USA). Additionally, cells were stained with PE-conjugated anti-calreticulin (FMC75; Abcam, Cambridge, UK) and Alexa Fluor 647-conjugated anti-CD47 monoclonal antibodies (B6H12; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for analysis. The median fluorescent intensities (MFIs) of CALR and CD47 were normalized and reported as ratios relative to the MFIs of T lymphocytes from the same healthy donor to minimize the variability of data obtained on different days. HSCs, common myeloid progenitors (CMPs), megakaryocyte-erythroid progenitors (MEPs) and granulocyte-macrophage progenitors (GMPs) were isolated according to the following profiles: HSC, Lin<sup>-</sup> CD34<sup>+</sup>CD38<sup>-</sup> CD45RA<sup>-</sup>; CMP, Lin<sup>-</sup> CD34<sup>+</sup>CD38<sup>+</sup>CD123<sup>+</sup>CD45RA<sup>-</sup>; MEP, Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>+</sup>CD123<sup>-</sup>CD45RA<sup>-</sup>; and GMP,

Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>+</sup>CD123<sup>+</sup>CD45RA<sup>+</sup>.

To detect phosphorylated signaling mediators, sorted HSCs, CMPs, MEPs and GMPs were stained with PE-conjugated anti-phosphorylated STAT5 (47), anti-phosphorylated MEK1 (J114-64) and anti-phosphorylated STAT1 (4a) as well as Alexa Fluor 647-conjugated anti-phosphorylated STAT3 (4) and anti-phosphorylated Erk1/2 (20A) monoclonal antibodies following fixation with BD Phosflow Fixation Buffer I and permeabilisation with BD Phosflow Perm Buffer III (all antibodies and reagents for these experiments were obtained from Becton, Dickinson and Company). The MFI of each molecule was normalized to that of T lymphocytes from healthy donors. The cells were analyzed and sorted on a FACS Aria 3 cell sorter (Becton, Dickinson and Company).

### ***In vitro* phagocytosis assays of progenitor cells and mature blood cells**

Macrophages derived from healthy volunteers were obtained as described previously<sup>41</sup>. BM HSPCs were sorted and opsonized with an anti-CD34 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Mature PB cells were opsonized as follows: neutrophils with anti-CD15 antibody, erythrocytes with anti-CD235a antibody and platelets with anti-CD42b antibody. Macrophages were activated as previously described<sup>41</sup>. Opsonized HSPCs or mature blood cells were co-incubated with activated macrophages in a 1:1 ratio at 37°C for 2 h, followed by mounting via cytopspin preparation. After May–Grünwald–Giemsa staining, the macrophages and engulfed cells were enumerated by a blinded observer. We calculated the phagocytosis index as previously described<sup>41</sup>.

### **Statistical analysis**



A statistical analysis was performed using JMP version 11 software (SAS Institute, Cary, NC, USA). Expression levels of CD47 and CALR as well as phagocytic index and phosphorylation levels of STATs were analyzed using Turkey's honest significant difference test following an analysis of variance (ANOVA). The phosphorylation levels of molecules in the JAK-STAT pathway were analyzed using Dunnett's test. P values of  $<0.05$  were considered to be statistically significant.

## Results

### **CALR mutation does not alter the cell surface expression of CALR in patients with ET**

We examined whether *CALR* mutation might affect the cell surface expression of CALR and thus alter sensitivity to phagocytosis by macrophages. We used flow cytometry to compare between the cell surface expression levels of CALR on each HSPC fraction derived from patients with ET and those on each HSPC fraction derived from normal controls. First, we examined 135 Japanese patients diagnosed with *BCR-ABL* negative MPNs, including PV (n = 29), ET (n = 82) and PMF (n = 24). In the study population, the *JAK2* V617F mutation was found in 80 patients (59.3%), *JAK2* exon 12 mutation in 2 patients (1.48%), *MPL* exon 10 mutation in 2 patients (1.48%) and *CALR* exon 9 mutation in 34 patients (25.2%). No mutations were detected in 17 patients (12.6%) and no patient had overlapping mutations. *CALR* mutations were heterozygous in all 34 patients (27 patients with ET and 7 with PMF). *JAK2* V617F mutations were found in 26 patients with PV, 39 with ET and 15 with PMF (Figure S1). Thus, we isolated HSPC fractions from patients with ET who harboured *JAK2* V617F (n = 13) or *CALR* mutations (n = 18) and compared the surface expression of CALR in those fractions from the abovementioned patients and those derived from normal controls (n = 14). However, we detected no significant changes in the expression levels of CALR on any HSPC, including HSC, CMP, GMP and MEP, derived from patients with ET regardless of gene mutation and their normal counterparts (Figure 1A and 1B). Expression levels of CALR was higher in mature blood cells than in HSPC, as previously reported<sup>41</sup>; however, no significant differences in the surface expression levels of CALR on mature blood cells between patients with MPN and healthy controls were observed (Figure 1C). Thus, *CALR* mutation did not change the cell surface expression of CALR in HSPC as well as mature

blood cells in patients with ET.

**Expression of CD47, an anti-phagocytic marker, in patients with MPN was comparable to that in normal control**

The macrophage-mediated phagocytosis of viable cells is known to be regulated by a balance between pro-phagocytic signals through cell surface CALR and anti-phagocytic signals through cell surface CD47<sup>32</sup>. Therefore, we also examined the surface expression of CD47 in patients with ET and compared it with that in normal controls (Figure 2A). However, no differences in the expression level of CD47 on the cell surface of HSPC and mature blood cells were observed between patients with ET and normal controls (Figure 2B and 2C). These results suggest that CD47 expression is not involved in the pathogenesis induced by *CALR* mutation.

**Sensitivity to macrophage engulfment was not affected by *CALR* mutation**

Compared with normal controls, we did not observe any differences in surface CALR expression in patients with ET who harboured *CALR* mutation. However, *CALR* mutation changes the intracellular distribution of the protein and may affect intracellular pro-phagocytic signaling. Therefore, we also performed an *in vitro* phagocytosis assay to determine whether the phagocytic threshold was affected by *CALR* mutation (Figure 3A). To evaluate the phagocytosis index, we co-incubated opsonised HSPCs and mature blood cells isolated from patients with ET and healthy donors with activated normal macrophages. As positive control, we performed the same procedures using hematopoietic stem cells with inhibitory antibodies against the interaction between signal regulatory protein alpha (SIRPA) and CD47 and observed significant phagocytosis, with the phagocytosis index being greater than 1 (data

not shown). As shown in Figure 3B and 3C, the phagocytic sensitivities of HSPCs (Figure 3B) and mature blood cells (Figure 3C) derived from normal controls and patients with *JAK2*-mutated and *CALR*-mutated ET were comparable, suggesting that *CALR* mutation did not affect sensitivity to phagocytosis by macrophages.

### ***JAK2*-mutated PV/ET or *CALR*-mutated ET uses a distinctive signaling pathway**

Our data suggests that phagocytic dysregulation is not involved in the pathogenesis of *CALR* mutated ET. Thus, it is still unknown what to be due to the differences of clinical phenotype between *JAK2*-mutated and *CALR*-mutated MPNs. Although previous reports have shown that *CALR* mutations activate the JAK-STAT pathway, we sought to investigate whether the activation of signaling molecules downstream of JAK2 differs at HSPC levels depending on gene mutation or disease type. We evaluated the phosphorylation statuses of JAK-STAT signaling pathway molecules in each HSPC fraction from BM specimens; normal BM (n=7), *JAK2*-mutated PV (n=5), ET (n=5) and *CALR*-mutated ET (n=5) by using flow cytometry, and illustrated as a heatmap in Figure 4A. STATs were clearly more strongly phosphorylated in HSPCs from patients than those from normal BM, regardless of disease type and gene mutation. However, no increase in phosphorylation of other signaling molecules, such as extracellular signal-regulated kinase (ERK) 1/2, Mitogen-activated protein kinase kinase (MEK) was observed in *JAK2*-mutated and *CALR*-mutated HSPCs, indicating that STAT phosphorylation was the main common pathway in MPNs even at HSPC levels, regardless of gene mutations (Figure 4A). A comparison of STAT phosphorylation statuses in PV- and ET-derived HSPCs revealed differences in the STAT1 and STAT5 phosphorylation patterns among patients with MPN (Figure 4B). In *JAK2*-mutated PV, the phosphorylation of STAT5 was more robust than that of STAT1 at the CMP stage. In contrast, the phosphorylation of

STAT1 was stronger than that of STAT5 at the CMP stage in *JAK2*-mutated or *CALR*-mutated ET. At the MEP stage, however, the phosphorylation of STAT5 was stronger than that of STAT1, regardless of disease type. Thus, we observed different phosphorylation pattern at HSPC levels between PV and ET, however, there were no difference between those of *JAK2*-mutated and *CALR*-mutated ET.

## Discussion

There was a lack of understanding regarding the mechanism by which *CALR* mutation contributed to the pathogenesis of MPNs. The universal activation of JAK2 signaling, regardless of the *JAK2* or *CALR* mutational status, was observed in a previously conducted gene expression study of PB granulocytes from patients with MPN<sup>11</sup>. Recent reports had shown that mutant *CALR* can bind to MPL, and activate JAK-STAT pathway<sup>12-15</sup>. Regarding which HSPC stage is most affected by JAK-STAT activation, several studies examined the clone sizes of mutated genes in each HSPC populations, and found that HSCs often featured a small *JAK2*-mutant clone size<sup>42-43</sup>. We also evaluated the mutational statuses at each differentiation stage from *JAK2*-mutated PV/ET and *CALR*-mutated ET BM specimens (Figure S2). We observed that the *JAK2* or *CALR*-mutant clone size in the HSC fraction was relatively small, but the clone size increase in accordance with differentiation in both *JAK2* and *CALR*-mutant MPNs, indicating that *JAK2* and *CALR* mutations confer a proliferative advantage upon mature HSPC fraction in a similar fashion. These results indicate that JAK-STAT pathway activation is a key pathogenesis in both of *CALR* and *JAK2* mutated MPNs. However, another unique function of cell-surface *CALR*, but not *JAK2*, is the transduction of pro-phagocytic signals to phagocytes via LRP1<sup>31-37</sup>. In this study, we investigated whether dysregulation of macrophage engulfment was actively involved in the pathogenesis of *CALR*-mutated ET.

The macrophage-mediated phagocytosis of viable cells is known to be regulated by a balance between pro-phagocytic *CALR*-LRP1 and anti-phagocytic CD47-SIRPA signals<sup>32</sup>. We previously showed that disruption of this balance, promoted by CD47 downregulation, induced engulfment by macrophages and caused hemophagocytic lymphohistiocytosis<sup>41</sup>.

Conversely, it was reported that CD47 expression is upregulated on human acute myeloid leukemia (AML) stem cells to a greater extent than on their normal counterparts and is critical for AML stem cells to evade macrophage phagocytosis<sup>44</sup>. Besides AML, CD47 was highly expressed on acute lymphoblastic leukemia (ALL)<sup>33</sup> and non-Hodgkin's lymphoma cells<sup>34</sup> and was responsible for their evasion of macrophage immunosurveillance. In myelodysplastic syndrome, the increased expression of CALR on GMP mediates prophagocytic signals, resulting in an enhanced phagocytosis of GMP and ineffective hematopoiesis<sup>45</sup>. These findings suggest that dysregulation of macrophage engulfment is actively involved in the pathogenesis of hematopoietic malignancies. As mutations in *CALR* affect the localization of CALR in the cytoplasm<sup>7,8</sup>, we hypothesized that these mutations also affect the cell surface expression level of CALR and intracellular signaling, which in turn results in a decreased sensitivity to phagocytosis and myeloid cell expansion. First of all, we evaluated cell surface expression of CALR, and our results indicated that the cell surface expression level of CALR on HSPCs and mature blood cells from *CALR*-mutated ET was equivalent to that on HSPCs from both *JAK2*-mutated ET and normal controls. This result is matched with the previous report describing that cell line transfected mutant CALR showed no significant increase in cell surface CALR expression. Additionally, the expression level of CD47 on the cell surface of HSPC and mature blood cells did not differ between patients with ET and normal controls. Furthermore, we also evaluated the phagocytic threshold by in vitro phagocytosis assay using macrophages. There are several subgroups in macrophages<sup>46-49</sup>. M1 macrophages are characterized with pro-inflammatory cytokine secretion and production of nitric oxide, and contribute to killing of intracellular pathogens. M2 macrophages are known to have high interleukin-10 production, and involved in clearing apoptotic cells. Previously, Verstovsek et al. showed that inflammatory cytokines, including interferon gamma,

interleukin-6, tumor necrosis factor alpha and macrophage inflammatory protein beta, are increasing in plasma of patients with *JAK2*-mutated MPNs<sup>50</sup>, thus we investigated phagocytic activity of pro-inflammatory M1 macrophages. As a result, we observed no *CALR*-mutation-dependent effect on the phagocytic sensitivity of HSPCs and mature blood cells. Taken together, although transduction of pro-phagocytic signals to phagocytes is a unique function of cell surface CALR, we conclude that phagocytic dysregulation is not involved in the pathogenesis of *CALR*-mutated ET.

Our results suggest that pathogenesis of *CALR*-mutated ET are not mediated by cell surface CALR, but by intracellular CALR. Previous reports described that mutant *CALR* causes activation of the JAK-STAT pathway and phosphorylation of STAT5, similar to those associated with *JAK2* V617F mutation. Therefore, we isolated HSC and progenitor fractions from *JAK2*-mutated PV/ET and *CALR*-mutated ET BM specimens and evaluated phosphorylation statuses of signaling molecules downstream of JAK2 at each differentiation stage to clarify the involvement of *CALR* mutation in the pathophysiology of ET. We reconfirmed JAK-STAT pathway activation not only in *JAK2*-mutated PV/ET but also in *CALR*-mutated ET. We next identified differences in the HSPC fraction-related STAT phosphorylation patterns between PV and ET. STAT5 phosphorylation was consistently increased in both disease subtypes; however, an individual investigation of the phosphorylation level for each fraction revealed that STAT5 phosphorylation was more robust than STAT1 phosphorylation in CMPs from *JAK2*-mutated PV, whereas the reverse was true in CMPs from *JAK2* and *CALR*-mutated ET. Previous studies involving *Stat1*-deficient mouse models have shown that STAT1 promotes megakaryopoiesis<sup>51</sup> and constrains erythroid differentiation<sup>52</sup>. Additionally, Chen et al. reported that increased STAT1 activity enhanced megakaryocytic differentiation and repressed erythroid differentiation in K562 cells<sup>53</sup>. These



results suggest that the increase in STAT1 phosphorylation prior to MEP differentiation might be critical in the ET phenotype, regardless of the mutation type. However, we did not find any unique phosphorylation patterns in *CALR*-mutated ET compared with those in *JAK2*-mutated ET, indicating that *CALR* mutations activate the JAK-STAT pathway in a manner similar to that associated with *JAK2* mutations in the pathogenesis of ET. Our results are well-matched to those of recent reports describing that mutant *CALR* activates JAK-STAT pathway via interaction with MPL and *JAK2*<sup>12-15</sup>.

In conclusion, we have revealed that the mutation in *CALR* does not change the surface expression of *CALR* and the threshold for phagocytosis by macrophages. We have also identified that *CALR*-mutated ET showed JAK-STAT pathway activation patterns at various HSPC stages similar to those associated with *JAK2*-mutated ET. An understanding of the precise mechanism of JAK-STAT activation is critical to the development of future therapeutic approaches for MPNs.

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**Conflicts of interest:** None

**Financial disclosure:** The authors have no financial interests to disclose.

## References

1. Dameshek, W. (1951) Some speculations on the myeloproliferative syndromes. *Blood*, **6**, 372-375.
2. Baxter, E.J., Scott, L.M., Campbell, P.J., et al. (2005) Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *The Lancet*, **365**, 1054-1061.
3. James, C., Ugo, V., Le Couédic, J., et al. (2005) A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*, **434**, 1144-1148.
4. Jones, A.V., Kreil, S., Zoi, K., et al. (2005) Widespread occurrence of the JAK2 V617F mutation in chronic myeloproliferative disorders. *Blood*, **106**, 2162-2168.
5. Kralovics, R., Passamonti, F., Buser, A.S., et al. (2005) A gain-of-function mutation of JAK2 in myeloproliferative disorders. *The New England Journal of Medicine*, **352**, 1779-90.
6. Levine, R.L., Wadleigh, M., Cools, J., et al. (2005) Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell*, **7**, 387-397.
7. Klampfl, T., Heinz, G., Ashot, H., et al. (2013) somatic mutations of calreticulin in myeloproliferative neoplasms. *The New England Journal of Medicine*, **369**, 2379-2390.
8. Nangalia, J., Massie, C.E., Baxter, E.J., et al. (2013) Somatic *CALR* mutations in myeloproliferative neoplasms with nonmutated *JAK2*. *The New England Journal of Medicine*, **369**, 2391-2405.
9. Michalak, M., Corbett, E.F., Mesaeli, N., Nakamura, K., Opas, M. (1999) Calreticulin: one protein, one gene, many functions. *Biochemical Journal*, **344**, 281-292
10. Michalak, M., Groenendyk, J., Szabo, E., Gold, L., Opas, M. (2009) Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum. *Biochemical Journal*,

**417**, 651-666.

11. Rampal, R., Al-Shahrour, F., Abdel-Wahab, O., et al. (2014) Integrated genomic analysis illustrates the central role of JAK-STAT pathway activation in myeloproliferative neoplasm pathogenesis. *Blood*, **123**, e123-e133.
12. Marty, C., Pecquet, C., Nivarthi, H., et al. (2016) Calreticulin mutants in mice induce an MPL-dependent thrombocytosis with frequent progression to myelofibrosis. *Blood*, **127**, 1317-1324
13. Chachoua, I., Pecquet, C., El-Khoury, M., et al. (2016) Thrombopoietin receptor activation by myeloproliferative neoplasm associated calreticulin mutants. *Blood*, **127**, 1325-1335
14. Araki, M., Yang, Y., Masubuchi, N., et al. (2016) Activation of the thrombopoietin receptor by mutant calreticulin in CALR-mutant myeloproliferative neoplasms. *Blood*, **127**, 1307-1316
15. Elf, S., Abdelfattah, N.S., Chen, E., et al. (2016) Mutant Calreticulin Requires Both Its Mutant C-terminus and the Thrombopoietin Receptor for Oncogenic Transformation. *Cancer Discovery*, **6**, 368-381
16. Chao, M.P., Gotlib, J. (2014) Two faces of ET: CALR and JAK2. *Blood*, **123**, 1438-1440.
17. Chen, C., Gau, J., Chou, H., et al. (2014) Frequencies, clinical characteristics, and outcome of somatic CALR mutations in JAK2-unmutated essential thrombocythemia. *Annals of Hematology*, **93**, 2029-2036.
18. Chi, J., Nicolaou, K.A., Nicolaidou, V., et al. (2013) Calreticulin gene exon 9 frameshift mutations in patients with thrombocytosis. *Leukemia*, **28**, 1152-1154.
19. Finazzi, G., Carobbio, A., Guglielmelli, P., et al. (2014) Calreticulin mutation does not modify the IPSET score for predicting the risk of thrombosis among 1150 patients with

essential thrombocythemia. *Blood*, **124**, 2611-2612.

20. Francesco, P., Domenica, C., Martherita, M. (2014) JAK Inhibitor in CALR-Mutant Myelofibrosis. *The New England Journal of Medicine*, **370**, 1167-1168.
21. Gangat, N., Wassie, E., Lasho, T., et al. (2014) Mutations and thrombosis in essential thrombocythemia: prognostic interaction with age and thrombosis history. *European Journal of Haematology*, **94**, 31-36.
22. Giona, F., Teofili, L., Capodimonti, S., et al. (2014) CALR mutations in patients with essential thrombocythemia diagnosed in childhood and adolescence. *Blood*, **123**, 3677-3679.
23. Li, B., Xu, J., Wang, J., et al. (2014) Calreticulin mutations in Chinese with primary myelofibrosis. *Haematologica*, **99**, 1697-1700.
24. Qiao, C., Sun, C., Ouyang, Y., et al. (2014) Clinical importance of different calreticulin gene mutation types in wild-type JAK2 essential thrombocythemia and myelofibrosis patients. *Haematologica*, **99**, e182-e184.
25. Rotunno, G., Mannarelli, C., Guglielmelli, P., et al. (2014) Impact of calreticulin mutations on clinical and hematological phenotype and outcome in essential thrombocythemia. *Blood*, **123**, 1552-1555.
26. Rumi, E., Pietra, D., Ferretti, V., et al. (2014) JAK2 or CALR mutation status defines subtypes of essential thrombocythemia with substantially different clinical course and outcomes. *Blood*, **123**, 1544-1551.
27. Rumi, E., Pietra, D., Pascutto, C., et al. (2014) Clinical effect of driver mutations of JAK2, CALR, or MPL in primary myelofibrosis. *Blood*, **124**, 1062-1069.
28. Storti, F., Andrikovics, H., Krahling, T., et al. (2014) Distinct clinical characteristics in myeloproliferative neoplasms with calreticulin mutations. *Haematologica*, **99**, 1184-1190.

29. Tefferi, A., Lasho, T.L., Tischer, A., et al. (2014) The prognostic advantage of calreticulin mutations in myelofibrosis might be confined to type 1 or type 1-like CALR variants. *Blood*, **124**, 2465-2466.
30. Tefferi, A., Wassie, E.A., Guglielmelli, P., et al. (2014) Type 1 versus Type 2 calreticulin mutations in essential thrombocythemia: A collaborative study of 1027 patients. *American Journal of Hematology*, **89**, e121-e124.
31. Gold, L.I., Eggleton, P., Sweetwyne, M.T., et al. (2010) Calreticulin: non-endoplasmic reticulum functions in physiology and disease. *The FASEB Journal*, **24**, 665-683.
32. Chao, M.P., Jaiswal, S., Weissman-Tsukamoto, R., et al. (2010) Calreticulin is the dominant pro-phagocytic signal on multiple human cancers and is counterbalanced by CD47. *Science Translational Medicine*, **2**, 63ra94-63ra94.
33. Chao, M.P., Alizadeh, A.A., Tang, C., et al. (2011) Therapeutic antibody targeting of CD47 eliminates human acute lymphoblastic leukemia. *Cancer Research*, **71**, 1374-1384.
34. Chao, M.P., Alizadeh A.A., Tang, C., et al. (2010) Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-hodgkin lymphoma. *Cell*, **142**, 699-713.
35. Chao, M.P., Majeti, R., Weissman, I.L. (2011) Programmed cell removal: a new obstacle in the road to developing cancer. *Nature Reviews Cancer*, **12**, 58-67.
36. Gardai, S.J., McPhillips, K.A., Frasch, S.C., et al. (2005) Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. *Cell*, **123**, 321-334.
37. Martins, I., Kepp, O., Galluzzi, L., et al. (2010) Surface-exposed calreticulin in the interaction between dying cells and phagocytes. *Annals of the New York Academy of*

*Sciences*, **1209**, 77-82.

38. Obeid, M., Tesniere, A., Ghiringhelli, F., et al. (2006) Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nature Medicine*, **13**, 54-61.
39. Tesniere, A., Apetoh, L., Ghiringhelli, F., et al. (2008) Immunogenic cancer cell death: a key-lock paradigm. *Current Opinion in Immunology*, **20**, 504-511.
40. Oku, S., Takenaka, K., Kuriyama, T., et al. (2010) JAK2 V617F uses distinct signalling pathways to induce cell proliferation and neutrophil activation. *British Journal of Haematology*, **150**, 334-344.
41. Kuriyama, T., Takenaka, K., Kohno, K., et al. (2012) Engulfment of hematopoietic stem cells caused by down-regulation of CD47 is critical in the pathogenesis of hemophagocytic lymphohistiocytosis. *Blood*, **120**, 4058-4067.
42. Dupont, S., Massé, A., James, C., et al. (2007) The JAK2 617V>F mutation triggers erythropoietin hypersensitivity and terminal erythroid amplification in primary cells from patients with polycythemia vera. *Blood*, **110**, 1013-1021
43. Stein, B.L., Williams, D.M., Rogers, O., et al. (2011) Disease burden at the progenitor level is a feature of primary myelofibrosis: a multivariable analysis of 164 JAK2 V617F-positive myeloproliferative neoplasm patients. *Experimental Hematology*, **39**, 95-101
44. Majeti, R., Chao, M.P., Alizadeh, A.A., et al. (2009) CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. *Cell*, **138**, 286-299.
45. Pang, W.W., Pluvinau, J.V., Price, E.A., et al. (2013) Hematopoietic stem cell and progenitor cell mechanisms in myelodysplastic syndromes. *Proceedings of the National Academy of Sciences*, **110**, 3011-3016.
46. Martinez, F.O., Sica, A., Mantovani, A. et al. (2008) Macrophage activation and

polarization. *Frontiers in Bioscience*, **13**, 453-461

47. Schwartz, Y.Sh., Svistelnik, A.V. (2012) Functional phenotypes of macrophages and the M1-M2 polarization concept. Part I. Proinflammatory phenotype. *Biochemistry (Moscow)*, **77**, 246-260
48. Murray, P.J., Allen J.E., Biswas, S.K., et al. (2014) Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity*, **41**, 14-20
49. Cassetta, L., Cassol, E., Poli, G., et al. (2011) Macrophage polarization in health and disease. *The Scientific World Journal*, **11**, 2391-2402
50. Verstovsek, S., Kantarjian, H., Mesa, R.A., et al. (2010) Safety and efficacy of INCB018424, a JAK1 and JAK2 inhibitor, in myelofibrosis. *The New England Journal of Medicine*, **363**, 1117-1127
51. Huang, Z., Richmond, T.D., Muntean, A.G., et al. (2007) STAT1 promotes megakaryopoiesis downstream of GATA-1 in mice. *Journal of Clinical Investigation*, **117**, 3890-3899.
52. Duek, A., Lundberg, P., Shimizu, T., et al. (2014) Loss of Stat1 decreases megakaryopoiesis and favors erythropoiesis in a JAK2-V617F-driven mouse model of MPNs. *Blood*, **123**, 3943-3950.
53. Chen, E., Beer, P.A., Godfrey, A.L., et al. (2010) Distinct clinical phenotypes associated with JAK2V617F reflect differential STAT1 signaling. *Cancer Cell*, **18**, 524-535.

## Figure legends

### **Figure 1. Flow cytometric analysis of surface expression of CALR on HSPCs and mature blood cells.**

The cell surface expression levels of CALR were evaluated by flow cytometry. The expression levels are shown as the MFI ratio to normal T cells. (A) The representative histograms of CALR expression on HSPCs. Black line: normal BM, red line: *JAK2*-mutated ET, blue line: *CALR*-mutated ET. (B) CALR expression levels on HSPCs. No significant differences in CALR expression levels among HSPC populations were observed. (C) CALR expression levels on mature blood cells. No significant differences in CALR expression levels among mature blood cells were observed.

### **Figure 2. Flow cytometric analysis of CD47 surface expression on HSPCs and mature blood cells.**

The cell surface expression levels of CD47 were evaluated by flow cytometry. The expression levels are shown as the MFI ratio to normal T cells. (A) The representative histograms of CD47 expression on HSPCs. Black line: normal BM, red line: *JAK2*-mutated ET, blue line: *CALR*-mutated ET. (B) CD47 expression levels on HSPCs. No significant differences in CD47 expression levels among HSPC populations were observed. (C) CD47 expression levels on mature blood cells. No significant differences in CD47 expression levels among mature blood cells were observed.

### **Figure 3. Phagocytosis assay with HSPCs and mature blood cells**

A phagocytosis assay was performed using BM HSPCs and mature blood cells to evaluate



the phagocytosis index. (A) A schematic of the *in vitro* phagocytosis assay. The red arrow indicates the engulfed HSC. (B) Phagocytosis assay with HSPCs. No significant changes in this threshold among HSPC populations were observed. (C) Phagocytosis assay with mature blood cells. No significant changes in this threshold among mature blood cells were observed.

#### **Figure 4. Phosphorylation statuses of molecules downstream of JAK2**

Each HSPC population was isolated from normal BM (n = 7), *JAK2*-mutated PV (n = 5), *JAK2*-mutated ET (n = 5) and *CALR*-mutated ET (n = 5) and subjected to flow cytometric analysis of phosphorylated molecules downstream of JAK2. Phosphorylation levels are shown as ratios of the MFI of each fraction to that of normal BM. (A) Phosphorylation is illustrated as a heat map. HSPCs from MPN BM exhibited increased STAT1 and STAT5 phosphorylation, regardless of disease subtype. (B) STAT1 and STAT5 phosphorylation profiles in MPN HSPCs. In *JAK2*-mutated PV, STAT5 was more robustly phosphorylated than STAT1 in all populations. In contrast, in *JAK2/CALR*-mutated ET, STAT1 was more strongly phosphorylated than STAT5 at the CMP stage, whereas the reverse pattern was observed at the MEP stage.

## Supplemental Materials

### Figure S1

***JAK2*, *MPL* and *CALR* mutation among the patients with *BCR-ABL* negative MPNs.**

### Figure S2

**Colony-forming cell assay and mutation detection in individual colonies.**

HSPCs derived from normal BM (n = 3), *JAK2*-mutated PV (n = 3), *JAK2*-mutated ET (n = 3) or *CALR*-mutated ET (n = 5) were sorted as a single cell to 35-mm plates and cultured in complete methylcellulose. Individual colonies were examined for *JAK2* or *CALR* mutation and the proportions of colonies carrying mutations were calculated. (A) For all disease types, colonies from MEPs or GMPs exhibited an increased frequency of mutations relative to colonies from HSCs; HSCs vs MEPs or GMPs in *JAK2*-mutated PV ( $11.3 \pm 4.2\%$  vs  $57.0 \pm 13.6\%$ ;  $p < 0.05$  and  $11.3 \pm 4.2\%$  vs  $80.3 \pm 1.6\%$ ;  $p < 0.01$ , respectively), HSCs vs MEPs or GMPs in *JAK2*-mutated ET ( $29.4 \pm 6.7\%$  vs  $92.7 \pm 3.0\%$ ;  $p < 0.001$  and  $29.4 \pm 6.7\%$  vs  $97.2 \pm 2.3\%$ ;  $p < 0.001$ , respectively), and HSCs vs MEPs or GMPs in *CALR*-mutated ET ( $43.8 \pm 6.5\%$  vs  $75.8 \pm 3.4\%$ ;  $p < 0.05$  and  $43.8 \pm 6.5\%$  vs  $81.7 \pm 4.0\%$ ;  $p < 0.01$ , respectively). *JAK2*-mutated ET also showed the increased frequency of mutant clones in CMPs (HSCs  $29.4 \pm 6.7\%$  vs CMPs  $67.8 \pm 5.1\%$ ;  $p < 0.01$ ). (B) The results were summarized as pie charts.

### Table S1

List of primers used to detect mutations