Mutant calreticulin knock-in mice develop thrombocytosis and myelofibrosis without a stem cell self-renewal advantage

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Key Points

1. Mutant CALR drives ET and MF in knock-in mice
2. Mutant CALR expression results in expansion of phenotypic HSCs without self-renewal advantage

ABSTRACT

Somatic mutations in the endoplasmic reticulum chaperone calreticulin (CALR) are detected in approximately 40% of patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF). Multiple different mutations have been reported, but all result in a +1bp frame shift and generate a novel protein C-terminus. In this study, we generated a conditional mouse knock-in model of the most common CALR mutation, a 52bp deletion. The mutant novel human C-terminal sequence is integrated into the otherwise intact mouse CALR gene and results in mutant CALR expression under the control of the endogenous mouse locus. $CALR^{del/+}$ mice develop a transplantable ET-like disease with marked thrombocytosis associated with increased and morphologically abnormal megakaryocytes and increased numbers of phenotypically-defined hematopoietic stem cells (HSCs). Homozygous $CALR^{del/del}$ mice developed extreme thrombocytosis accompanied by features of myelofibrosis including leukocytosis, reduced hematocrit, splenomegaly and increased bone marrow reticulin. $CALR^{del/+}$ HSCs were more proliferative *in vitro*, but neither $CALR^{del/+}$ nor $CALR^{del/del}$ displayed a competitive transplantation advantage in primary or secondary recipient mice. These results demonstrate the consequences of heterozygous and homozygous CALR mutations and provide a powerful model for dissecting the pathogenesis of CALR-mutant ET and PMF.

INTRODUCTION

The myeloproliferative neoplasms (MPNs) are chronic hematological malignancies that arise in the hematopoietic stem cell (HSC) compartment and share a variable propensity to transform to acute myeloid leukemia.[1-3] The BCR-ABL-negative 'classic' MPNs are described as falling into three categories: polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). PV and ET are chronic phase disorders with a relatively benign prognosis – major complications include thrombosis and myelofibrotic transformation. PMF is less common and is a more advanced disease. It closely resembles myelofibrotic transformation of PV and ET, and in many cases is likely to represent presentation in accelerated phase of an undiagnosed pre-existing ET.[4,5]

Most patients with one of the three classical MPNs harbor phenotypic driver mutations that affect cytokine signaling through JAK family kinases.[6] The most common lesion is an acquired JAK2V617F mutation.[7-10] With the subsequent discovery of JAK2 exon 12 mutations, it is now clear that the vast majority of PV patients harbor a gain-of-function mutation in JAK2.[11] In ET and PMF, only 50% of patients carry a JAK2 mutation. Of the JAK2 non-mutated patients, approximately 5-10% carry a gain-of-function mutation in THPO receptor (MPL), the gene for the thrombopoietin (THPO) receptor[12], and a further 70-84% possess mutations in CALR, which encodes an endoplasmic reticulum chaperone.[6,13]

Though multiple different CALR mutations have been described,[2,6,13] all are deletions or insertions within exon 9 that results in a +1bp frame shift and thereby generate a protein with a novel C-terminus. Recent structure/function studies have elegantly shown that mutant CALR interacts with

the extracellular domain of THPO receptor to activate downstream signaling and that the novel C-terminus is essential for this process.[14-16] Retroviral studies[14,17] and transgenic studies[18] (in which mutant CALR cDNA was driven by the H-2K^b promoter and Mo-MuLV LTR) have shown that mutant CALR can generate a myeloproliferative phenotype in mice. However, the pathogenetic consequences of expressing mutant CALR in an appropriate cell type-specific manner and at physiological levels are unknown. Here, we describe the generation of knock-in mice expressing the most common form of mutant CALR (52bp deletion) under the control of the mouse CALR locus.

MATERIALS AND METHODS

Generation of hematopoietic-specific conditional knock-in mice for mutant calreticulin

A 9.3 kb region spanning exons 2-9 of mouse CALR (corresponding to nucleotides 16143-25460 in the reverse complementary sequence of the mouse BAC clone RP24-341A18, accession number AC155163) was manipulated to include the targeting cassettes. A LoxP site was inserted into nucleotide position 19478 of the 5' homology arm (nucleotides 16143-20881) which was followed downstream by a PGKNeo-poly(A) minigene and another LoxP site. The 3' homology arm (20141-25460) is preceded by sequence (19499-10782) including part of the mouse intron 7 and of exon 8, which was fused at the BsmBI site with the mutant human CALR cDNA sequence (corresponding to nucleotides 1095-1350, accession number BC020493). A thymidine kinase (TK) minigene located downstream of the 3' homology arm was also included in the targeting vector. The targeting plasmid DNA linearized with NotI was used for gene targeting in embryonic stem cells (ES cells, JM8 in C57BL/6N background) and ES clones resistant to G418 and FIAU were selected. ES clones with correctly targeted alleles (designated $CALR^{fl/+}$) were injected into albino C57BL/6 blastocysts and chimeras were bred with C57BL/6 mice to generate germline heterozygous $CALR^{fl/+}$ mice. All mice were kept in specific pathogen free conditions and all procedures performed according to UK Home Office regulations.

Bone marrow transplantation assays

For transplantation assays, $Kit^{W41/W41}$ (CD45.1) recipients were irradiated with 400 cGy and C57BL/6 (CD45.1) recipients were irradiated with 2 x 550 cGy. The $Kit^{W41/W41}$ strain is derived from C57BL/6 which requires only a sub-lethal dose of radiation and is more permissive for donor cell engraftment since the endogenous HSCs are >10-fold less functional. For non-competitive

transplantation assays, 2 x 10^6 nucleated BM cells were injected into recipient mice. Peripheral blood was obtained every four weeks when full blood counts were determined and donor cell repopulation was examined by flow cytometric analysis using CD45.1 and CD45.2 antibodies (BD Biosciences). For competitive repopulation assays, 1 x 10^6 nucleated competitor BM cells (a 1:1 ratio) were obtained from CD45.1/CD45.2 F_1 mice and injected into recipient mice. For secondary BM transplantation, 5 x 10^6 nucleated BM cells from primary recipients were injected into recipient mice. In all cases, peripheral blood was obtained and analyzed by flow cytometry for donor contribution to myeloid (Ly6g and Mac1) and lymphoid (B220 and CD3e) cells.

Single HSC *in vitro* Cultures

In vitro culture of single E-SLAM HSCs was performed as previously described.[19,20] E-SLAM HSCs were sorted into round-bottom 96-well plates, preloaded with 50 µL serum-free Stemspan medium (STEMCELL Technologies). A further 50 µL of medium was subsequently added to each well to a final concentration of 10% FCS, 1% Pen/Strep, 1% L-Glut, 300 ng/mL SCF (STEMCELL Technologies), 20 ng/mL IL-11 (STEMCELL Technologies) and 0.1 mM β-mercaptoethanol. For cell division kinetics, the number of cells in each well was counted manually every day for up to 4 days in culture. To evaluate the number and proportion of mature cell types produced by single E-SLAM HSCs *in vitro*, after 7 days in culture, clonal cells were stained with antibodies for lineage markers (CD3, Ly6G/Ly6C, CD11b, CD45R/B220, Ter-119), Sca-1 BV605 and c-Kit APC/Cy7 to assess the frequencies of LSK (Lineage$^-$Sca-1$^+$c-Kit$^+$) and lineage positive cells. Lineage output of single cell cultures was also analyzed after 14 days using flow cytometry with antibodies for Mac1 APC, CD41 PE, and CD71 FITC.

Statistics

The statistical differences between control and CALR$^{del/+}$ mice were assessed using a two-tailed, unpaired Student's *t*-test unless otherwise indicated.

RESULTS

Generation of conditional mutant calreticulin knock-in mice

To study the role of calreticulin mutations in the pathogenesis of MPNs, we generated a conditional knock-in allele in murine ES cells using homologous recombination for the most common CALR mutation (52 bp deletion) (Figure 1A). Species-specific differences in codon preferences meant that simply generating an analogous 52bp deletion within the mouse sequence would generate a novel C-terminus substantially different from that seen in ET patients (Figure 1B). We therefore adopted a strategy in which the targeted allele (CALR^{fl}) contains a LoxP site in intron 7, and a PGKNeo-polyA LoxP cassette followed by mouse exon 8 fused with mutant human calreticulin 3' cDNA sequence downstream of exon 9 (Figure 1A; supplemental Figure 1A). This approach allowed the generation of a conditional allele expressing a mutant calreticulin protein with a carboxy-terminal amino acid sequence identical to that found in MPN patients, whilst keeping the mouse sequence otherwise intact (Figure 1B). The targeted allele (CALR^{fl}) is predicted to express wild-type mouse calreticulin and can be induced to express humanized mutant calreticulin under the control of murine CALR regulatory elements upon removal of the floxed mouse CALR exons and PGKNeo-polyA by Cre-mediated recombination. Correctly targeted ES clones were characterized by genomic PCR (Figure 1C, 1D; supplemental Figure 1B). Targeted ES clones were expanded and then subjected to transient Cre expression using a PGK-Cre plasmid. Genomic PCR confirmed the recombination of the allele (Figure 1E). RT-PCR was performed on total RNA from ES cells after Cre recombination and the PCR product was cloned and sequenced to demonstrate expression of CALR mRNA with the 52bp deletion (Figure 1F; supplemental Figure 1C). Correctly targeted ES cells were then injected into C57BL/6 albino blastocysts to produce chimeras. The resulting chimeric mice were bred to C57BL/6 mice to generate germline

transmission (i. e. CALR$^{fl/+}$ mice). Quantitative RT-PCR demonstrated that CALR$^{fl/+}$ mice expressed levels of CALR comparable with CALR$^{+/+}$ mice (supplemental Figure 1D).

CALR$^{del/+}$ mice develop a myeloproliferative phenotype resembling human essential thrombocythaemia

CALR$^{fl/+}$ mice were crossed with Mx1Cre transgenic mice[21] and expression of Cre recombinase was induced with polyinosine-polycytosine (pIpC) injection 6-8 weeks after birth. Mice carrying the recombined allele with mutant calreticulin expression were denoted CALR$^{del/+}$. Without pIpC treatment, recombination did not occur at detectable levels and blood counts were normal in Mx1Cre positive CALR$^{fl/+}$ mice (supplemental Figures 2A, B). By 4 weeks after pIpC, genomic PCR showed recombination in more than 80% of nucleated blood cells (supplemental Figure 2C). Mutant CALR RNA expression was confirmed by RT-PCR in bone marrow cells (supplemental Figure 2D). In HSPCs, transcript levels of mutant CALR were 65-72% (mean 68%) of transcript levels from the WT allele as analysed by fragment analysis (Supplemental Figure 2E). A reduction in the level of mutant transcripts was also seen using several different expression constructs (supplemental Figure 2F) and in a previously reported transgenic model,[18] indicating that lower levels of mutant CALR transcripts are not specific to the targeting construct used to generate our knock-in mice, and may instead reflect altered stability associated with the novel 3' sequence. Levels of the mutant protein in CALR$^{del/del}$ mice were much lower than levels of WT protein in control cells (supplemental Figure 2G), an observation consistent with the previous demonstration that the mutant protein is unstable[51,52] and also consistent with the results from a retroviral transplantation model.[17]

CALR$^{del/+}$ mice developed a striking thrombocytosis compared with wild-type littermate controls. White blood cell (WBC) counts were mildly elevated in some but not all cohorts, and red blood cell (RBC) parameters were not affected (Figure 2A). No difference in survival was observed in cohorts maintained for up to 8 months (supplemental Figure 3A). We also crossed CALR$^{fl/+}$ mice with VavCre transgenic mice[22]. Similar to results with Mx1Cre, VavCre resulted in high rates of recombination in peripheral blood and mice developed thrombocytosis (supplemental Figure 3B and C).

In JAK2^{V617F} knock-in mice, platelet reactivity to collage-related peptide (CRP) and thrombin agonists was increased.[23] We therefore studied platelet reactivity to several agonists including CRP, thrombin and ADP, but observed no significant increase in response to any agonist using platelets of the CALR$^{del/+}$ mice (supplemental Figure 3D).

Bone marrow cellularity and spleen weight were not significantly affected in CALR$^{del/+}$ mice 3-4 months after pIpC injection (supplemental Figure 4A and B). Histological analysis of BM from CALR$^{del/+}$ mice showed megakaryocytic hyperplasia with megakaryocytes displaying large and hyperlobated nuclei and increased clustering (Figure 2B). Spleen architecture was not affected, although a degree of extra-medullary hematopoiesis was observed in some cases, and there was no significant increase in reticulin staining in either bone marrow or spleen at this time point (Figure 2B; supplemental Figure 4C). Compared to littermate controls, the bone marrow of CALR$^{del/+}$ mice contained increased numbers of CFU-MKs (Figure 2C) and megakaryocytes (Figure 2D) together with reduced numbers of CD71^{+}Ter119^{+} erythroblasts (Figure 2E). Numbers of myeloerythroid progenitor populations and more mature myeloid cells (Mac1^{+}Ly6g^{+}) in the

bone marrow were not significantly altered (supplemental Figure 4D). In the spleen, there was no significant alteration in the proportion of megakaryocytes, myeloid cells or erythroblasts (supplemental Figure 4E). Taken together, these data demonstrate that CALR$^{del/+}$ mice displayed a marked thrombocytosis accompanied by increased megakaryopoiesis and compromised erythropoiesis in the bone marrow.

Thrombocytosis in CALR$^{del/+}$ mice is transplantable

Thrombopoietin (THPO) levels were not raised in CALR$^{del/+}$ mice and were, if anything, reduced (although this did not reach statistical significance), suggesting that the thrombocytosis was not THPO-driven and was likely to be cell-intrinsic (Figure 3A). To confirm this inference, wild-type recipient mice were transplanted with bone marrow cells from CALR$^{del/+}$ mice 2 months post pIpC. At both one month and four months after transplant, the level of chimerism was approximately 80% in peripheral blood mononuclear cells, myeloid cells (Ly6g^{+} and Mac1^{+}) and lymphoid cells (B220^{+} and CD3e^{+}) (Figure 3B; supplemental Figure 5A). Recipients of CALR$^{del/+}$ bone marrow developed marked thrombocytosis with platelet numbers similar to donor mice by 4 months post-transplantation (Figure 3C). Following secondary transplants, recipients also developed thrombocytosis (Figure 3D) with donor chimerism at 4 months post transplantation around 60% for peripheral blood mononuclear cells, myeloid cells and lymphoid cells (Figure 3E, supplemental Figure 5B) indicating that thrombocytosis was still evident in the presence of 40% normal hematopoietic cells. These data demonstrate that the thrombocytosis in CALR$^{del/+}$ mice is transplantable and reflects a bone marrow-cell intrinsic mechanism.

Mutant CALR is associated with increased proliferation of single HSCs *in vitro* but no self-renewal advantage *in vivo*

The bone marrow of CALR$^{del/+}$ mice contained increased numbers of HSCs, both Lin$^-$ Sca1$^+$cKit$^+$CD150$^+$CD48$^-$ HSCs (Figure 4A) and E-SLAM HSCs (CD45$^+$EPCR$^+$CD150$^+$CD48$^-$, supplemental Figure 6A). To examine the effect of mutant CALR on the behaviour of HSCs, we studied the survival and cell division kinetics of single HSCs together with their ability to proliferate and differentiate.[19,20] Compared to wildtype controls, the proportion of single CALR$^{del/+}$ HSCs that gave rise to clones of over 50 cells after 7 days in culture was significantly higher (Figure 4B). Single HSCs from CALR$^{del/+}$ mice showed significantly earlier cell division compared with those from littermate controls (Figure 4C) and formed larger clones after 7 days in culture (Figure 4D, supplemental Figure 6B). Although clones from CALR$^{del/+}$ HSCs were larger, flow cytometric analysis showed that they contained unaltered percentages of LSK cells, lineage positive, Mac1$^+$, CD41$^+$ and CD71$^+$ cells (supplemental Figure 6C-E).

To investigate the functional consequences of mutant calreticulin on HSCs, competitive bone marrow transplantation was performed using cells from mice at 2 months after pIpC. Bone marrow cells from CALR$^{del/+}$ or littermate control mice (both CD45.2) were mixed with equal numbers of CD45.1/45.2 F1 competitor bone marrow cells and transplanted into irradiated C57Bl/6 recipients (CD45.1). The contribution of CALR$^{del/+}$ cells to recipient peripheral blood, myeloid cells (Ly6g$^+$ and Mac1$^+$) and lymphoid cells (B220$^+$ and CD3e$^+$) was similar to littermate controls at both 1 and 4 months following transplantation (Figure 4E). Recipients of CALR$^{del/+}$ bone marrow did not develop thrombocytosis (supplemental Figure 6F); probably because donor chimerism was usually lower than 50% in this setting. Analysis of the recipient bone marrow 4 months after transplant

revealed a similar picture. Compared to control cells, CALR$^{del/+}$ cells showed no increase in their contribution to E-SLAM HSCs (Figure 4F), myeloid and lymphoid lineages (Figure 4G). Secondary transplants were performed, and at 4 months after transplantation, CALR$^{del/+}$ cells again showed no significant repopulation advantage (Figure 4H). Together, these data show that CALR$^{del/+}$ bone marrow contains more phenotypically defined HSCs, and these cells exhibit increased proliferation *in vitro*, but the long term *in vivo* repopulating activity of CALR$^{del/+}$ bone marrow is normal.

Homozygosity for CALR mutation (CALR$^{del/del}$) results in severe thrombocytosis and development of myelofibrosis

To assess the pathological consequences of homozygous expression of mutant calreticulin, CALR$^{fl/+}$ mice on an Mx1Cre background were crossed with each other to generate CALR$^{fl/fl}$ mice. Upon Cre recombination, mice with homozygous expression of mutant calreticulin (denoted as CALR$^{del/del}$) developed extreme thrombocytosis with platelet counts around 9x 10^6/uL by 3-4 months after pIpC (Figure 5A). This was accompanied by a modest but significant increase in WBC numbers and reduced hematocrit (Figure 5A). By 6-8 months post pIpC, CALR$^{del/del}$ mice showed splenomegaly (Figure 5B, supplemental Figure 7A) and reduced bone marrow cellularity (supplemental Figure 7B). Macroscopically, bone marrow samples from the CALR$^{del/del}$ were paler and their spleen cell samples were redder in colour compared with the littermate controls (supplemental Figure 7C). Histological analysis of bone marrow showed almost complete effacement of normal hematopoiesis by megakaryocytes displaying nuclear atypia and accompanied by increased levels of reticulin (Figure 5C, supplemental Figure 7D). H&E staining of sections of the spleen showed destruction of normal splenic architecture with markedly

increased numbers of atypical megakaryocytes without increased levels of reticulin (Figure 5C, supplemental Figure 7E).

Flow cytometric analysis demonstrated that CALR$^{del/del}$ bone marrow harboured substantially increased numbers of HSCs, both Lin^{-}Sca1^{+}cKit^{+}CD150^{+}CD48^{-} HSCs (Figure 5D) and E-SLAM HSCs (supplemental Figure 7F). Marked increases in the proportion of megakaryocyte progenitors (MkP, Lin^{-}Sca1^{-}cKit^{+}CD150^{+}CD41^{+}) were noted in both bone marrow and spleen (Figures 5E). Myeloid progenitors (Lin^{-}Sca1^{-}cKit^{+}), PreGM (Lin^{-}Sca1^{-}cKit^{+}CD41^{-}CD16/32^{-}CD105^{-}CD150^{-}) and GMP (Lin^{-}Sca1^{-}cKit^{+}CD41^{-}CD16/32^{+}CD150^{-}) were expanded in CALR$^{del/del}$ mice (Figure 5F; supplemental Figure 7G), and erythroblasts (CD71^{+}Ter119^{+}) were reduced in bone marrow but erythroid progenitors (PreCFU-E, Lin^{-}Sca1^{-}cKit^{+}CD41^{-}CD16/32^{-}CD105^{+}CD150^{+}) were increased in the spleen (Figure 5G; supplemental Figure 7G). Taken together, these data demonstrate that homozygosity for mutant CALR produces a dramatic phenotype with extreme thrombocytosis, a marked increase in megakaryopoiesis, myelofibrosis and HSC expansion.

Non-competitive bone marrow transplants were performed to see if this extreme thrombocytosis is transplantable. Wild-type recipient mice were transplanted with bone marrow cells from CALR$^{del/del}$ mice. At 4 months after transplant, the level of chimerism was approximately 70% in peripheral blood mononuclear cells, myeloid cells (Ly6g^{+} and Mac1^{+}) and lymphoid cells (B220^{+} and CD3e^{+}) (Figure 6A). Recipients of CALR$^{del/del}$ bone marrow developed a similar degree of thrombocytosis with platelet numbers similar to the CALR$^{del/del}$ donor mice by 5 months post-transplantation as well as leukocytosis (Figure 6A). These data demonstrate that the extreme

thrombocytosis in CALR$^{del/del}$ mice is transplantable. To investigate the functional consequences of mutant calreticulin homozygosity on HSCs, competitive bone marrow transplantation was performed. Bone marrow cells from CALR$^{del/del}$ or littermate control mice (both CD45.2) were mixed with equal numbers of CD45.1/45.2 F1 competitor bone marrow cells and transplanted into irradiated Kit$^{W41/W41}$ C57Bl/6 recipients (CD45.1). The contribution of CALR$^{del/del}$ cells to recipient peripheral blood, myeloid cells (Ly6g^{+} and Mac1^{+}) and lymphoid cells (B220^{+} and CD3e^{+}) was analysed by flow cytometry and was similar to littermate controls (Figure 6B). At 4 months post transplantation, secondary transplants were performed, analysis showed that CALR$^{del/del}$ cells exhibit similar competitive repopulating capacity in the secondary recipients (Figure 6C). Together, these data demonstrate that CALR$^{del/del}$ bone marrow contains strikingly expanded phenotypically defined HSCs, but the long term *in vivo* repopulating activity of CALR$^{del/del}$ bone marrow are comparable to the wildtype controls.

DISCUSSION

In this paper, we describe a knock-in mouse model of the most common CALR mutation, an approach that allows us to explore the consequences of mutant CALR without the confounding effects of additional mutations present in samples from MPN patients. The targeting strategy was chosen to result in expression of a murine CALR protein in which the wild type C-terminus is replaced by the mutant human C-terminus found in MPN patients. Following Cre-mediated recombination, the mutant transcript is expressed under the control of the full complement of endogenous CALR regulatory elements.

Heterozygous $CALR^{del/+}$ mice develop an ET-like phenotype with marked thrombocytosis, increased megakaryopoiesis and abnormal bone marrow megakaryocyte morphology. Hemoglobin and red cell levels were normal and there was no increased reticulin staining in the bone marrow. The results contrast with the phenotype of JAK2V617F knock-in mice in which platelets, hemoglobin and leukocytes are all elevated and the bone marrow contains increased erythroid, myeloid and megakaryocytic progenitors.[24-27] Moreover, these differing phenotypes are consistent with what is observed in JAK2-mutant compared to CALR-mutant MPN patients. The isolated thrombocytosis seen in $CALR^{del/+}$ mice resembles that seen in CALR-mutant ET patients and, compared to JAK2-mutant ET patients, those carrying a CALR mutation have higher platelet levels with lower levels of hemoglobin and white cells.[28-31] Furthermore, the effect of mutant CALR on platelet reactivity was different from that previously observed with JAK2 mutant cells.[23] Whilst the former showed normal responses to platelet agonists, JAK2 mutant platelets were hyper-reactive. These results are in keeping with the increased thrombosis rate observed in patients bearing JAK2 mutations.[30]

Homozygosity for CALR mutations has been observed in patients with ET,[13,31,32] but its pathogenetic consequences are unclear. Our results demonstrate that homozygosity for mutant CALR causes extreme thrombocytosis associated with increased white cell counts, reduced hemoglobin levels, splenomegaly and increased levels of bone marrow reticulin, a constellation of features that resemble myelofibrotic transformation of ET. This phenotype contrasts with that seen in mice homozygous for JAK2V617F, which show extreme elevation of hemoglobin levels and a fall in platelet counts relative to mice heterozygous for JAK2V617F.[33] Together, our data demonstrate clear differences in the consequences of CALR and JAK2 mutations, and that these differences are more pronounced in the homozygous setting.

Our results accord with observations made using retroviral transplants or transgenic mice expressing the human mutant CALR.[14,17,18] Both approaches resulted in thrombocytosis and megakaryocytic hyperplasia, but myelofibrosis was only seen in mice harboring retroviral constructs,[17] perhaps reflecting varying levels of expression achieved by these different approaches. This concept is consistent with our finding of myelofibrosis is in $CALR^{del/del}$ but not in $CALR^{del/+}$ mice.

Our results shed light on the role of mutant CALR within the HSC compartment. Bone marrow transplant studies demonstrated that the ET-like phenotype seen in $CALR^{del/+}$ mice could be propagated in both primary and secondary recipients. These results demonstrate that CALR-mutant long-term repopulating HSCs are able to give rise to features of ET even in secondary recipients where chimerism levels were reduced to 60%. These observations accord with studies of patients with CALR-mutant ET, which showed that the CALR mutation is present in highly

purified HSCs.[6] Moreover, analyses of individual hematopoietic colonies showed that the CALR mutation was present in the first mutant phylogenetic node in all 5 patients studied, indicating that it was likely to be an initiating mutation in these patients.[6]

However, our data show that mutant CALR does not confer a competitive HSC advantage in serial transplants in either heterozygous or homozygous settings. This result is consistent with the report of a transgenic mouse model which concluded that HSCs expressing mutant CALR exhibited normal self-renewal.[18] In a retroviral transplant model, phenotypic GFP^+ LSK and GFP^+ SLAM LSK cells expanded over time in primary recipient mice.[17] However, secondary transplants were not performed and therefore the impact on long term HSC function remains unclear. Both transgenic and retroviral approaches are known to result in dysregulated expression, both in terms of transcript levels and pattern of expression, which may contribute to the distinct phenotypes.

It is interesting that our knock-in mutant CALR mice (and three independent mutant JAK2 knock-in mice[20,24,34,35]) all show no HSC advantage in serial transplantation studies, despite mutant CALR (or mutant JAK2) being found as a sole driver mutation in a substantial proportion of MPN patients. There are several potential explanations (not mutually exclusive) for this apparent conundrum: (i) genetic background may cooperate with mutant CALR — there is mounting evidence that genetic background influences HSC function in mice[36,37] and also the development of both MPNs[38-40] and clonal hematopoiesis[41]; (ii) unidentified somatic driver mutations may cooperate with mutant CALR — it seems unlikely that many additional coding mutations remain to be discovered, but there is much less known about the role of somatic non-coding mutations affecting regulatory elements in tumors[42-44]; (iii) age-related changes affecting the

microenvironment (eg niche or inflammatory signaling) may explain the association of MPNs with increasing age[45-47]; and (iv) lineage tracing studies suggest that steady state hematopoiesis is supported by long-lived progenitors that may not read out in transplantation assays.[48,49]

Levels of the mutant protein were much lower than levels of WT protein, consistent with previous findings that the mutant protein is unstable[50,51] and less abundant compared to WT controls in a retroviral expression model.[17] We also noted that, in HSPCs from heterozygous knock-in mice, mutant transcripts were expressed at lower levels (mean 68%) relative to WT transcripts. We considered the possibility that this might reflect a technical problem with our construct and explain the lack of a clonal advantage. Although our data do not formally exclude this scenario, we believe this is highly unlikely for multiple reasons: (1) The knock-in construct gives rise to strong HSC, progenitor, megakaryocytic and platelet phenotypes. HSC, progenitor and platelet numbers are similar to or higher than those seen in the retroviral or transgenic mouse models.[14,17,18] Thus, our knock-in construct clearly has functional consequences in multiple cell types and stages of differentiation. (2) The lack of an HSC clonal advantage is consistent with results obtained from the transgenic CALR model where similar serial competitive transplants were performed (Fig 4c in Shide et al[18]). (3) In homozygous mice, which have double the dose of the mutant allele, we still see no clonal advantage in secondary transplant recipients. This is inconsistent with the concept that a 32% decrease in mutant transcript levels is responsible for the lack of an HSC repopulation advantage. (4) Our results demonstrate that several mutant CALR expression constructs (which are completely different from our knock-in construct; supplemental Fig. 2F) also give rise to lower transcript levels compared to WT controls. In addition, the transcript levels of mutant CALR in the transgenic model are reported to be 64% of those of WT levels (Fig 3B in

Shide et al[18]). Together, these data indicate that lower levels of mutant CALR transcript are not specific to the targeting construct for our knock-in mice and may represent altered stability associated with the novel 3' sequence in mice. It remains to be seen if lower mutant CALR mRNA levels are also present in patient samples.

It is informative to compare the consequences of CALR and JAK2 mutations on HSCs. CALR$^{del/+}$ mice displayed an increased number of phenotypic HSCs, but whole bone marrow serial transplantation experiments showed no functional alteration in competitive repopulation ability. Across several reported heterozygous JAK2^{V617F} knock-in mouse models, phenotypic HSC frequency was variable, with some models having increased HSC numbers and others reporting a reduction.[20,35,52,53] Nevertheless, where secondary transplants were performed, none of these models had a competitive self-renewal advantage compared to WT cells and, if anything, there was a functional decline in repopulating ability for JAK2$^{V617F/+}$ cells in secondary recipients.[20,24,35,54] *In vitro*, single E-SLAM HSCs from CALR$^{del/+}$ mice entered cell division earlier and produced substantially larger clones (3 fold increase in size) but did not display aberrant differentiation characteristics compared with their wild-type counterparts. In contrast, single HSCs from heterozygous JAK2 mice did not show changes in early cell divisional kinetics but did give rise to larger, more differentiated clones. Notably, these clones contained increased lineage positive cells and reduced stem/progenitor (LSK) cells.[20] Thus, while both mutant JAK2 and CALR can drive a myeloproliferative phenotype, their HSCs have distinct features both *in vivo* and *in vitro*, suggesting differences in downstream signaling pathways and in mechanisms of clonal expansion.

Taken together, our results support the concept that ET consists of biologically distinct entities associated with different mutations. It seems likely that it will become increasingly important to subclassify MPNs on the basis of their underlying causal mutations instead of relying on their phenotypic consequences (e.g. levels of hematocrit or megakaryocytic morphology). Recent results suggest that the different consequences of CALR and JAK2 mutations reflect, at least in part, differential interaction of mutant CALR and JAK2 with the homodimeric cytokine receptors EPOR, THPO receptor and GCSFR, with mutant CALR preferentially binding to and activating THPO receptor.[14,15] Our mouse model provides a powerful tool to further dissect the mechanisms by which mutant CALR contributes to MPN pathogenesis.

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AUTHORSHIP CONTRIBUTIONS

Contribution: J.L. designed and performed experiments and analyzed the data with input from D.P. on lineage and single HSC analysis, H.J.P. on flow cytometry analysis, C.G.A. on MK colony assays, J.G. on histology, O.M.D. on help in generating the model, T.K. for help in fragment analysis and C.B. on platelet reactivity analysis. T.L.H., D.C.P., R.S., M.W and J.A. provided assistance in generation and phenotypic analysis of the mice cohorts; C.G. collaborated on platelet analysis; G.S.V. collaborated in the generation of the knock-in model. D.G.K. collaborated on HSC analysis; J.L. and A.R.G. wrote the paper with input from D.P., S.L. and D.G.K.; A.R.G. directed the research.

DISCLOSURE OF CONFLICT INTERSTS

The authors declare no competing financial interests.

FIGURE LEGENDS

Figure 1. Generation of a conditional humanized mutant CALR knock-in mouse model. (A) Diagrams showing the endogenous mouse CALR gene locus ($CALR^{+}$), the targeting construct, the targeted conditional knock-in allele ($CALR^{fl}$) and the recombined allele ($CALR^{del}$) following Cre recombination to remove the PGKNeopAstop cassette. The recombined allele is designed to carry the mouse CALR genomic region including mouse exons 1-7 followed by the 5' part of mouse exon 8 fused to the 3' part of the mutant human CALR cDNA sequence (orange). LoxP sites are indicated by red arrows. (B) Amino acid alignment of wild type and mutant calreticulin proteins. There is a 94% identity between the entire amino acid sequences of human and mouse proteins; alignments show amino acid 343 to the C-terminus of human mutant CALR and the designed humanized mutant CALR knock-in product (top panel) and the predicted product if the corresponding 52bp region is deleted in the mouse CALR gene. Alignment was performed using an online program (www.expasy.org). Sites of the mouse/human junction and the 52bp deletion are indicated. (C) Diagram showing location of the primers for PCR genotyping. (D) Characterization of ES cell targeting. PCR performed on genomic DNA from ES clones (lanes 1-5) using primers F and R shown in (C). The ES clone in lane 5 is correctly targeted. M; DNA size marker. (E) Characterization of Cre/LoxP mediated recombination of targeted ES cells. PCR was performed on genomic DNA from ES cells upon Cre recombinase expression using primers F and R shown in (C). M, DNA marker; fl/+, targeted ES clone; +/+, wild type ES clones; lanes 1-2, Cre recombined ES clones. (F) Sequencing traces of RT-PCR product showing correct humanized mutant CALR expression with the 52bp deletion indicated (52bp del). RT-PCR was performed on total RNA from an ES clone that was Cre recombined and the PCR product was cloned and sequenced.

Figure 2. CALR$^{del/+}$ mice develop a myeloproliferative disease. (A) Time course of blood parameters of CALR$^{del/+}$ and control mice showing significantly increased platelets, normal hematocrit and hemoglobin. A mildly elevated white blood cell count is shown and is only seen in some cohorts. The CALR$^{del/+}$ mice were CALR$^{fl/+}$ Mx1Cre^{+} and the littermate control mice CALR$^{+/+}$ were CALR$^{fl/+}$ Mx1Cre^{-} or CALR$^{+/+}$ Mx1Cre^{+}. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; mean ± SEM are shown. (B) Histological analysis showing megakaryocytic hyperplasia with increased clustering and hyperlobated nuclei without fibrosis. H&E staining was performed on bone marrow (left panel) from mice at 3-4 months after pIpC injection; bone marrow staining for reticulin (middle panel) showing no fibrosis and H&E staining of spleen (right panel) showing normal splenic architecture. (C) CALR$^{del/+}$ mice show increased megakaryocyte colonies. Bar graphs showing significantly increased number of CFU-MK colonies in bone marrow. CFU-MK assay was performed in media with or without TPO. (D) CALR$^{del/+}$ mice show increased megakaryocytes. Flow cytometry was performed to assess the frequency of bone marrow megakaryocytes (MK, CD41^{+}CD42^{+} or CD41^{+}CD61^{+}). (E) CALR$^{del/+}$ mice show reduced erythroblasts in bone marrow. Flow cytometry was performed to assess the frequency of erythroblasts (CD71^{+}Ter119^{+}). * $P < 0.05$; ** $P < 0.01$; data are shown as mean ± SEM.

Figure 3. Thrombocytosis in CALR$^{del/+}$ mice is transplantable. (A) Plasma THPO level is not significantly altered in CALR$^{del/+}$ mice. (B) Donor chimerism is comparable in the primary non-competitive bone marrow transplants. Bone marrow cells from CALR$^{del/+}$ or control mice (2 x 10^{6} per recipient) were transplanted into irradiated (2 x 550 rads) CD45.1 C56BL/6 recipients respectively. Donor chimerism in peripheral blood was analyzed using flow cytometry with CD45.1 and CD45.2 antibodies and are derived as percentage of CD45.2-positive cells in whole

nucleated blood. (C) Time course of blood counts showing significantly increased platelet counts in the recipients of CALR$^{del/+}$ bone marrow. * P <0.05; ** P <0.01; *** P <0.001; data are shown as mean ± SEM. (D) Time course of blood counts showing significantly increased platelet counts in the secondary transplant recipients of CALR$^{del/+}$ bone marrow. Bone marrow cells from the primary recipients of CALR$^{del/+}$ or control mice bone marrow (5 x 10^6 per recipient) were transplanted into irradiated (1 x 400 rads) *Kit*$^{W41/W41}$ (CD45.1) recipients respectively. * P < 0.05; data are shown as mean ± SEM. (E) Donor chimerism is comparable in the secondary non-competitive bone marrow transplants. Donor chimerism in peripheral blood was analyzed using flow cytometry as above.

Figure 4. Mutant CALR increases phenotypic HSCs with altered behavior *in vitro* but no self-renewal advantage *in vivo*. (A) CALR$^{del/+}$ mice show increased frequency of HSCs in the bone marrow. Flow cytometry was performed on bone marrow from mice at 3-4 months post pIpC injection and HSC is defined as lineage$^-$Sca-1$^+$c-Kit$^+$CD150$^+$CD48$^-$. **, P<0.01; data are shown as mean ± SEM. (B) CALR$^{del/+}$ E-SLAM HSCs have a significantly higher cloning efficiency than wild-type E-SLAM HSCs. Single E-SLAMs were defined as successfully forming a clone if assayed to have more than 50 cells at day 7 as assessed by flow cytometry. Both CALR$^{+/+}$ and CALR$^{del/+}$ represent 72 individual E-SLAMs HSCs from 3 independent experiments; **, P<0.01; p-value was calculated from a chi-squared test. (C) E-SLAM HSCs from CALR$^{del/+}$ mice exhibit early cell division in a single cell *in vitro* assay. Line graphs showing significantly higher proportion of E-SLAM HSCs completed first division during the 4 days of single cell *in vitro* analysis. *, P<0.01; p-value was calculated from a chi-squared test; three independent experiments were performed. (D) E-SLAM HSCs from CALR$^{del/+}$ mice form larger clones *in vitro*.

After 7 days in culture, E-SLAM clones (irrespective of size) were analyzed using flow cytometry and the average number of cells per clone was measured; *, $P<0.01$; data are shown as mean ± SEM. (E) Bone marrow cells from CALR$^{del/+}$ mice show similar repopulating capacity in peripheral blood of primary competitive transplants. Donor repopulation was assessed using flow cytometry of nucleated peripheral blood with antibodies for CD45.1 and CD45.2 to distinguish donor origin; Ly6g and Mac1 for myeloid; and B220 and CD3e for lymphoid lineages. Competitive repopulating ability is presented as the percentage of repopulated cells derived from test donor cells among the total number of donor-derived cells (i.e. test/(test + competitor)). Bar graphs showing bone marrow cells from CALR$^{del/+}$ mice exhibit comparable repopulating capacity to both myeloid and lymphoid cells in peripheral blood. Mean ± SEM are shown. (F) CALR$^{del/+}$ bone marrow primary recipients show similar frequency of donor derived E-SLAM HSCs compared to the control recipient mice. Flow cytometry was performed using antibodies for CD45.1 and CD45.2 together with antibodies for E-SLAM HSC markers. Data are shown as mean ± SEM. (G) Bone marrow cells from CALR$^{del/+}$ mice show similar repopulating capacity in bone marrow of primary competitive transplants. Primary recipients were culled 4 months after transplantation and flow cytometry was performed as above to assess donor competitive repopulating ability in the bone marrow. Bar graphs showing bone marrow cells from CALR$^{del/+}$ mice exhibit comparable repopulating capacity to both myeloid and lymphoid lineages in the bone marrow of the primary recipients; data are shown as mean ± SEM. (H) Bone marrow cells from CALR$^{del/+}$ mice show similar repopulating capacity in secondary competitive transplants. Donor repopulation was assessed using flow cytometry of peripheral blood as in (E). Bar graphs showing comparable repopulating capacity to multiple lineages in peripheral blood in the secondary competitive transplantation recipients; data are shown as mean ± SEM.

Figure 5. CALR$^{del/del}$ mice develop an extreme myeloproliferative disease with myelofibrosis. (A) CALR$^{del/del}$ mice develop marked thrombocytosis. Bar graphs showing significantly increased platelets, elevated WBC counts and reduced hematocrit in CALR$^{del/del}$ mice at 3-4 months post pIpC. The CALR$^{del/del}$ mice were CALR$^{fl/fl}$ Mx1Cre^{+} and CALR$^{del/+}$ mice were CALR$^{fl/+}$ Mx1Cre^{+} and the control mice (CALR$^{wt/+}$) were CALR$^{fl/+}$ Mx1Cre^{-} or CALR$^{+/+}$ Mx1Cre^{+}. *, P<0.01; ***, P<0.001; data are shown as mean ± SEM. (B) CALR$^{del/del}$ mice show splenomegaly. (C) CALR$^{del/del}$ mice show striking megakaryocytic hyperplasia. Tissues from mice at 7-8 months post pIpC were taken for histological analysis. H&E staining of bone marrow (left panel) showing almost complete effacement of normal haematopoiesis by megakaryocytes, displaying nuclear atypia in CALR$^{del/del}$ mice; silver staining for reticulin (middle panel) showing bone marrow fibrosis in CALR$^{del/del}$ mice; H&E staining of spleen (right panel) showing destruction of splenic architecture in CALR$^{del/del}$ mice. (D) CALR$^{del/del}$ mice show markedly increased frequencies of HSCs. Flow cytometry was performed and HSCs were defined as Lin^{-}cKit^{+}Sca1^{+}CD150^{+}CD48^{-}. (E) CALR$^{del/del}$ mice show markedly increased frequencies of megakaryocytic progenitors (MkP, Lin^{-}cKit^{+}Sca1^{-}CD150^{+}CD41^{+}). (F) CALR$^{del/del}$ mice show markedly increased frequencies of myeloid progenitors in spleen. Flow cytometry was performed and myeloid progenitors were defined as Lin^{-}cKit^{+}Sca1^{-} population. (G) CALR$^{del/del}$ mice show significantly reduced numbers of erythroblasts. Flow cytometry was performed and erythroblasts were defined as CD71^{+}Ter119^{+}. *, P<0.05; **, P<0.01; data are shown as mean ± SEM.

Figure 6. Mutant CALR homozygosity results in a transplantable extreme thrombocytosis but does not confer competitive repopulating advantage in serial transplants. (A) Donor chimerism is comparable in the primary non-competitive bone marrow transplants. Bone marrow

cells from CALR$^{del/del}$ or control mice at 7-8 months post pIpC (2 x 10^6 per recipient) were transplanted into irradiated (1 x 400 rads) Kit$^{W41/W41}$ C56BL/6 recipients (CD45.1+) respectively. Bar graphs show donor chimerism in peripheral blood which was analyzed using flow cytometry with CD45.1 and CD45.2 antibodies and is derived as percentage of CD45.2-positive cells in whole nucleated blood. Time course of blood counts are shown and significantly increased platelet counts were seen in the recipients of CALR$^{del/+}$ bone marrow. * P <0.05; ** P <0.01; *** P <0.001; data are shown as mean ± SEM. (B) Bone marrow cells from CALR$^{del/del}$ mice show similar repopulating capacity in peripheral blood of primary competitive transplants. Donor repopulation was assessed using flow cytometry detailed as in Figure 4E; data are shown as mean ± SEM. (C) Bone marrow cells from CALR$^{del/del}$ mice show similar repopulating capacity in secondary competitive transplants. Donor repopulation was assessed using flow cytometry of peripheral blood as in Figure 4E and bar graphs showing comparable repopulating capacity to multiple lineages in peripheral blood in the secondary competitive transplantation recipients are shown; data are shown as mean ± SEM.

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