

Mechanism underlying the development of myeloproliferative neoplasms through mutant calreticulin

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Abstract

Deregulation of cytokine signaling is frequently associated with various pathological conditions, including malignancies. In patients with myeloproliferative neoplasms (MPNs), recurrent somatic mutations in the *calreticulin* (*CALR*) gene, which encodes a molecular chaperone that resides in the endoplasmic reticulum, have been reported. Studies have defined mutant *CALR* as an oncogene promoting the development of MPN, and deciphered a novel molecular mechanism by which mutant *CALR* constitutively activates thrombopoietin receptor *MPL* and its downstream molecules to induce cellular transformation. The mechanism of interaction and activation of *MPL* by mutant *CALR* is unique, not only due to the latter forming a homomultimeric complex through a novel mutant-specific sequence generated by frameshift mutation, but also for its ability to interact with immature asparagine-linked glycan for eventual engagement with immature *MPL* in the endoplasmic reticulum. The complex formed between mutant *CALR* and *MPL* is then transported to the cell surface, where it induces constitutive activation of downstream kinase *JAK2* bound to *MPL*. Refined structural and cell biological studies can provide an in-depth understanding of this unusual mechanism of receptor activation by a mutant molecular chaperone. Mutant *CALR* is also involved in modulation of the immune response, transcription, and intracellular homeostasis, which could contribute to the development of MPN. In the present article, we comprehensively review the current understanding of the underlying molecular mechanisms for mutant molecular chaperone-induced cellular transformation.

KEYWORDS

calreticulin, chaperone protein, *JAK2 V617F*, myeloproliferative neoplasm, thrombopoietin receptor

Yoko Edahiro and Marito Araki contributed equally to this work.

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1 | INTRODUCTION

Philadelphia-negative myeloproliferative neoplasms (MPNs) are characterized by the clonal proliferation of hematopoietic stem and progenitor cells, resulting in an increase of one or more lineages of hematopoietic cells occasionally associated with bone marrow fibrosis and/or splenomegaly.¹ In patients with MPN, somatic mutations in Janus kinase 2 (*JAK2*), myeloproliferative leukemia protein (*MPL*), and calreticulin (*CALR*) are found in a mutually exclusive manner.^{2,3} In addition to *JAK2* and *MPL* mutations that induce constitutive activation of the cytokine receptor signaling cascade,⁴ identification of mutations in *CALR* that encode a molecular chaperone was unexpected, as alteration of the *CALR* gene had, to the best of our knowledge, never been implicated in any malignancy. A series of studies have defined mutant *CALR* as a driver for the development of MPNs, and as an oncogene that induces constitutive activation of the JAK-STAT signaling cascade in an *MPL*-dependent manner. In this review, we discuss this unique mutant molecular chaperone that shows oncogenic properties.

2 | CALR PROTEIN

The *CALR* gene encodes a 46-kDa soluble protein that consists of a signal peptide (SP), amino-terminal(N) domain, proline-rich(P) domain, and a carboxy-terminal(C) domain that includes the endoplasmic reticulum (ER) retention signal KDEL (Lys-Asp-Glu-Leu) (Figure 1). *CALR* predominantly localizes in the ER, a reservoir for intracellular calcium, where it plays the role of a storage protein due to the strong calcium-binding capacity of its C-domain. *CALR* also functions as a molecular chaperone, due to the ability of its N- and P-domains to promote proper folding of glycoproteins in the ER.⁵ In addition to the ER, *CALR* localizes in the nucleus and cell surfaces, where it plays a role in transcriptional regulation and phagocytosis, respectively, under various physiological and pathological conditions.⁵ Despite its involvement in various cellular functions, no apparent connection had been reported between aberration of *CALR* and any malignancy until 2013.

3 | DISCOVERY OF CALR MUTATIONS IN MYELOPROLIFERATIVE NEOPLASMS

In 2013, after a long search for a common genetic lesion in patients with MPNs, who are negative for a gain-of-function mutation in the *JAK2* or *MPL* gene, 2 seminal studies reported unique frameshift mutations in *CALR* in patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF), which are 2 subgroups of MPNs.^{2,3} To date, more than 50 types of *CALR* mutations have been reported, including 2 major mutations, a 52-base deletion (del52) and a 5-base insertion (ins5), all of which being small insertions or deletions in exon 9, the last exon of *CALR*. Of interest, all frameshift mutations found in the patients are +1 frameshift mutations localized

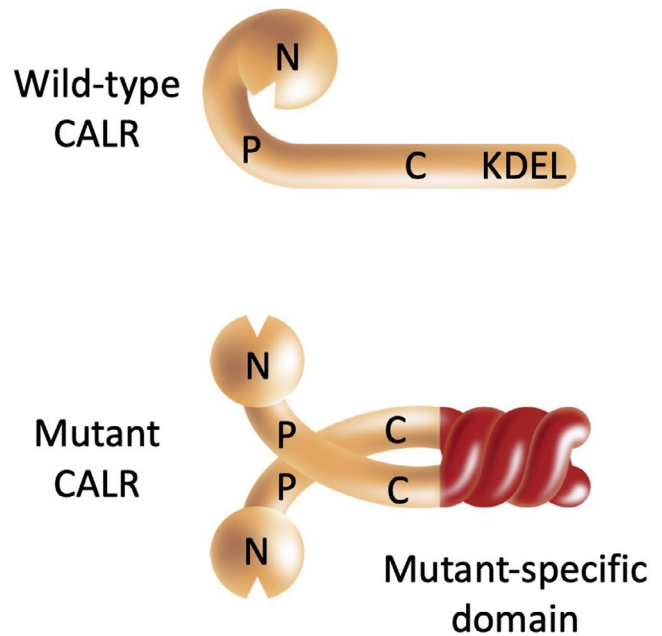


FIGURE 1 A model of WT and mutant calreticulin (*CALR*) protein. Wild-type *CALR* consists of the following domains: amino-terminal (N)-domain, proline-rich (P)-domain, and carboxyl-terminal (C)-domain. KDEL is the endoplasmic reticulum retention signal. In mutant *CALR*, a portion of the C-domain is replaced by a novel domain generated by a +1 frameshift mutation, which includes a mutant-specific sequence commonly found in all mutant *CALR* in myeloproliferative neoplasm (MPN) patients. Mutant *CALR* forms a homomultimeric complex through interaction between mutant-specific sequences, which induces presumptive structural changes and enables N-domain binding to immature N-glycan on myeloproliferative leukemia protein (*MPL*) for activation. In WT *CALR*, the interaction between the N-domain and *MPL* is suppressed by the P-domain. Figure is adopted and modified from Araki et al¹⁴

in a narrow region, generating a novel amino acid sequence at the carboxy-terminus of *CALR* that is common to all mutant *CALRs* (Figure 1). The 44 amino acid-long mutant-specific sequence mostly contains positively charged amino acids, and replaces a cluster of negatively charged amino acids in the C-domain of WT *CALR*. In addition to this, the KDEL sequence is also removed in the mutant *CALR* (Figure 1). These unique features strongly suggest that the *CALR* mutations found in patients are gain-of-function mutations.

4 | MUTANT CALR CAUSES MPN PHENOTYPES IN ANIMAL MODELS

To examine the oncogenic property of mutant *CALR* in vivo, mouse models were created. Retrovirus transduction of human *CALR* del52 and ins5 into lineage-negative or c-KIT⁺ mouse bone marrow cells, and subsequent transplantation to lethally irradiated mice, induced an increase in platelet count, one of the features of ET.^{6,7} An increase in platelets was also observed in a transgenic mouse harboring human *CALR* del52 driven by *H-2Kb* promoter,⁸

or in a knock-in mouse conditionally expressing *CALR* del52 by endogenous promoter.⁹ In the mouse transplanted with *CALR* del52 cells, the number of red and white blood cells was reduced later, along with an increase in spleen weight and fibrosis in both bone marrow and spleen, thus showing the phenotype of secondary myelofibrosis, and indicating the potential of mutant *CALR* in the development of bone marrow fibrosis observed in PMF. These observations suggest that mutant *CALR* causes MPNs in animal models, hence defining mutant *CALR* as an oncogene leading to the development of MPNs.

5 | THROMBOPOIETIN RECEPTOR ACTIVATION BY MUTANT CALR

The molecular mechanism underlying the oncogenic property of mutant *CALR* was characterized primarily by using in vitro model systems. The expression of *CALR* del52 and ins5 in cytokine-dependent cell lines confers on them the capacity to grow in the absence of cytokines in a manner that is dependent on the expression of a thrombopoietin (TPO) receptor (ie MPL), yet is not dependent on the expression of other cytokine receptors such as erythropoietin or granulocyte colony stimulating factor receptors.^{7,10,11} Such context-dependent transformation is associated with an activation of JAK2, a downstream molecule of MPL. Reciprocally, mutant *CALR*-dependent megakaryocytic cell growth was suppressed when the expression of MPL was knocked down^{10,11} or eliminated.⁶ Consistent with this, *MPL* KO diminished the increase of platelet count, induced by the expression of mutant *CALR* in the bone marrow transplantation model in vivo.⁶ The evidence showed that mutant *CALR* induced constitutive activation of MPL, resulting in an activation of JAK2 downstream molecules for the transformation of cells (Figure 2 top right). As MPL played a dominant role in the expansion of megakaryocytes, with subsequent production of platelets, constitutive activation of MPL by mutant *CALR* explained the reason for the ectopic expansion of megakaryocytes, commonly observed in patients with ET and PMF harboring *CALR* mutation. These results aligned mutant *CALR* on the MPL-JAK2 axis, thus rationalizing the fact that gain-of-function mutations of *JAK2*, *MPL*, or *CALR* are found in a mutually exclusive manner in ET and PMF, and solidifying the concept of activation of MPL downstream molecules promoting the development of ET and PMF.

6 | PRESUMPTIVE STRUCTURAL CHANGE CONFERS MPL-BINDING CAPACITY TO MUTANT CALR

Considering the strong genetic interaction between mutant *CALR* and *MPL*, biochemical interaction between their gene products was examined. Studies showed that mutant *CALR* showed much stronger binding to MPL compared to WT *CALR*.¹⁰ Domain analysis identified the N-domain, commonly existing in both WT and

mutant *CALR*, as a binding surface for MPL, which was negatively regulated by the P-domain in the context of WT *CALR*.¹⁰ In the mutant, however, the presence of a mutant-specific sequence enables the N-domain to interact with MPL, regardless of the P-domain, thereby suggesting a structural change, induced by a mutant-specific sequence, for suppression of the activity of the P-domain (Figure 1).^{10,12} A subsequent study revealed that the mutant-specific sequence serves as a domain for the formation of a homomultimer mutant of *CALR*,¹³ and is likely the cause of the dynamic change in structure that enables the N-domain in mutant *CALR* to bind to MPL (Figures 1 and 2).¹⁴

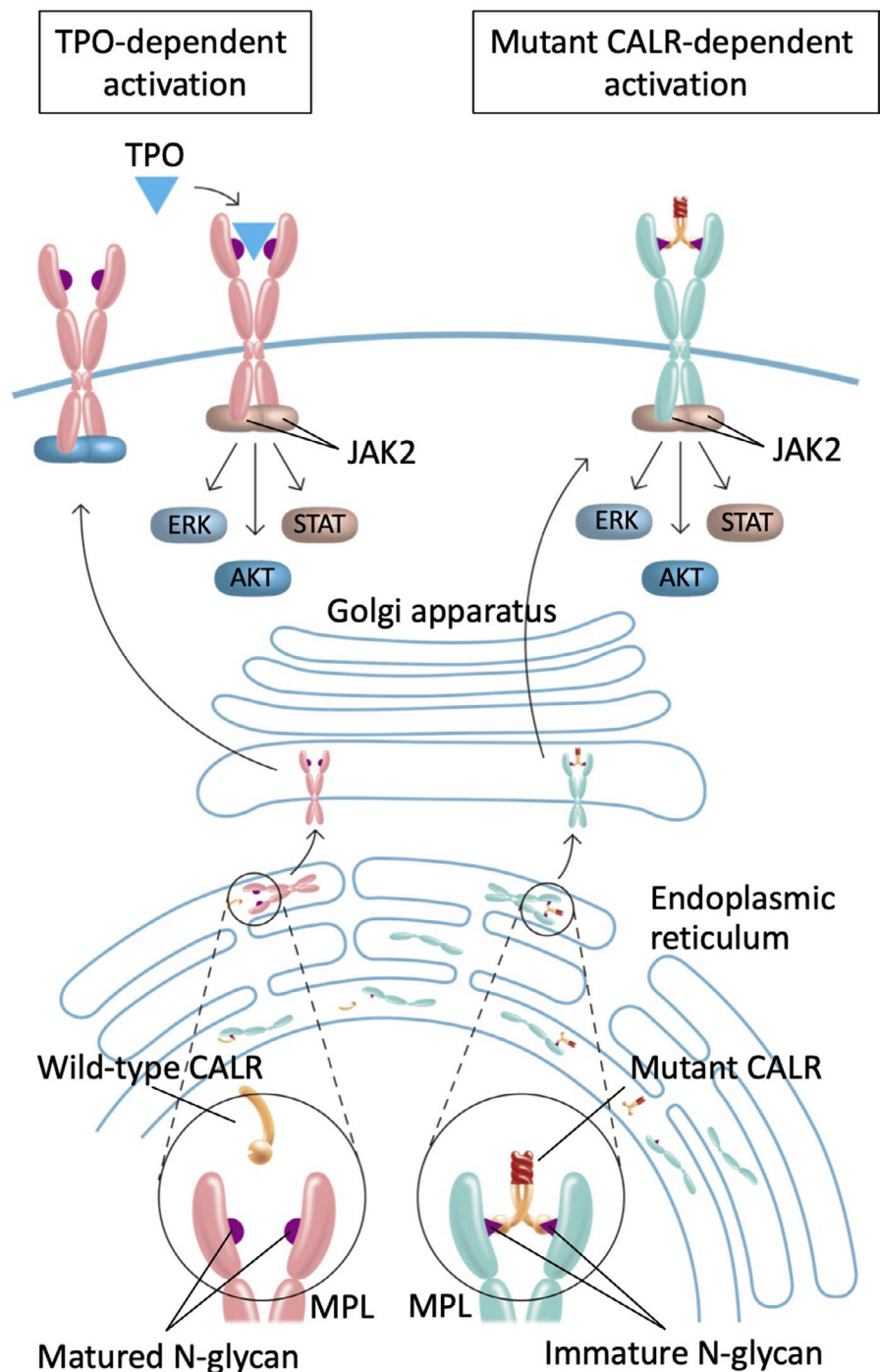
7 | ENGAGEMENT OF MUTANT CALR AND MPL IN THE SECRETORY PATHWAY

The N-domain of *CALR* is equipped with oligosaccharide-binding capacity, which initiates the folding process of newly synthesized unfolded glycoproteins by recognizing and binding the immature asparagine-linked glycan (N-glycan) attached to the proteins. As a glycoprotein, MPL is believed to be a substrate of WT *CALR* in the ER (Figure 2), which raises the possibility that the process of initial engagement between mutant *CALR* and MPL would resemble the one with WT *CALR*, recognizing the substrate as a molecular chaperone. This hypothesis has been supported by the following experimental results: the oligosaccharide binding capacity of mutant *CALR* was found to be indispensable for MPL binding and activation.^{11,15,16} Mutant *CALR*-bound *MPL* largely presents immature N-glycan^{11,15}; and an asparagine residue at 117, located in the extracellular domain of MPL, is required for the binding of mutant *CALR* and subsequent activation by it.^{11,16} In agreement with these observations, mislocalization of mutant *CALR* outside the secretory pathway, due to deletion of SP, was seen to result in a loss of MPL binding and transformation capacity.^{15,17} These findings implied that the first interaction between mutant *CALR* and MPL occurs in the ER, where MPL harbors immature N-glycan.

8 | MECHANISM OF ACTIVATION OF MPL BY MUTANT CALR

Type I cytokine receptors, including MPL, are thought to be activated by the interaction of a ligand and subsequent conformational change, which induces intermolecular phosphorylation of JAK2 molecules bound to each receptor for its own and downstream activation (Figure 2). As the mutant *CALR* binding site is mapped to the extracellular domain of MPL (see above), mutant *CALR* was hypothesized to gain 2 or more binding sites for simultaneous interaction with multiple molecules of MPL. Indeed, mutant *CALR*—not WT *CALR*—that forms a homomultimeric complex through the intermolecular interaction between mutant-specific sequences (Figure 1), eventually confers multiple MPL-binding sites to the mutant *CALR* homomultimeric complex.¹³ The homomultimeric

FIGURE 2 Mutant calreticulin (CALR) interacts with myeloproliferative leukemia protein (MPL) in the secretory pathway for activation on the cell surface. Homomultimerized mutant CALR interacts with MPL through the recognition of immature N-glycan on MPL in the endoplasmic reticulum (ER); the complex then moves to the cell surface, where downstream activation takes place. As an ER-resident molecular chaperone, WT CALR interacts with MPL in the ER, but it dissociates after MPL maturation. This does not happen in mutant CALR, presumably due to its strong affinity to the immature form of MPL. TPO, thrombopoietin



complex formation is required for MPL binding and activation,¹³ thus suggesting that homomultimerization induces the conformational change required for the binding of the N-domain to MPL (see above). A recent study showed that homomultimeric mutant CALR stabilizes MPL multimer.¹⁷ Despite the similarity in terms of stabilization of MPL multimer by mutant CALR, the activation mechanism seems to be different from that of its natural ligand TPO, as mutant CALR is capable of activating MPL D261A/L265A that is defective in TPO-dependent activation.¹¹ Nevertheless, these results support a model in which mutant CALR serves as a fake ligand to activate MPL (Figure 2).

9 | CELL SURFACE ACTIVATION OF MPL BY MUTANT CALR

As mutant CALR and MPL interact in cells, the question of whether the former activates the latter before reaching the cell surface needs to be investigated. Prolonged activation of internalization-defective, but not WT, MPL is observed in cells expressing CALR del52, when cell surface transportation of MPL is blocked.¹⁷ Similarly, MPL activation persisted much longer, which associated with higher levels of MPL on the surface of cells expressing mutant CALR compared to that on cells expressing WT CALR, when cell

surface transportation of MPL was blocked.¹⁵ Mutant CALR even induced a cell surface presentation and activation of maturation-defective MPL that failed to be transported to the cell surface due to defective maturation.¹⁷ These lines of evidence imply that mutant CALR is engaged with MPL in the ER, where N-glycan is in an immature state; the findings also suggest that, due to a strong affinity to the immature N-glycan and lack of chaperone activity,¹⁸ the complex formed between mutant CALR and MPL is subsequently transported to the cell surface for activation. In agreement with this, protease-mediated removal of MPL from the cell surface in mutant CALR-expressing cells induced a rapid decrease of mutant CALR-dependent downstream activation,¹⁵ implying that mutant CALR activates MPL on the cell surface. Proximal ligation assays detected an interaction between mutant CALR and MPL on the cell surface.^{15,17} Collectively, a model was proposed in which a complex of homomultimerized mutant CALR and MPL becomes active on the cell surface and transmits an oncogenic signal through activation of JAK2 (Figure 2).

10 | MUTANT CALR AS A SOLUBLE LIGAND FOR MPL

Considering its capacity to activate the cytokine receptor, the potential of mutant CALR as a soluble ligand in the extracellular environment has been studied. Presumably, due to a lack of KDEL sequence, mutant CALR is secreted out of cells¹⁹; however, it does not activate mature MPL expressed on the surface of cells not expressing mutant CALR.^{10,19} This is probably due to its unique capacity to interact with immature N-glycan (see above), which should be converted into mature form when MPL reaches the surface of normal cells. Recently, mutant CALR was reported to induce MPL activation from outside the cells expressing mutant CALR.²⁰ This strongly suggests that the immature form of MPL, expressed on the surface of CALR mutant-expressing cells, is activated by mutant CALR. However, whether such activation from outside the cells could play a dominant role in the transformation of cells remains elusive.

11 | MODULATION OF INTRACELLULAR HOMEOSTASIS BY MUTANT CALR

As CALR has been shown to be involved in calcium homeostasis and protein quality control, the interaction between mutant CALR and these pathways has been studied. In CALR-mutant megakaryocytes, a defective interaction between mutant CALR and store-operated calcium entry (SOCE) machinery that regulates calcium flux, and spontaneous cytosolic calcium ion flows has been observed.²¹ The calcium flux-mediated transmission of the MPL downstream signal, and the SOCE-dependent proliferation of CALR-mutant megakaryocytes, are also shown.²¹ Collectively, these results imply that mutant CALR-induced spontaneous calcium flux by activating SOCE enhances MPL signaling and thus promotes megakaryopoiesis.²¹

Mutant CALR shows defective interaction with its partner Erp57, which function together in protein folding,²² suggesting the possibility of an increased level of unfolded protein causing ER stress in the cells. Consistent with this observation, transcriptomic data showed that the unfolded protein response (UPR) pathway is activated in hematopoietic cells harboring mutant CALR.^{23,24} Mutant CALR-induced ER stress seems to promote apoptosis in K562 cells,²⁵ but this is not evident in UT-7 or HEK293T cells, presumably due to the suppression of mutant CALR accumulation by ER-associated degradation.²⁶ Although studies show that mutant CALR induces UPR in CALR-mutant cells, its involvement in the development of MPN remains elusive.

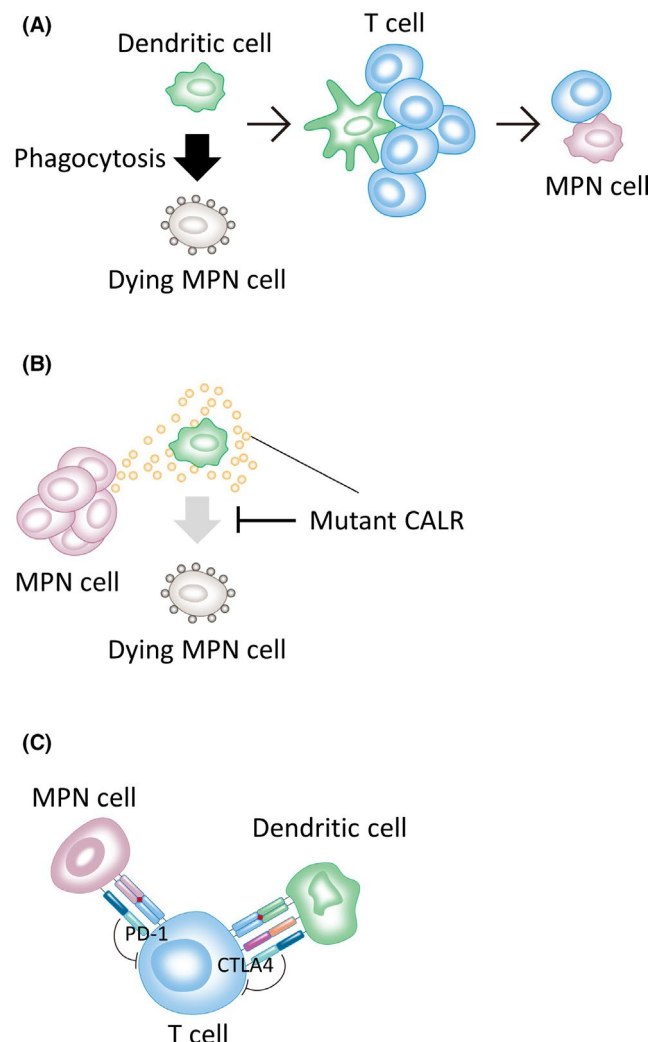


FIGURE 3 Models of immune-suppression in calreticulin (CALR) mutant myeloproliferative neoplasm (MPN). A, A model for the T cell activation by dendritic cell. CALR localized on the surface of dying MPN cell promotes phagocytosis by dendritic cells that later stimulate T cells to kill target cells. B, A model for the interference of phagocytosis by secreted mutant CALR. Mutant CALR binds to surface molecules of dendritic cells, interferes with phagocytosis, and thus promotes the immune suppression. C, A model for immune suppression of T cells by immune checkpoint receptors such as programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA4)

12 | INVOLVEMENT OF MUTANT CALR IN TRANSCRIPTIONAL REGULATION

Proteomic analysis revealed that mutant CALR preferentially interacts with FLI1, a transcription factor regulating hematopoiesis. More abundant mutant CALR and FLI1 bind to the promoter region of MPL, which contributes to the expression of MPL in CALR-mutant cells.²² Wild-type CALR has been shown to play roles in the regulation of transcription through binding to nuclear shuttling proteins, nuclear receptors, or other nuclear proteins.²⁷⁻³² Modulation of nuclear function of CALR might also contribute to the development of MPN.

13 | IMMUNE SUPPRESSION IN CALR-MUTANT MPN

Although CALR is mainly localized in the ER, CALR translocates to the cell surface in apoptotic cells and promotes phagocytosis by macrophages or dendritic cells (Figure 3A).³³ As mentioned above, mutant CALR is secreted out of cells, and detected in the plasma of patients.²⁰ Soluble CALR has been shown to interfere with dendritic cell-mediated phagocytosis of dying cancer cells both in vitro and in vivo (Figure 3B).³⁴ This blockade of phagocytosis compromised the immune-cell response against cancer cells and promoted cancer cell expansion in vivo.³⁴ In contrast, cell surface-expressed mutant CALR did not modulate phagocytosis of hematopoietic cells harboring CALR mutations.³⁵

In the ER, CALR is involved in a peptide-loading process for MHC-I that presents a self-antigen as well as a tumor-associated antigen. Mutant CALR did not retain this property and suppressed MHC-I surface expression if no WT CALR was expressed.¹⁸ Despite the CALR mutation generally being heterozygous in MPN patients, a monoallelic CALR mutation might downregulate MHC-I cell surface expression and promote an escape of CALR-mutant cells from immune surveillance.

In addition to the immune-suppressive effect of mutant CALR, canonical immune-suppression of T cells in MPN patients harboring a CALR mutation was observed. A mutant-specific sequence generated by a frameshift mutation has been reported as a neoantigen for CD4⁺ T cells, but this response was reduced in cells derived from MPN patients harboring a CALR mutation.³⁶⁻³⁸ T cells in those patients presented elevated levels of immune-checkpoint receptors including programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA4) compared to those from healthy individuals (Figure 3C).³⁸ Despite a low frequency, restoration of the T cell response against CALR neoantigen was observed when patient-derived cells were treated with mAbs inhibiting the immune-checkpoint in vitro.³⁸ This restoration was also observed in a patient treated with pembrolizumab, a PD-1 inhibitor,³⁸ implying that conventional immune suppression could play a role in the development of CALR-mutant MPN.

14 | CONCLUSIONS

In conclusion, a series of studies have uncovered the unexpected role of a mutant molecular chaperone as a fake ligand for a cytokine receptor, which functions to induce constitutive activation of the receptor and promote the development of hematopoietic malignancies. Structural analysis and refined cellular imaging analysis are required for an in-depth understanding of such a unique action triggered by a simple nucleotide insertion or deletion at the end of the gene encoding calreticulin. Finally, further understanding of the mechanism of evasion of CALR-mutant cells from immune surveillance could lead to the development of novel therapeutic approaches against MPN.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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