Novel molecular mechanism of cellular transformation by a mutant molecular chaperone in myeloproliferative neoplasms

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Biological processes are influenced by the concentration of cytokines in tissues and the expression of their receptors in cells. In tumors, such regulation is disrupted, most commonly as a result of alterations in genes encoding the receptors or downstream effector molecules. Mutant proteins either lose a tumor suppressive property or gain an oncogenic property, and thus promote oncogenic transformation of cells. Recurrent somatic mutations of Janus kinase 2 (JAK2) and myeloproliferative leukemia protein (MPL) were identified in 2005 and 2006, respectively, in patients with Philadelphia-chromosome negative myeloproliferative neoplasms (MPN). These mutations were found to generate a +1 frameshift in the reading frame on exon 9, which encodes the carboxy (C)-terminus end of CALR, and thus conferred a common mutant-specific sequence in all the CALR mutants. The mutant CALR (but not the wild-type) constitutively activates the thrombopoietin (TPO) receptor, myeloproliferative leukemia protein (MPL), even in the absence of TPO to induce cellular transformation. Preferential interaction between the mutant CALR and MPL is achieved by a presumptive conformational change induced by the mutant-specific C-terminus domain, which allows N-domain binding to MPL. Even though mutant CALR is expressed on the cell surface and is secreted out of cells, it only presents autocrine capacity for MPL activation. These findings define a novel molecular mechanism by which the mutant molecular chaperone constitutively activates the cytokine receptor to induce cellular transformation.

JAK2 is a key downstream effector molecule for cytokine receptors such as MPL and the erythropoietin receptor, EPOR, which promote megakaryopoiesis and erythropoiesis, respectively. Therefore, the identification of gain-of-function mutations in JAK2 and MPL in patients with MPN has provided a rational model for the development of MPN that are characterized with an increase in the number of mature cells of one or more blood cell lineages. Although these findings have greatly enhanced our understanding of the pathogenesis of MPN, the etiology in about 50% of ET and PMF patients remains unknown, because neither JAK2 nor MPL mutations could be identified in these patients. The present review focuses on the role of CALR mutations in the oncogenic transformation observed in MPN and defines a novel molecular mechanism by which a mutant molecular chaperone, CALR, constitutively activates the cytokine receptor to induce cellular transformation. The findings from this review can also be used to drive studies on the molecular mechanisms underlying oncogenic transformations in other tumors.
**Calreticulin and its Mutations in MPN**

In 2013, recurrent somatic mutations in the *calreticulin* (CALR) gene that encodes a molecular chaperone residing in the endoplasmic reticulum (ER) were identified in JAK2- and MPL-unmutated patients with ET and PMF.\(^{(14,15)}\) The two most common CALR mutations, comprising 80% of all CALR mutations, are 52 base-pair deletion (Type 1: c.1092_1143del, hereafter called del52) and 5 base-pair insertion (Type 2: c.1154_1155insTTGTC, hereafter called ins5). So far, more than 50 different types of CALR mutations have been identified, all of which have been small insertions or deletions that cause a frameshift in exon 9, which encodes the carboxyl (C)-terminus end of CALR. Interestingly, the mutations are exclusively +2/-1 base-pair frameshifts that cause a +1 frameshift in the reading frame, and thus generate a novel amino acid sequence common to all mutant CALR proteins on their C-terminal end (Fig. 1a). Furthermore, the unique C-terminus amino acid sequence found in the mutant CALR protein comprises multiple positively charged amino acids; in contrast, in the wild type, the corresponding region consists of mostly negatively charged amino acids (Fig. 1a). These unique features of CALR mutations strongly suggest that they are gain-of-function mutations.\(^{(14,15)}\)

CALR was first identified in 1974 as a calcium-binding protein from the ER in rabbit skeletal muscles,\(^{(16)}\) and the gene was later cloned and named to reflect its ability to bind calcium and its abundance in the ER.\(^{(17,18)}\) CALR plays crucial roles in various cellular and biological events inside and outside of the ER, including quality control of newly synthesized proteins,\(^{(19,20)}\) calcium storage,\(^{(21)}\) focal adhesion disassembly,\(^{(22)}\) antigen presentation,\(^{(23)}\) clearance of cells by phagocytosis,\(^{(24,25)}\) and efficient targeting of cytotoxic T-lymphocytes to target cells.\(^{(26)}\)

The CALR protein consists of multiple domains. The first 17 amino acids comprise a signal peptide sequence that is cleaved upon entering the ER.\(^{(27)}\) This sequence is followed by the amino-terminal domain (N-domain), which contains amino acids required for carbohydrate binding, zinc binding, and chaperon activity.\(^{(28-31)}\) Next comes the proline-rich P-domain, which binds to calcium and ERp57 that cooperates with CALR for protein folding.\(^{(32-34)}\) The last C-domain shows a capacity for calcium storage and includes an ER retention signal sequence, the KDEL\(^{(18,21,34)}\) (Fig. 1). The three-dimensional structure of the entire CALR protein has not been clarified so far; therefore, its structure has been envisaged based on the structure of the ER-membrane associated homologue, calnexin,\(^{(35)}\) and the structural information of various parts of the CALR protein.\(^{(36-38)}\) However, these structural, biochemical, and functional data about CALR cannot explain the oncogenic potential of the frameshift mutations found in patients with MPN.

**Oncogenic Property of Mutant CALR**

Because of the mutual exclusion of JAK2 and MPL mutations from the CALR mutation in patients with MPN who presented similar biological and clinical features, the mutant CALR is suspected to play a role in the MPL-JAK2 pathway. In fact, although the underlying mechanism is unclear, expression of mutant CALR was found to induce cytokine-independent JAK2 activation in Ba/F3 cells.\(^{(14)}\) Consistent with this observation, bone marrow cells from patients with ET and PMF harboring CALR mutations have been shown to form endogenous megakaryocytic colonies; these colonies have been thought to reflect the cell-autonomous activation of MPL or its downstream molecule.\(^{(39)}\) These observations have pushed researchers to examine the oncogenic property of mutant CALR by transducing CALR del52 or ins5 into cell lines where the proliferation depends on the presence of the cytokine. The resulting studies have shown that mutant CALR induces cytokine-independent growth in a manner dependent on the expression of MPL but independent of the expression of other class I cytokine receptors such as EPOR and GCSFR/CSF3R.\(^{(40-42)}\)

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Fig. 1. (a) Mutant and wild-type calreticulin (CALR). Mutations found in patients with myeloproliferative neoplasms are either deletions or insertions in a specific region in exon 9 of the CALR gene. These mutations cause a +1 frameshift in the reading frame, which results in all mutant CALR proteins possessing the same amino acid sequence at the carboxyl (C)-terminal. Amino acid sequences of the C-terminus of wild-type CALR and the two most important mutants, CALR del52 (type 1) and ins5 (type 2). Arrowheads indicate the boundary between the amino acid sequences unaffected and affected by the frameshift mutation. (b) Domain structures of wild-type (CALR\(^{wt}\)) and mutant CALR (CALR\(^{mut}\)). CALR proteins consist of the following domains: a signal peptide (SP), amino-terminal N-domain (N), proline rich P-domain (P), carboxy-terminal C-domain (C) that includes an endoplasmic reticulum retention signal protein, KDEL, in the wild type. Model for the preferential myeloproliferative leukemia protein (MPL)-binding of mutant CALR is presented: the P-domain blocks binding of the N-domain to MPL in wild-type CALR, whereas in mutant CALR, this capacity of the P-domain is blocked by the mutant-specific C-terminus domain.
The factor-independent growth was also found to be associated with elevated levels of ERK1/2 and STAT5 phosphorylation, which was abolished upon treatment of the cells with a JAK2 inhibitor. Furthermore, the mutant CALR-dependent megakaryopoiesis observed in human hematopoietic stem/progenitor cells harboring CALR mutations can also be abrogated by knocking down MPL using shRNA. These data imply that mutant CALR activates JAK2 signaling, presumably by activating MPL in the absence of TPO (Fig. 2).

The oncogenic property of mutant CALR has also been demonstrated in vivo using animal models. Lineage-negative or c-kit-positive bone marrow cells overexpressing mutant CALR have been engrafted into lethally irradiated mouse transplantation models. The mice transplanted with hematopoietic cells expressing CALR del52 and ins5 were found to show increased platelet counts in the peripheral blood, which was associated with an increase in the number of megakaryocytes in the bone marrow; both these findings are the hallmarks of patients with ET. In another study, the expression of CALR del52 induced by a ubiquitous promoter H2-K resulted in similar phenotypes in a transgenic model. Mutant CALR expression induced thrombocytosis but not erythrocytosis or leukocytosis, which is consistent with the results of the in vitro studies that indicated that mutant CALR exclusively activates MPL whose expression and function is limited to megakaryocytes and stem cells, but does not occur in cells of other lineages.

In the transplantation model study, CALR del52 resulted in stronger phenotypes than ins5 in terms of increased platelet and megakaryocyte counts and the induction of fibrosis in the bone marrow and spleen. Consistent with this, hematopoietic stem and progenitor cells harboring CALR del52 expand more in transplanted mice compared with those harboring wild-type CALR or CALR ins5. These data indicate that CALR del52 confers growth advantages to hematopoietic stem and progenitor cells. However, in the study with the transgenic model, bone marrow cells harboring CALR del52 did not exhibit growth advantages over the control cells. The discrepancy between the results obtained for the two models may be, at least partly, as a result of differences in promoter activities. In addition, the transplanted model showed that CALR del52 possesses a stronger oncogenic property than ins5 in vivo, which is contradictory to the clinical features seen in patients, in the sense that CALR ins5 has been found to be associated with higher platelet counts than CALR del52. Contrary to this, CALR del52 showed increased frequency of fibrosis in mouse bone marrow, which is consistent with the clinical features observed in patients with MPN. Nevertheless, the mechanisms underlying the differences in the oncogenic capacity between CALR del52 and ins5 are currently unknown and need to be investigated.

Why does Mutant CALR, but not Wild-Type CALR, Possess Oncogenic Properties?

Because MPL is required for the oncogenic property of mutant CALR, studies have examined the physical interaction between MPL and mutant CALR, revealing a preferential interaction between MPL and mutant CALR, but not wild-type CALR. This interaction was suspected to occur between MPL and the mutant-specific sequence that exists exclusively in the C-terminus of the mutant but not wild-type CALR (Fig. 1b). However, examination of a series of truncated versions of mutant CALR in terms of their MPL-binding capacity revealed that the N-domain of mutant CALR, which exists even in wild-type CALR, is the binding site for MPL (Fig. 1b). Furthermore, the P-domain blocks binding of the N-domain to MPL in wild-type CALR, whereas this capacity of the P-domain is blocked by the mutant-specific C-terminus domain in mutant CALR (Fig. 1b). Based on these lines of evidence, a model has been proposed that the differences in the N-domain between the mutant and wild-type CALR, in terms of MPL-binding ability, result from structural alterations caused by the mutant-specific C-terminus amino acid sequence. Although more detailed structural studies are required to come to a definite conclusion, this is, so far, the only rational model that can explain why mutant CALR but not wild-type CALR has oncogenic properties, in particular, preferential interaction with and constitutive activation of MPL.

Molecular Mechanism Underlying the Action of MPL by Mutant CALR

Upon determining the MPL-dependent oncogenic property of mutant CALR, studies were carried out to determine the molecular mechanism underlying the activation of MPL and...
its downstream molecules such as JAK2 by mutant CALR. Homodimerization of MPL is required for activation of MPL-bound JAK2, but we found that mutant CALR did not promote MPL homodimerization. The extracellular domain of MPL is indispensable for mutant CALR-dependent downstream activation and interacts with mutant CALR (Araki M et al., unpublished data). Furthermore, mutant CALR interacts with JAK2 through MPL. Based on these data, we proposed that the association between mutant CALR and the extracellular domain of MPL induces structural changes in the MPL-JAK2 complex that are required for activation (Fig. 2); these structural changes are thought to occur upon the cytokine-mediated activation of receptor-bound JAK2.

Furthermore, because of the role of wild-type CALR in folding glycosylated proteins in the ER, involvement of glycosylation in the activation of MPL by mutant CALR has been studied. Amino acid substitutions (D135L/Y109F) of residues required for binding to the N-glycosylation modification on mutant CALR abrogate MPL activation. Reciprocally, mutant CALR fails to activate MPL harboring a substitution in the N-glycosylation site (N117Q). These data strongly suggest that mutant CALR recognizes N-glycosylated MPL for activation. Interestingly, mutant CALR activates MPL harboring D261A/L265A substitutions that abolish TPO-binding capacity. Taken together, these data indicate that although mutant CALR becomes “sticky” for those receptors, in agreement with this, weak activation of GCSFR/CSF3R has been observed in a reporter assay that is more sensitive than the method by which structural changes are induced in MPL differs between mutant CALR and TPO.

As strong receptor preference was observed in mutant CALR-dependent oncogenic transformation in hematopoietic cells, mutant CALR is expected to preferentially bind to MPL but not to EPOR and GCSFR/CSF3R. However, mutant CALR strongly interacts with all these receptors (supplementary information in authors’ publication), which implies that mutant CALR becomes “sticky” for those receptors. In agreement with this, weak activation of GCSFR/CSF3R has been observed in a reporter assay that is more sensitive than the cell proliferation assay defining a capacity for factor-independent growth. Although MPL, EPOR, and CSF3R are grouped as class I cytokine receptors because of their structural similarities, the extracellular domains show distinctive differences. Therefore, the presumptive structural change induced in the receptors by mutant CALR may only position MPL (and GCSFR/CSF3R to a lesser extent) in a manner sufficient for JAK2 activation.

**Mutant CALR Induces Autocrine Activation, but not Paracrine Activation, of MPL**

Because wild-type CALR is localized on the cell surface and is secreted from cells, a question of whether mutant CALR possesses a capacity for paracrine activation of MPL expressed in adjacent cells not harboring mutant CALR was raised. Although accumulation of mutant CALR on the cell surface and its secretion from the cells have been confirmed, no paracrine capacity of MPL-activation has been detected in coculture experiments and conditioned media experiments (Fig. 3). These observations further raise a question as to why the secreted or surface-localized mutant CALR cannot activate MPL on the cell surface. This is an as yet unanswered question, but based on the potential involvement of MPL glycosylation in mutant CALR-dependent MPL activation, it is likely that mutant CALR interacts with a certain form of MPL during receptor maturation in the ER and in the Golgi apparatus, but not with the matured form on the cell surface. In concordance with this hypothesis, the levels of matured MPL decreased in Ba/F3 cells expressing mutant CALR presumably because the binding of mutant CALR blocks MPL maturation.

Another important question related to the cell-autonomous activation of MPL is the location where MPL is activated by...
mutant CALR in the cells. In mouse embryonic cells, mutant CALR predominately accumulates in the ER to Golgi intermediate compartment (ERGIC),\(^{4,45}\) where the glycosylated proteins including MPL pass through for maturation, presumably as a result of a loss of ER retention signal. Therefore, mutant CALR is suggested to engage with MPL and subsequently induce premature MPL activation in ERGIC or in cellular compartment(s) before reaching the cell surface (Fig. 4).\(^{4,43}\) Similarly, oncogenic mutant forms of receptor tyrosine kinases Flt3-ITD and c-kit (D814Y) found in hematological malignancies become prematurely active and phosphorylate STAT5 at the ER.\(^{5,54}\) However, in megakaryocytic cells, MPL and mutant CALR accumulate on the cell surface, where MPL activation is induced by TPO in normal hematopoiesis.\(^{4,40}\) Therefore, the model for cell surface activation of MPL by mutant CALR has been proposed (Fig. 4).\(^{4,40}\) To date, there is insufficient information to confirm where the activation of MPL by mutant CALR occurs.

**Conclusions**

Identification and characterization of mutant CALR has provided a novel insight into oncogenic transformation: constitutive activation of the receptor by a mutant molecular chaperone for cellular transformation. Molecular mechanisms similar to the one described in the present review may be found in tumors other than MPN. Although recent studies have provided a rational explanation for why unique CALR mutations are found in patients with JAK2- and MPL-unmutated MPN and for what mutant CALR confers to the cells, many questions are left unanswered, such as what triggers mutant CALR and MPL interaction, how mutant CALR activates MPL, where this interaction occurs in cells, why secreted mutant CALR does not activate MPL on the cell surface, and why cells expressing mutant CALR are not eliminated by the immune system of patients. Answers to these questions will lead to a deeper understanding of this novel oncogenic mechanism and to the development of novel therapeutic strategies against MPN.

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**Disclosure Statement**

Authors declare no conflicts of interest for this article.

**Abbreviations**

- **C-domain**: C-terminus domain
- **ER**: endoplasmic reticulum
- **ET**: essential thrombocytthemia
- **MPN**: myeloproliferative neoplasm
- **PMF**: primary myelofibrosis
- **TPO**: thrombopoietin

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Oncogenic transformation by mutant chaperone

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