

IN THE SPOTLIGHT

Molecular Mechanism of Mutant CALR-Mediated Transformation

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Summary: Elf and colleagues used an elegant series of functional and biochemical assays to investigate the molecular mechanism of mutant calreticulin (CALR)-driven cellular transformation in myeloproliferative neoplasms (MPN). Mutant CALR is sufficient to induce MPN in mouse transplantation experiments, and transformation is dependent upon physical interaction mediated by the positive electrostatic charge of the mutant CALR C-terminal domain and the thrombopoietin receptor MPL. *Cancer Discov*; 6(4); 344–6. ©2016 AACR.

See related article by Elf et al., p. 368 (8).

Myeloproliferative neoplasms (MPN) are a group of clonal hematopoietic stem cell malignancies that give rise to discrete hematologic diseases (1). BCR-ABL-negative MPNs comprise a group of molecularly related but phenotypically distinct myeloid malignancies that include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). Patients with PV present clinically with predominant erythrocytosis and myeloid hyperplasia, and patients with ET or PMF present with predominant megakaryocytic hyperplasia and either thrombocytosis or bone marrow fibrosis, respectively. BCR-ABL-negative MPNs are united by aberrant cytokine signaling caused by recurrent oncogenic driver mutations that lead to downstream JAK-STAT activation and cellular transformation. The most common driver mutation in MPN is *JAK2*^{V617F}, which is present in >95% of PV cases and 50% to 60% of ET and PMF cases (1). Additional driver mutations include activating mutations in *MPL* in 3% to 7% of cases of ET and PMF (1–3) and, less commonly, deactivating mutations in negative regulators of JAK-STAT signaling (4).

Recently, in an attempt to identify additional driver mutations in MPN, whole-exome sequencing of patients with ET and PMF wild-type for both *JAK2* and *MPL* (double-negative patients) identified mutually exclusive *CALR* mutations in 67% of patients with ET and 88% of patients with PMF with double-negative disease (5, 6). To date, 36 different insertion or deletion (indels) *CALR* mutants have been identified (5). All mutations are located within exon 9 and cause a one base-pair (+1 bp) reading frameshift mutation that generates a novel C-terminal peptide (5, 6).

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The wild-type *CALR* gene is highly conserved and encodes a 417-amino acid multifunctional protein that localizes predominately to the lumen of the endoplasmic reticulum (ER), where it functions as a protein-folding chaperone and a Ca²⁺ storage molecule (7). *CALR* is made up of three protein domains: the N-terminal and proline-rich domains are primarily responsible for most of the protein-folding functions, whereas the C-terminal domain binds to Ca²⁺ with a series of negatively charged amino acids and has an ER retention signal (KDEL). Notably, the novel C-terminal domain found in patients with mutant *CALR* MPN lacks the KDEL ER retention motif and codes for a series of positively charged amino acids. *CALR* has never been found to be mutated in cancer prior to its identification in MPNs, and this has prompted several important questions concerning the role of mutant *CALR* in the pathogenesis of these diseases: Can mutant *CALR* alone drive oncogenic transformation? What is the mechanism by which the *CALR*-mutant C-terminus is involved in disease pathogenesis? Why are *CALR* mutations exclusive from *JAK2* mutations?

To investigate whether mutant *CALR* is capable of inducing an MPN phenotype in mice, Elf and colleagues (8) transduced wild-type c-KIT-enriched primary bone marrow cells with retroviral vectors expressing either empty vector, wild-type human *CALR* cDNA (*CALR*^{WT}), or human mutant *CALR* cDNA (*CALR*^{MUT}; the most commonly found *CALR* mutation L367fs*46; results in a 52-bp deletion) and transplanted these cells into lethally irradiated congenic recipient mice. Sixteen weeks after transplantation, *CALR*^{MUT} recipient mice developed an ET-like phenotype, including thrombocytosis and megakaryocytic hyperplasia, suggesting specific involvement of the megakaryocytic lineage. Furthermore, analysis of *CALR* expression in primary bone marrow cells from patients with *CALR*-mutant MPN showed high expression in the megakaryocyte lineage and in immature myeloid cells and was largely absent from erythroid or mature myeloid lineages.

The megakaryocyte lineage-specific phenotype observed in *CALR*^{MUT} transplantation experiments and the megakaryocytic lineage restricted expression of *CALR* in patients prompted the authors to look at the potential role of *MPL* in *CALR*^{MUT}-mediated transformation. Using an established cellular transformation assay, first pioneered by Daley and

colleagues (9), Elf and colleagues show that CALR^{MUT}-expressing Ba/F3 murine hematopoietic cells could not convert to IL3 independence alone and instead required coexpression of MPL but not other hematopoietic cytokine receptors, including the granulocyte-colony stimulating factor receptor (G-CSFR) or the erythropoietin receptor (EPOR). This effect was reproduced in UT-7 megakaryocytic cells in which expression of CALR^{MUT}, but not CALR^{WT}, allowed for GM-CSF-independent growth with MPL coexpression.

Given that the results of the cellular transformation assay were obtained with ectopic expression of CALR variants, the authors recapitulated the transforming ability of mutant CALR under endogenous expression levels. CRISPR/Cas9 gene editing technology was used to generate a +1-bp frameshift mutation in exon 9 of murine *Calr*. Ba/F3-MPL cells stably expressing Cas9 were infected individually with two distinct small guide RNAs (sgRNA), which led to IL3-independent growth that was not observed in *Calr*-targeted parental Ba/F3-Cas9, or *Calr*-targeted Ba/F3-Cas9 cells overexpressing either EPOR or G-CSFR. Sequencing of genomic DNA from these experiments revealed that each distinct sgRNA was able to generate indels that led to a +1-bp frameshift mutation. Together, these data provide definitive evidence that generation of a heterozygous

1-bp frameshift mutation (as seen in human MPNs; refs. 5 and 6) in the endogenous *Calr* locus coupled with MPL expression is sufficient for cellular transformation.

RNA sequencing of CALR^{MUT}-transformed Ba/F3-MPL cells showed significant enrichment for STAT3 and STAT5 gene signatures, and Western blotting confirmed activation of the JAK-STAT signaling pathway. Notably, cellular transformation and JAK-STAT activation were not observed in parental Ba/F3, Ba/F3-EPOR, or Ba/F3-G-CSFR-expressing CALR^{MUT} cells. Furthermore, knockdown of *Jak2* by shRNA led to a significant decrease in proliferation of Ba/F3-MPL cells with CALR^{MUT}, and treatment with the JAK inhibitor ruxolitinib abolished downstream STAT3 activation. This suggests that mutant CALR is dependent on MPL for transformation and that coexpression leads to downstream JAK-STAT signaling, which can be abrogated by targeting JAK2.

To study the structural and functional basis of oncogenic transformation, Elf and colleagues generated individual CALR domain mutants. They found that ectopic expression of either the wild-type N-terminal, proline-rich, or C-terminal domains alone, or the mutant CALR C-terminal domain alone, was not sufficient to confer IL3-independent growth to Ba/F3-MPL cells, suggesting that the mutant CALR

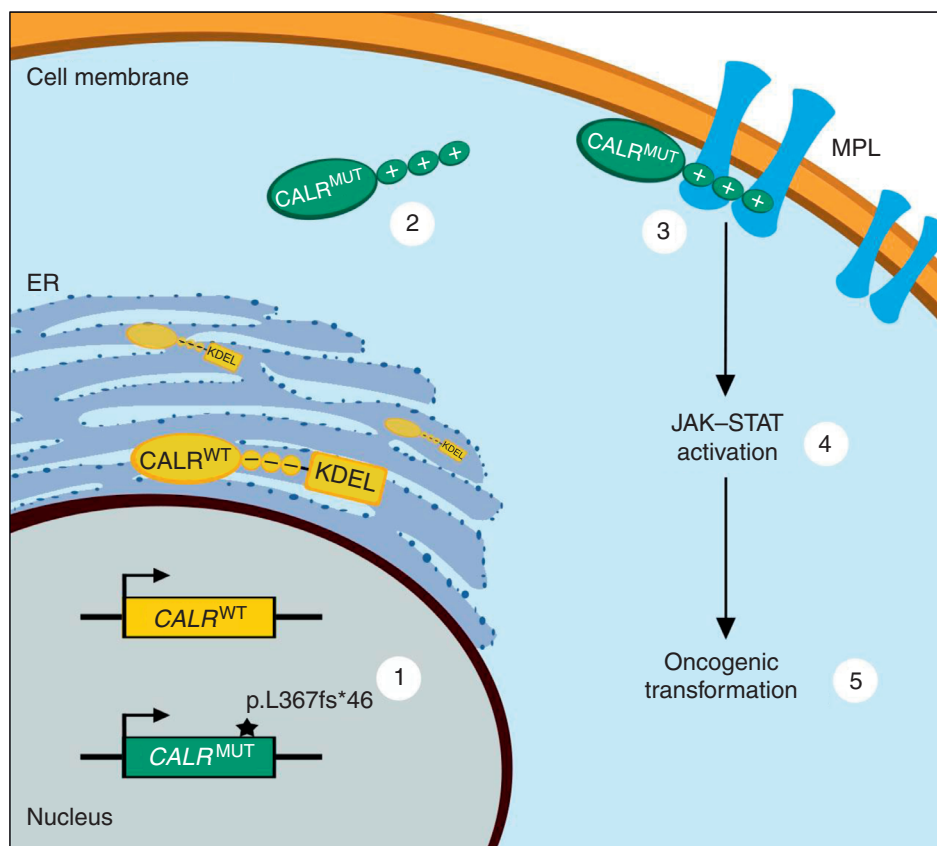


Figure 1. Schematics of the mechanism of mutant CALR-mediated oncogenic transformation. 1, frameshift mutations (here: L367fs*46) in exon 9 of the calreticulin (*CALR*) gene code for mutant CALR (CALR^{MUT}) with a positively charged C-terminal domain instead of the negatively charged C-terminus of wild-type CALR (CALR^{WT}) protein. 2, CALR^{MUT} also lacks the C-terminal ER retention signal (KDEL) present in CALR^{WT} protein. 3, the positive charge of the CALR^{MUT} C-terminus is required for direct binding to the thrombopoietin receptor MPL. 4 and 5, binding of CALR^{MUT} to MPL is required for downstream JAK-STAT activation and oncogenic transformation. Illustration by Barbara Walter.

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C-terminal domain is necessary, but alone it is not sufficient to transform cells. Furthermore, removal of the C-terminal KDEL sequence alone did not lead to cellular transformation.

Next, Elf and colleagues created a series of C-terminal deletion mutants in which 8 to 10 amino acids were individually deleted. All four mutants led to oncogenic transformation and equal outgrowth of Ba/F3-MPL cells, suggesting that the transforming capabilities of CALR^{MUT} are not due to specific residues within the C-terminus but rather are a shared property of the mutant C-terminal tail. CALR mutations in patients with MPN encode for a positively charged C-terminus with a series of lysine (K) and arginine (R) residues that replace this normally negatively charged region (5). Strikingly, a C-terminal CALR domain mutant in which each K and R residue was substituted with neutral glycine (G) residues (CALR^{MUT}-neutral) was no longer capable of transforming Ba/F3-MPL cells, whereas a control mutant in which each non-K and non-R residue was substituted with G (CALR^{MUT}-positive) retained its transforming ability. Collectively, these data revealed an absolute requirement for MPL expression and for the positively charged C-terminal domain of mutant CALR for oncogenic transformation, which suggested a direct interaction between the two molecules. Elf and colleagues then used FLAG-tagged CALR variant immunoprecipitation experiments to definitively show that CALR^{MUT}, but not CALR^{WT}, physically interacts with MPL. Importantly, these results were recapitulated in CRISPR-targeted Ba/F3-MPL-Cas9 cells expressing pathophysiologically relevant levels of mutant CALR, showing that this differential binding is not due to overexpression of mutant CALR. Finally, the authors showed that the positively charged amino acids of the mutant CALR C-terminal domain are required for physical interaction with MPL.

In summary, through extensive mutagenesis-based structure-function experiments and biochemical assays, Elf and colleagues provide a detailed mechanism for mutant CALR-mediated oncogenic transformation and offer explanations to many aspects of mutant CALR-mediated MPNs (Fig. 1). Specifically, this study shows that mutant CALR is sufficient to initiate an ET-like phenotype *in vivo*; that direct physical interaction between mutant CALR and MPL is essential for mutant CALR-mediated transformation; and that this interaction is dependent upon the positive electrostatic charge of the mutant CALR C-terminus.

The observation that mutant CALR activates downstream JAK-STAT signaling provides a biologic basis as to why *JAK2* and *CALR* mutations are mutually exclusive in patients with MPN and offers an explanation as to why *CALR*-mutated

patients respond to JAK inhibition. The study by Elf and colleagues solidify JAK-STAT activation as the central pathway driving oncogenic transformation in MPNs. Despite improvements in understanding the initiation of this process, JAK inhibition does not significantly alter the course of disease, highlighting the urgent need for novel disease-modifying drugs (1). Importantly, the mechanistic findings by Elf and colleagues provide a biologic rationale to develop novel therapeutic inhibitors targeting the required physical interaction between mutant CALR and MPL in mutant CALR-mediated MPNs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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