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Brief Report

MYELOID NEOPLASIA

Defining the requirements for the pathogenic interaction between mutant calreticulin and MPL in MPN

Shannon Elf,[1,*] Nouran S. Abdelfattah,[1,*] April J. Baral,[2,*] Danielle Beeson,[2] Jeanne F. Rivera,[2] Amy Ko,[1] Natalie Florescu,[1] Gabriel Birrane,[3] Edwin Chen,[2,†] and Ann Mullally[1,4,5,†]

[1]Division of Hematology, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA; [2]School of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds, United Kingdom; [3]Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA; [4]Broad Institute, Cambridge, MA; and [5]Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA

KEY POINTS

- **The binding of mutant calreticulin to MPL can be uncoupled from MPL activation.**
- **The lectin activity but not the chaperone functionality of mutant CALR is required for cytokine-independent growth.**

Mutations in calreticulin (*CALR*) are phenotypic drivers in the pathogenesis of myeloproliferative neoplasms. Mechanistic studies have demonstrated that mutant CALR binds to the thrombopoietin receptor MPL, and that the positive electrostatic charge of the mutant CALR C terminus is required for mutant CALR-mediated activation of JAK-STAT signaling. Here we demonstrate that although binding between mutant CALR and MPL is required for mutant CALR to transform hematopoietic cells; binding alone is insufficient for cytokine independent growth. We further show that the threshold of positive charge in the mutant CALR C terminus influences both binding of mutant CALR to MPL and activation of MPL signaling. We find that mutant CALR binds to the extracellular domain of MPL and that 3 tyrosine residues within the intracellular domain of MPL are required to activate signaling. With respect to mutant CALR function, we show that its lectin-dependent function is required for binding to MPL and for cytokine independent growth, whereas its chaperone and polypeptide-binding functionalities are dispensable. Together, our findings provide additional insights into the mechanism of the pathogenic mutant CALR-MPL interaction in myeloproliferative neoplasms. (*Blood*. 2018;131(7):782-786)

Introduction

Recurrent mutations in calreticulin (*CALR*), an endoplasmic reticulum (ER) resident chaperone protein, represent the second most common mutation in patients with myeloproliferative neoplasms (MPNs) after JAK2V617F.[1-4] *CALR* mutations in MPN occur as a heterogeneous set of indel mutations in exon 9 of *CALR* that all result in a +1 bp frameshift in the CALR reading frame.[5,6] Although the mutant CALR C-terminal alterations vary, all *CALR* mutations lead to a loss of most of the C-terminal acidic domain and a concomitant gain of a novel C terminus consisting of 36 amino acids that are enriched for positively charged residues. Mechanistic studies have demonstrated that mutant CALR ($CALR^{MUT}$) binds to the thrombopoietin receptor, MPL, to activate the MPL-JAK-STAT signaling axis,[7-10] and that the positive charge of the $CALR^{MUT}$ C terminus is required to mediate this interaction.[9]

Several unanswered questions remain regarding the molecular and functional basis of $CALR^{MUT}$ oncogenic activity. In this report, we use a series of mutagenesis experiments to address some of these questions, focusing on the structural determinants of $CALR^{MUT}$ and MPL that are necessary for hematopoietic cell transformation.

Study design

Ba/F3 cell growth assays

Ba/F3 cells expressing $CALR^{MUT}$ or MPL variants were generated by retroviral transduction and assayed for cytokine-independent growth as previously described.[9]

In vitro binding assay

V5-tagged purified recombinant wild-type CALR ($CALR^{WT}$), $CALR^{MUT}$, $CALR^{MUT}$-D135L, and $CALR^{MUT}$-D317A proteins were incubated with purified recombinant ERp57 or MPL for 30 minutes at 4°C, and washed with *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid acetate buffer. Bound proteins were eluted from beads and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Results and discussion

To resolve which amino acids within the $CALR^{MUT}$ C terminus are required for $CALR^{MUT}$ activity, we generated $CALR^{MUT}$ variants harboring serial truncations of the mutant C-terminal tail in blocks of 8 to 10 amino acids (Figure 1A) and tested their ability to associate with MPL in pull-down assays. All $CALR^{MUT}$ variants examined retained the ability to bind MPL (Figure 1B). However,

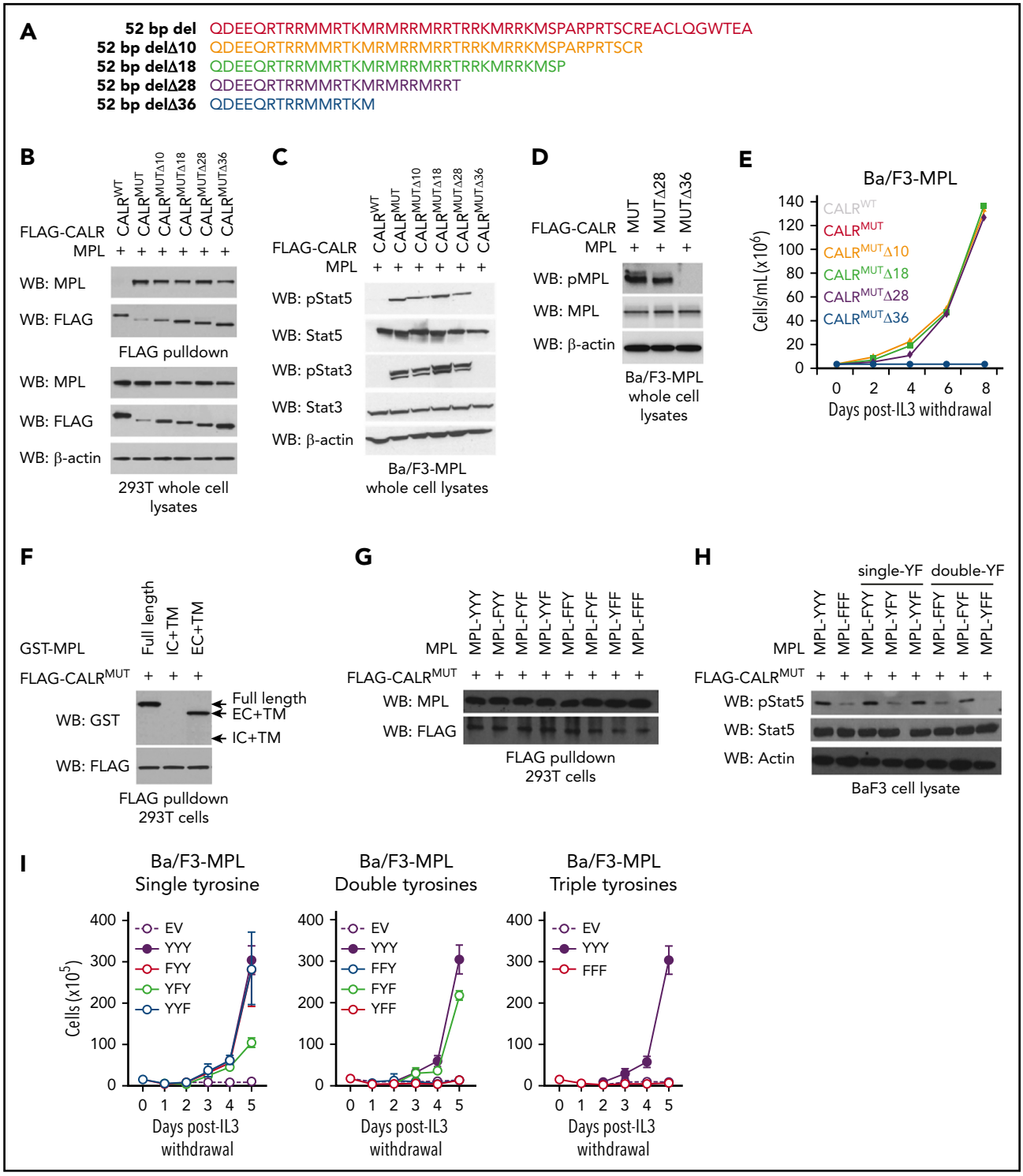

Figure 1. Binding of mutant calreticulin to MPL is required to transform hematopoietic cells but binding alone is insufficient for cytokine-independent growth. (A) Schema depicting serial C-terminal truncation mutants of mutant CALR. (B) Immunoblotting of FLAG-immunoprecipitated proteins and whole cell lysates from 293T cells cotransfected with wild-type FLAG-CALR ($CALR^{WT}$), FLAG-CALR 52-bp deletion ($CALR^{MUT}$), or FLAG-CALR 52-bp deletion serial C-terminal truncation mutants ($CALR^{MUT}$ Δ10-Δ36) demonstrates that mutant CALR truncated up to Δ36 still binds to MPL. (C) Immunoblotting demonstrates phosphorylation of Stat5 and Stat3 in Ba/F3-MPL cells expressing $CALR^{MUT}$ and truncation variants Δ10, Δ18, and Δ28, but not Δ36. (D) Immunoblotting demonstrates phosphorylation of MPL in Ba/F3-MPL cells expressing $CALR^{MUT}$ and Δ28, but not Δ36. (E) Growth curves in Ba/F3-MPL cells expressing $CALR^{WT}$, $CALR^{MUT}$, or $CALR^{MUT}$ C-terminal truncation variants demonstrates that only severe truncation of the mutant CALR C terminus (Δ36) abolishes the transforming capacity of mutant CALR. (F) Immunoblotting of FLAG immunoprecipitated proteins from 293T cells co-transfected with FLAG-CALR 52 bp deletion ($CALR^{MUT}$) and glutathione *S*-transferase (GST)–tagged full-length MPL, GST-tagged MPL intracellular + transmembrane domains, or GST-tagged MPL extracellular + transmembrane domains demonstrates that mutant CALR binds to the extracellular domain of MPL. (G) Immunoblotting of FLAG immunoprecipitated proteins from 293T cells coexpressing FLAG-tagged mutant CALR and MPL YF variants demonstrates that mutations of intracellular tyrosine residues on MPL does not affect the ability of mutant CALR to bind to MPL. (H) Immunoblotting demonstrates that phosphorylation of Stat5 is abrogated in Ba/F3 cells expressing MPL YF variants harboring loss of Y626. (I) Growth curves in Ba/F3 cells stably expressing MPL-YF variants demonstrate that all 3 intracellular tyrosines play a role in supporting cytokine independent growth in Ba/F3 cells mediated by mutant CALR. WB, western blot.

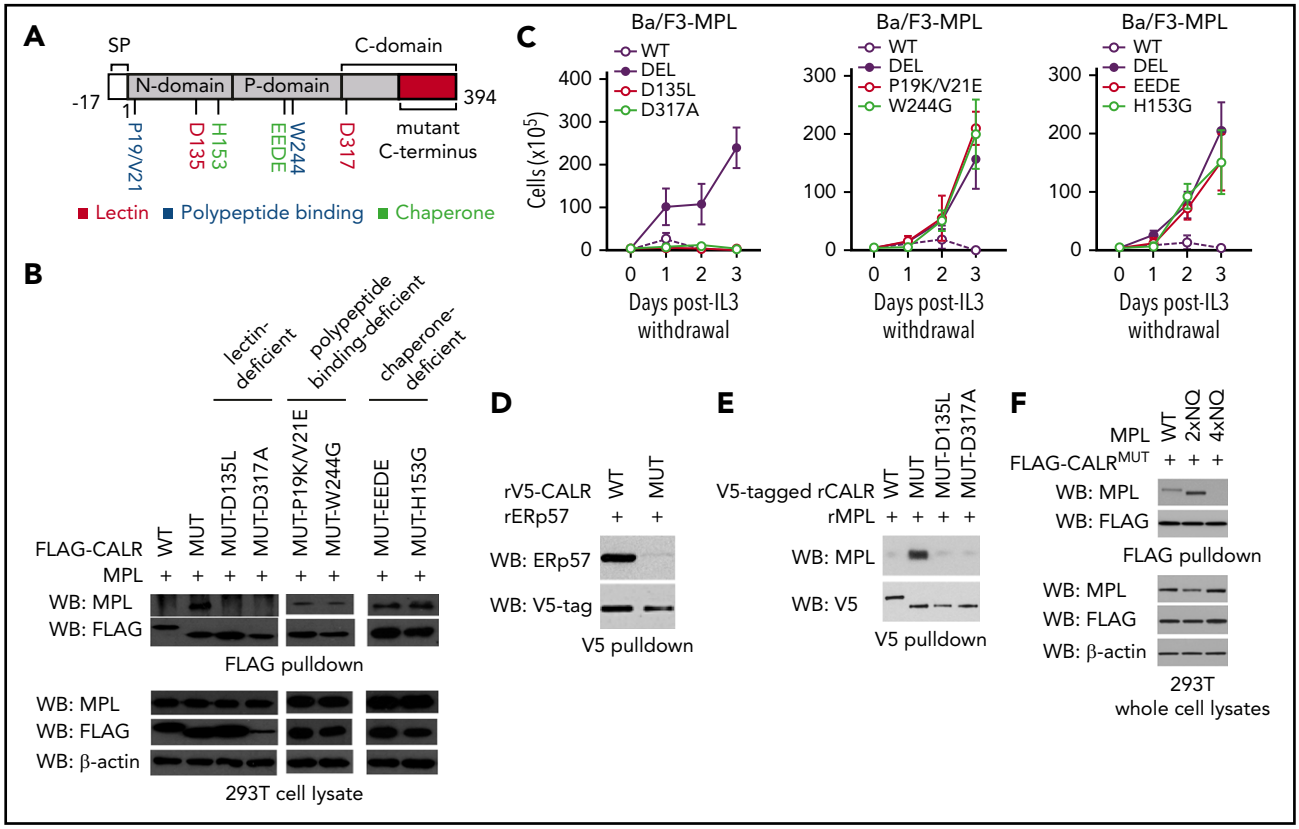

Figure 2. The lectin-dependent function of mutant CALR is required for cytokine-independent growth, whereas its chaperone and polypeptide binding functionalities are dispensable. (A) Schema depicting mutations introduced into the lectin (dark red), polypeptide binding (blue), and chaperone (light green) domains of mutant CALR. (B) Immunoblotting of FLAG immunoprecipitated proteins from 293T cells cotransfected with wild-type CALR, mutant CALR, or mutant CALR lectin-, chaperone-, and polypeptide binding-deficient variants demonstrates that binding between mutant CALR and MPL is lost when residues required for lectin binding are mutated, but binding is retained when residues critical for CALR chaperone and polypeptide binding functionality are mutated. (C) Growth curves in Ba/F3-MPL cells expressing wild-type CALR, mutant CALR, or mutant CALR lectin- (left), chaperone- (center), and polypeptide binding-deficient (right) variants demonstrates that mutant CALR loses its ability to drive cytokine-independent growth when residues required for lectin binding are mutated but retains its ability to drive cytokine-independent growth in when residues critical for CALR chaperone and polypeptide-binding functionality are mutated. (D) In vitro binding assay between purified recombinant CALR^{WT} or CALR^{MUT} and purified recombinant ERp57 demonstrates that CALR^{WT} binds directly to ERp57 but CALR^{MUT} does not. (E) In vitro binding assay between purified recombinant CALR^{WT}, CALR^{MUT}, CALR^{MUT}-D135L, or CALR^{MUT}-D317A demonstrates that CALR^{MUT} binds directly to MPL but not CALR^{WT} or altered CALR^{MUT} variants. (F) FLAG pulldown in 293T cells coexpressing CALR^{MUT} and MPL glycosylation mutants (2xNQ = N117/178Q; 4xNQ = N117/178/298/358Q), shows CALR^{MUT} binding to MPL-2xNQ but not to MPL-4xNQ.

the most severely truncated form (CALR^{MUT}Δ36) failed to activate JAK-STAT signaling (Figure 1C), stimulate phosphorylation of MPL (Figure 1D), and transform Ba/F3-MPL cells to interleukin-3 independence (Figure 1E). Further truncation of an 11 amino acid positively charged stretch (QRTRRMMRTKM) that is also present in CALR^{MUT} protein species generated by the 52-bp deletion (CALR^{MUT}Δ47; see supplemental Figure 1A , available on the *Blood* Web site) led to loss of MPL binding (supplemental Figure 1B) and inability to transform Ba/F3-MPL cells (supplemental Figure 1C). A CALR^{MUT} variant in which these 11 residues are deleted but the distal 36 mutant-specific amino acids are retained (CALR^{MUT}Δ37-47) was still able to bind to MPL and transform Ba/F3-MPL cells (supplemental Figure 1A-C), suggesting that the QRTRRMMRTKM stretch is sufficient but not strictly required for MPL binding. Our data also suggest that although CALR^{MUT}Δ36 can bind to MPL, this binding is insufficient to activate MPL signaling. To our knowledge, these data provide the first evidence that physical interaction between CALR^{MUT} and MPL is not ipso facto sufficient to activate MPL. Rather, our data argue for a model whereby different thresholds of positive charge in the CALR^{MUT} C terminus are required to enable binding of CALR^{MUT} to MPL and to activate MPL signaling. Because MPL phosphorylation is dependent on homodimerization of single MPL chains, these data may suggest that CALR^{MUT}Δ36 retains the ability to interact with single MPL chains but is unable to induce homodimerization, which is required for receptor activation. Further studies are warranted to fully resolve the 3-dimensional structure of the CALR^{MUT}-MPL interaction.

We next sought to elucidate the regions of MPL that are essential to support CALR^{MUT} activity. We observed that CALR^{MUT} binds to full-length MPL and to the extracellular and transmembrane fragment of MPL, but not to the intracellular and transmembrane fragment (Figure 1F). Furthermore, an MPL variant in which the thrombopoietin (TPO) binding site is mutated (D235A/L239A)[11] was still able to bind to CALR^{MUT} (supplemental Figure 1D) and could support CALR^{MUT}-mediated cytokine-independent growth in Ba/F3 cells (supplemental Figure 1E). This suggests that CALR^{MUT} does not occupy the same binding pocket as TPO and is consistent with CALR^{MUT}-driven hematopoietic transformation being a TPO-independent process.[7]

Analysis of the intracellular portion of MPL reveals 3 tyrosine residues (Y591, Y626, and Y631) that may also be important for CALR^{MUT}-MPL signaling.[12] MPL variants were therefore generated where these residues were systematically mutated to phenylalanine

individually (FYY, YFY, YYF), in tandem (FFY, FYF, YFF), or altogether (FFF). As expected, all MPL variants were able to physically interact with CALR^{MUT} in FLAG pull-down assays (Figure 1G). However, we found differences in their ability to support CALR^{MUT} signaling. MPL variants harboring intact Y626 (MPL-FYY, MPL-YYF, MPL-FYF) supported robust Stat5 phosphorylation (Figure 1H) in association with cytokine-independent growth (Figure 1I), but not MPL variants in which the Y626 is mutated (MPL-YFY, MPL-YFF, MPL-FFY, MPL-FFF). These data indicate that Y626 plays a more prominent role than Y591 and Y631 in MPL signaling downstream of CALR^{MUT}, which is consistent with a previous report identifying Y626 as the major signaling tyrosine in canonical TPO-MPL signaling.[12] Our data therefore highlight the importance of tyrosine-mediated MPL signaling as a pathway coopted by CALR^{MUT} to effect cytokine-independent proliferation. Our studies do not rule out a role for nontyrosine residues of MPL, which may be required for CALR^{MUT}-mediated activation of other downstream signaling pathways (eg, extracellular signal-regulated kinase)[13]; future studies to explore this question are warranted.

Finally, we sought to gain functional insights into how CALR^{MUT} interacts with MPL to confer cytokine-independent growth. Wild-type calreticulin is an ER-resident chaperone that interacts with glycoproteins by binding to Glc1Man9GlcNAc2 oligosaccharides and the polypeptide backbone to facilitate proper protein folding. We therefore created variants of CALR^{MUT} harboring mutations in critical residues implicated in 3 key functionalities of wild-type CALR: (1) polypeptide binding, (2) chaperone activity, and (3) lectin activity. We then tested their capacity to bind to MPL and confer cytokine independence (Figure 2A).

We observed that both polypeptide binding-deficient variants of CALR^{MUT} (CALR^{MUT}-P19K/V21E and CALR^{MUT}-W244G) and chaperone-deficient variants of CALR^{MUT} (CALR^{MUT}-H153G and CALR^{MUT}-EEDE)[14,15] retained MPL binding ability (Figure 2B) and conferred cytokine-independent growth (Figure 2C). Consistent with the nonessentiality of chaperone functionality in CALR^{MUT} oncogenic activity, CALR^{WT} exhibits strong, direct binding to the ERp57 cochaperone, whereas CALR^{MUT} does not (Figure 2D). In contrast, lectin-deficient CALR^{MUT} variants harboring mutations in Asp-135 and Asp-317 (CALR^{MUT}-D135L and CALR^{MUT}-D317A)[16,17] were both unable to bind to MPL or confer cytokine independence (Figure 2B-C). In accordance, we also found that only recombinant CALR^{MUT} protein directly binds to recombinant MPL in an in vitro binding assay, whereas neither CALR^{WT} nor lectin-deficient CALR^{MUT} do (Figure 2E). These data explain the previously reported essential role for Asp-135 in mediating CALR^{MUT}-driven STAT5 activation[8] as being from a requirement for Asp-135 in mediating binding between CALR^{MUT} to MPL. Finally, to determine the requisite glycosylation status of MPL that enables CALR^{MUT} binding, we tested CALR^{MUT} binding to MPL mutants where either 2 (2xNQ = N117/178Q) or all 4 (4xNQ = N117/178/298/358Q) glycosylation sites in the extracellular domain of MPL were abolished. We found that CALR^{MUT} can still bind to MPL-2xNQ but not to MPL-4xNQ (Figure 2F). These data are consistent with a previous report that demonstrated that MPL variants devoid of the same 4 *N*-glycosylation sites failed to support STAT5 activation by CALR^{MUT}[8] and suggests that this defect is due to an inability of unglycosylated MPL to bind CALR^{MUT}.

In conclusion, our data provide additional insights into the molecular mechanism by which CALR^{MUT} interacts with MPL to induce MPN (supplemental Table 1). Specifically, we (1) uncouple the binding of CALR^{MUT} to MPL from MPL activation, (2) define the key properties of MPL required for CALR^{MUT} binding and for its activation, and (3) decipher the key functionalities of CALR^{MUT} required for its oncogenic activity.

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Authorship

Contribution: S.E., N.S.A., E.C., and A.M. designed the study; G.B. provided guidance on experimental design; S.E., N.S.A., A.J.B., D.B., G.B., J.F.R., A.K., and N.F. performed experiments and collected the data; S.E., N.S.A., A.J.B., and D.B. analyzed the data; and S.E., N.S.A., E.C., and A.M. wrote the manuscript.

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Correspondence: Ann Mullally, Harvard Institutes of Medicine Building, Room 738, 77 Ave Louis Pasteur, Boston, MA 02115; e-mail: amullally@partners.org; or Edwin Chen, School of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Woodhouse Ln, Leeds LS2 9JT, United Kingdom; e-mail: e.chen@leeds.ac.uk.

Footnotes

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*S.E., N.S.A., and A.J.B. contributed equally to this work.

†E.C. and A.M. contributed equally to this work.

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