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Mechanism of Mutant Calreticulin-Mediated Activation of the Thrombopoietin Receptor in Cancers

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November 2, 2020

Re: JCB manuscript #202009179

Dr. Malini Raghavan
University of Michigan-Ann Arbor
6706A Medical Science Building II
Ann Arbor, Michigan 48109

Dear Dr. Raghavan,

Thank you for submitting your manuscript entitled "Mpl activation by dimers of MPN calreticulin mutants stabilized by disulfides and ionic interactions". We apologize for the delay in communicating our decision to you. The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers were positive about the identification of protein structural elements in mutated calreticulin that mediate dimer formation/stabilization. However, Reviewer #3 questioned the dimerization model, and their points should be addressed to the best of your ability. We'd also encourage you to seriously tackle all of the other comments from Reviewers #1 and #2 to strengthen your model and bolster the conclusions about the features of CRT-Mpl interactions participating in oncogenic signaling.

Please let us know if you have any questions or anticipate any issues addressing the reviewers' points. We would be happy to discuss the revisions further as needed. While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations

are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Ira Mellman, Ph.D.
Editor, Journal of Cell Biology

Melina Casadio, Ph.D.
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This is an elegant study that identifies key structural elements of C-terminal mutated calreticulin (CRT) that are involved in activating Mpl signaling. It is established that C-terminal mutated CRT (CRTDel52) binds and activates the transmembrane receptor Mpl which activates JAK signaling to drive myeloproliferative neoplasms. However, the specific mechanisms by which CRT binding to Mpl have not been elucidated previously. The current studies show that dimers of CRTDel52 are required for binding and identifies key cysteine residues necessary for disulfide-linked dimer/multimer formation. Using molecular modeling and mutated protein expression studies, the authors established that additional N-terminal domain ionic residues are necessary for dimer formation. These studies are very credible and well controlled and represent a significant contribution to this important interaction that impacts human disease. Furthermore, the authors also investigate the importance of various C-terminal domain cysteine residues and N-terminal ionic residues in mediating Mpl binding and cell proliferation.

The manuscript would benefit from clarification of the cellular compartment in which CRTDel52-Mpl interactions occur. Because CRTDel52 lacks the KDEL ER retention sequence and Mpl is a transmembrane receptor, (as shown in the model in Figure 8), the supposition is that CRTDel52 is secreted and binds to the extracellular domain of Mpl. Has this been proven in the literature? Does

CRTDel52 bind Mpl in the ER and is co-trafficked with Mpl as it is incorporated into the plasma membrane? I ask because the relative expression and Mpl interactions of the various Mpl mutants are analyzed in total cell lysates (1% Triton-X 100 for protein expression; 1% digitonin for immunoprecipitations). However, there are no studies to show that the various mutants are actually trafficked properly and secreted so that interactions between CRT mutants and the extracellular domain are possible. Data showing secretion of the various mutants by western blotting and/or immunohistochemical localization (using antibody to the tag) of the constructs in the ER/golgi and/or cell membrane with MPL would enhance confidence in the authors' interpretation of the data. Furthermore, a discussion of the localization of the CRTDel52-Mpl interaction in cells should be included for the readers' benefit.

Minor Concerns
It is interesting that the antibody to the N-terminal domain does not recognize multimers. In agreement with the N-terminal mutation studies, this observation might suggest that epitopes for this N-terminal antibody are inaccessible in multimerized CRT.
Figure 1E-F: why are blots detecting hemoglobin with 2 different primary antibodies?
Why are the C-domain constructs not directly detectable in Ba/F3 lysates (page 8)?
Figure 7B: The experimental variability of the Del52-2CA and Del52-3CA constructs in mediating cell proliferation is substantial, which complicates interpretation of the biological role of these cysteine mutations. Does this variability reflect differences in transfection efficiency/trafficking/secretion of this mutant?

Reviewer #2 (Comments to the Authors (Required)):

Venkatesan et al. confirmed that CRT mutants form dimer and/or multimer species not just in HEK293 cells expressing CRT variants but also in MPN patient platelets. Based on the reported crystal structure and new biochemical data, they suggest that the CRT dimerization is mediated by two-site interactions: 1) the natural low-affinity interface at N-domain with support by an intermolecular disulfide bonding (C163), and 2) a pair of disulfide bonds at the newly generated C-domain (C400 and C404 for del52 mutant). The authors finally proposed a model for the 2:2 ligand-receptor complex for this oncogenic signaling. The results are interesting and give a new insight into this therapeutically important system. Experiments and interpretations are generally convincing, but several points need to be commented on or clarified before publication.

Major points
For the platelet samples, it is unclear if the dimeric CRT mutants were buried behind the hemoglobin bands or there are only oligomer species. Can the author attempt hemoglobin depletion before running gels?

In non-reducing SDS-PAGEs and native gels, it is possible that the slower migrations were partly from the complex formation with Mpl (as shown by IP), or cysteine cross-linking with Mpl (*extracellular region has 15 cysteine residues, which should yield at least one unpaired cysteine) or even other endogenous proteins. Can the authors assess this by showing Mpl blot for several representative gel analysis?

The authors say that: In the crystal structure of the "N-N" dimer, two C163 are not in direct contact, but could move closer to each other and form a disulfide bond following minor loop rearrangements. As the authors pointed out, in Figure 4B and E (non-reducing SDS-PAGEs), Del52-2CA shows

multimer bands although there is only one free cysteine in the construct, and in Figure 4B, Del52-3CA still shows dimers and multimer bands. The authors concluded that the intramolecular C105-C137 bridge is disrupted, and those cysteines can also mediate multimer formation, but C163 still plays a deterministic role for the N-domain association to form the ordered dimer. However, in the case of wild-type CRT structures, C163 sidechain seems to be facing to the N-domain core. Can the authors show the mobility of this loop experimentally or by simulation?

The study initially looked at the ability of the MPN-specific CALR mutations to form higher order structures by forming disulfide-stabilized multimers. They showed that their anti-CRT(Cmut) that was raised against the CALR-C-term domain was highly specific to the mutants and convincingly show the multimerization of CALR under non-reducing conditions and native-page, revealing the role of disulfide-bonds in forming multimers. It is interesting that they could not detect CALR with the N-term. commercial Ab (anti-CRT(N)) in the sera of MPN patients, but only with the anti-CRT(Cmut) for the IP experiment in Figure D. They also note that some platelet preparations show a possible Hb contamination band in the native page, and unfortunately it is around the size we may see other oligomers; perhaps a further sample clean-up can help clear that up.

In Figure 2, by making several CALR truncations and testing their specificity and ability to bind to MPL, the authors claim that as the C-term. domain is progressively shortened (truncated) compared to the Del52, you get a progressive loss of binding to MPL. Given, and as they noted, that each of the truncations had varying levels of the actual protein being expressed (possibly less stability/proteasomal degradation), it is difficult to conclude (from Figure 2B) that there is a difference in the amount of CALR protein associated with MPL between the mutants. Though it is clear that compared to WT, all mutants (except for Del52-36), show increased binding to MPL. In Figure 2C, they show compellingly that this dimerization is due to the cysteine residues by mutating them. The protein normalization in Figure 3A, is what I would have liked to have seen in 2B.

From the native page in Figure 3D, the authors claim that the CRTDel52□28 truncation is needed to partially destabilize CRTDel52 multimers, but the formation/detection of monomers shown is very minimal. Though it is clear that the CRTDel52□36 truncation is indeed needed to fully destabilize the CRTDel52 multimers.

In Figure 4B, they claim that the 'Ala- substitution of this residue in the CRTDel52-CA (CRTDel52(C163A)) mutant resulted in a dimer and multimer pattern similar to CRTDel52', but it seems the pattern more closely resembles the Del52-2CA mutant with the multimer band pattern, monomer and dimer intensity.

Based on the dimer model (from Figure 5A), the D165K mutation in combination with the CRTDel52-3CA mutations was claimed to induce more monomeric species in non- reducing blots (Figures 6A and 6B), but its visualization in 6A seems rather minimal. Though I would agree that there is more multimerization with the D166K mutations, which allowed for enhanced levels of disulfide-linked species.

In Figure 7, S8, it seems the only mutant constructs expressed are Del52 and Del52-12, and at much lower levels than WT. So assuming that the untagged constructs expressed in Ba/F3 will have similar expression, it is not surprising that you get no cell growth from the other truncated proteins. It may be the lack of cell proliferation does not have to with the actual biology, per se, but the lack of protein even in the system. However, the Del52-19 does not show any protein in Sup 8, yet cell growth in the proliferation assay. Still, they showed convincing that a larger truncation is needed to completely abolish CRTDel52 mediated proliferation. Additionally, based on the IPs in

Figure 7G, that CRTdel52 has a significantly reduced ability to bind to MPL and induce proliferation of Ba/F3 cells when the cysteine-residues and H170 site is mutated, strongly supports the necessity for both disulfide bonds and ionic interactions at both N-domain and C-domain dimerization interfaces to mediate the cell proliferation and oncogenic transformation previously noted with CALR-del52 in MPN.

Minor points
(1)
As authors cited, Elf et al. Blood 2018 reported that CRTdel52 Δ10, Δ18, and Δ28 are as active as full-length CRTdel52, which is in contrast to the observation in this manuscript and against the hypothesis that the newly derived C-terminal cysteines are important for the abnormal Mpl activation. A more explicit discussion about this deviation is encouraged to be added.

(2)
...indeed the mutants but not wild type CRT are detectable in patient serum by coimmunoprecipitation analyses (Figure 1D).

Is it possible to identify if the secreted form includes dimer/multimer species? Although CRT's affinity for surface Mpl proteins would be lower with mature glycans, are the secreted CRT mutants able to activate the Mpl signaling on the neighboring cells?

(3)
Do the authors suggest the abnormal signaling is mainly from the ordered dimer unit (proposed in Figure 5A and 8 or multimers of this dimer unit exampled in in Figure S6C), against random cross-linking of CRT/Mpl that would produce many different forms of dimer units with a spectrum of signaling strengths? Or, is the dimer model one of the signaling unit compatible with Mpl activation?

(4)
It is encouraged to report more details and state material availability of the anti-CRT mutant C-terminal antibody generated in this work, if possible.

Reviewer #3 (Comments to the Authors (Required)):

Myeloproliferative neoplasms (MPNs) involve the amplification of myeloid cells and platelets. Two mutations in the CALR gene are associated with MPN that result in a frameshift caused by the deletion of 52 bp (Del52) or the insertion of 5 bp (Ins5). Calreticulin is a major calcium binding protein of the endoplasmic reticulum that also acts as a carbohydrate-dependent molecular chaperone. These frameshift mutations change the C-termini of calreticulin resulting in a loss of its C-terminal ER retention sequence (KDEL) and calcium binding sites but maintain its N-glycan binding site. MPL is activated by these CALR mutations, leading to the overproduction of myeloid lineage lines. Previous studies have demonstrated that the binding of the mutant calreticulin to Mpl is at least in part mediated by an N-glycan of Mpl (Asn117) and these truncated forms of calreticulin oligomerize or aggregate. As calreticulin binding to Mpl and its activation appears to be central to MPN, the current study explores the nature of oligomeric mutant calreticulin binding to Mpl in cells. Three different cell-based assays are used to query protein interactions and activity: (1) oligomerization of calreticulin on denaturing (non-reducing and reducing) and native gel electrophoresis; (2) co-immunoprecipitations of calreticulin and Mpl; and (3) cell proliferation after the expression of Mpl with various calreticulin constructs. The main discovery was that the two novel Cys in the C-

terminus of calreticulin created by shift in the reading-frame along with a natural lone Cys, support disulfide mediate interactions between calreticulin monomers to form dimeric and higher order oligomeric species. Available structures of full length oligomeric calreticulin were used to model a predicted dimeric structure for the truncated Del52 mutant. This structural model that predicts C- and N-termini dimerization interfaces was tested using additional site mutations to disrupt dimerization, as well as there ability to support cell proliferation. This model potentially helps to explain the nature of the calreticulin and Mpl interactions that leads to Mpl activation associated with MPN.

Overall the manuscript is well written with a strong introduction that sets up an important biological problem that addresses the etiology of a subset of MPNs. My main issue is with the over reliance on the calreticulin dimer structural model of Del52 (Figure 5A) that is used to explain how MPNs exploits a natural dimerization interface of calreticulin. This model is based on extrapolating a dimeric structure of the full-length protein that has a different C-terminus and appears to be a result of crystal packing. Is there any evidence that full length calreticulin exists as a homodimer? Furthermore, extending this structure to the dimerization and activation of Mpl (Figure 8), while an attractive model, is highly speculative at this stage. Is there evidence for a calreticulin dimer-Mpl dimer heterotetrameric structure? The deletion or addition of Cys frequently creates protein aggregates that are not expected to be uniform but rather heterogenous poorly behaved complexes.

While the bands designated as calreticulin homodimers throughout the study are likely homodimers of calreticulin, this has not been directly tested or demonstrated.

A strength of the study is the use human MPN patient platelets to examine calreticulin oligomerization in diseased samples. In Figure 1, patient donor platelets expressing mutant calreticulin show higher oligomeric species but these species do not appear to be dimeric. How does this inform or support the model depicted in Figure 8?

Figure 2A would be aided by highlighting the novel Cys and designating where the sequence is altered in the MPN associated calreticulin mutants. How does a 52bp deletion and a 5bp insertion produce apparently a similar reading frame as the C-termini for both these mutants have similar sequences?

In Figure 2B and C, some of the truncated protein bands do not appear to be sufficiently separated from the endogenous calreticulin band to accurately quantify the monomeric fraction. Delta36 is not expressed and delta 28 is poorly expressed so it is difficult to draw any conclusions about their binding to MPL in the IP/IB experiment in Figure 2B.

We thank the reviewers for their valuable and important questions and suggestions that have strengthened the study. Our point-by-point responses are indicated below.

Editorial Comments

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures.

Response: To conform to this requirement, we have made changes to supplemental figure and Table files by:

a) Original Figure S1 is delected. Antibody specificity data are shown in Figure 1 and throughout the manuscript. Original Figures S2 and S3 are Figures S1 and S2 in the revised manuscript
b) Original Figure S4 is merged with Figure 2 of the revised manuscript.
c) Original Figure S5 is shown in S3B and S3C of the revised manuscript.
d) Original Figure S6 is merged with Figure 5 of the revised manuscript. To accommodate this change, original Figures 5B and 5C are Figures 6A and 6B of the revised manuscript
e) Previous Figure S7 is Figure S4C of the revised manuscript
f) Previous Figure S8 is S5A of the revised manuscript
g) Table I is also moved to the main manuscript and updated with the patient information for new Figure 1G of the revised manuscripts

Reviewer #1 (Comments to the Authors (Required)):

This is an elegant study that identifies key structural elements of C-terminal mutated calreticulin (CRT) that are involved in activating Mpl signaling. It is established that C-terminal mutated CRT (CRTDel52) binds and activates the transmembrane receptor Mpl which activates JAK signaling to drive myeloproliferative neoplasms. However, the specific mechanisms by which CRT binding to Mpl have not been elucidated previously. The current studies show that dimers of CRTDel52 are required for binding and identifies key cysteine residues necessary for disulfide-linked dimer/multimer formation. Using molecular modeling and mutated protein expression studies, the authors established that additional N-terminal domain ionic residues are necessary for dimer formation. These studies are very credible and well controlled and represent a significant contribution to this important interaction that impacts human disease. Furthermore, the authors also investigate the importance of various C-terminal domain cysteine residues and N-terminal ionic residues in mediating Mpl binding and cell proliferation.

Response: We thank the reviewer for pointing out the significance of the work.

The manuscript would benefit from clarification of the cellular compartment in which

CRTDel52-Mpl interactions occur. Because CRTDel52 lacks the KDEL ER retention sequence and Mpl is a transmembrane receptor, (as shown in the model in Figure 8), the supposition is that CRTDel52 is secreted and binds to the extracellular domain of Mpl. Has this been proven in the literature? Does CRTDel52 bind Mpl in the ER and is co-trafficked with Mpl as it is incorporated into the plasma membrane?

Response: The interaction is thought to initiate in the ER, where CRT_{Del52} acts as a chaperone for Mpl, and the complexes are co-trafficked to the cell surface with partially mature Mpl, via the secretory pathway (Masubuchi et al., 2019; Pecquet et al., 2019b).. These points are expanded in the revised introduction (page 4).

I ask because the relative expression and Mpl interactions of the various Mpl mutants are analyzed in total cell lysates (1% Triton-X 100 for protein expression; 1% digitonin for immunoprecipitations). However, there are no studies to show that the various mutants are actually trafficked properly and secreted so that interactions between CRT mutants and the extracellular domain are possible. Data showing secretion of the various mutants by western blotting and/or immunohistochemical localization (using antibody to the tag) of the constructs in the ER/golgi and/or cell membrane with MPL would enhance confidence in the authors' interpretation of the data. Furthermore, a discussion of the localization of the CRTDel52-Mpl interaction in cells should be included for the readers' benefit.

Response: Per the reviewer's suggestion, we measured localization by comparing the media/cell ratios of different CRT_{Del52} mutants. The new data are described on page 15 and with new Figure S5B: "Since the secretory efficiencies of mutants could affect function, we also assessed levels of secretion by comparing the media/cell ratios of different CRT_{Del52} mutants. We found that secretion efficiencies were reduced for several single mutants, although only significantly for $CRT_{Del52\text{-}H170A}$ (Figure S5B). These mutants, however, were largely functional for mediating cell proliferation (Figure 7C and 7D). Furthermore, secretion efficiencies of the functionally-defective $CRT_{Del52\text{-}3CA/D165K,}$ $CRT_{Del52\text{-}3CA/D166K,}$ $CRT_{Del52\text{-}3CA/2DK,}$ $CRT_{Del52\text{-}3CA/H170A}$ mutants (Figure 7) were not impaired relative to CRT_{Del52} (Figure S5B). Thus, the functional impairments of these mutants did not relate to low secretory efficiencies".

While conducting experiments for new Figure S5B, a slow-migrating band was visualized for constructs containing the $CRT_{Del52\text{-}D166K}$ mutant and to a lesser extent with constructs containing the $CRT_{Del52\text{-}H170A}$ mutant. This was also previously observed in the analyses of Figures 7G and 7F. On further investigation, we found that the slower migrating bands were sensitive to both Endo H and PNGase F digestions (new Figure S5C), suggesting that they correspond to glycosylated forms of the CRT_{Del52} proteins. The only predicted N-linked glycosylation site on CRT_{Del52} is N344, modeled to be buried within the C-C dimer of Del52 (Figure 5B). Thus, the disruption of predicted N-N interactions via the D166K and H170A mutations influences the conformational stability of C-C interactions, exposing a buried glycosylation site in the C-helix of CRT_{Del52}, finding that further support the multimer model of Figure 5D. These points are included in the revised manuscript pages 15-16.

Minor Concerns

It is interesting that the antibody to the N-terminal domain does not recognize multimers. In agreement with the N-terminal mutation studies, this observation might suggest that epitopes for this N-terminal antibody are inaccessible in multimerized CRT.

Response: The antibody to the N-terminal domain recognizes multimers, but more weakly than anti-CRT(C_{mut}). This is particularly apparent in Figure 4D/E. It is hard to determine the basis for these differences, which could relate to the higher specificity of the mutant-specific antibody.

Figure 1E-F: why are blots detecting hemoglobin with 2 different primary antibodies?

Response: The high density of hemoglobin contamination may have caused this effect. We undertook a new set of blots with two new patient samples. Precautions were taken to minimize hemoglobin contamination of the platelets preparations, as indicated in the revised methods. These new data are shown in new Figure 1G.

Why are the C-domain constructs not directly detectable in Ba/F3 lysates (page 8)?

Response: This is because of the lower level of expression of C-domains in Ba/F3 cells where we used a retroviral expression vector which has a weaker promoter (LTR-based) compared to the pcDNA vector promoter (CMV-based) used in HEK cells. However, it is important to note that the CRT_{Del52} C-domain interaction with Mpl is intact and detectable, in Ba/F3 cells (Figure 2D of the revised manuscript).

Figure 7B: The experimental variability of the Del52-2CA and Del52-3CA constructs in mediating cell proliferation is substantial, which complicates interpretation of the biological role of these cysteine mutations. Does this variability reflect differences in transfection efficiency/trafficking/secretion of this mutant?

Response: In the original submission, standards error of the mean (SEM) values were shown in all graphs of Figure 7 except panel 7B, which had used standard deviations (SD). This is changed to depict plots with SEM values in all graphs including 7B. A large number of experimental replicates are included in the Figure 7, as indicated in the legend. Importantly, separate sets of Del52-3CA experiments were performed and are reported in panels B, C and D, as indicated in the legends. Based on the numerous replicates of the proliferation assays with multiple independent infections of BaF3 cells for protein expression, we are confident in the conclusion that the cysteine mutants *per se* do not impair activity, and that additional globular domain mutations are required to abrogate functional activity.

Reviewer #2 (Comments to the Authors (Required)):

Venkatesan et al. confirmed that CRT mutants form dimer and/or multimer species not just in HEK293 cells expressing CRT variants but also in MPN patient platelets. Based on the reported crystal structure and new biochemical data, they suggest that the CRT dimerization is mediated by two-site interactions: 1) the natural low-affinity interface at N-domain with support by an intermolecular disulfide bonding (C163), and 2) a pair of disulfide bonds at the newly generated C-domain (C400 and C404 for del52 mutant). The authors finally proposed a model for the 2:2 ligand-receptor complex for this oncogenic signaling. The results are interesting and give a new insight into this therapeutically important system. Experiments and interpretations are generally convincing, but several points need to be commented on or clarified before publication.

Response: We thank the reviewer for the positive comments about the insights and interpretations.

Major points

For the platelet samples, it is unclear if the dimeric CRT mutants were buried behind the hemoglobin bands or there are only oligomer species. Can the author attempt hemoglobin depletion before running gels?

Response: We have included blots without Hemoglobin contamination in new Figure 1G, based on a recent patient blood collections, where precautions were taken to minimize hemoglobin contamination of the platelets preparations. In native blots of platelet lysates, we detect Del52 signals at the very top of the gels adjacent to stacking gels, but not other higher mobility species visualized in the non-reducing gels. This is also seen in transfected HEK cells when CRT_{Del52} and Mpl are co-expressed (new Figure S3A, native blot, lane 3). We thus believe that the bulk of Del52 in platelets migrates as a slow mobility species.

*In non-reducing SDS-PAGE and native gels, it is possible that the slower migrations were partly from the complex formation with Mpl (as shown by IP), or cysteine cross-linking with Mpl (*extracellular region has 15 cysteine residues, which should yield at least one unpaired cysteine) or even other endogenous proteins. Can the authors assess this by showing Mpl blot for several representative gel analysis?*

Response: In the non-reducing and native gels of HEK-derived proteins (Figure 2-4 and 6), cells were transfected only with vectors encoding Del52 and its mutants, but not Mpl, and thus could not correspond to complex formation with Mpl. Nonetheless, the point raised is an important one, on whether mixed disulfides can be formed between Del52 and Mpl. To examine whether mixed disulfides can be formed between Mpl and $CRT_{Del52,}$ we analyzed lysates from cells expressing Mpl, Del52 or both. We did not observe distinct new disulfide-linked species in cells expressing Mpl+CRT_{Del52} compared with CRT_{Del52} alone or in cells expressing Mpl+ CRT_{Del52} compared to Mpl alone or with

(new Figures S4A and S4B). Related discussions are on page 11 of the revised manuscript.

The authors say that: In the crystal structure of the "N-N" dimer, two C163 are not in direct contact, but could move closer to each other and form a disulfide bond following minor loop rearrangements. As the authors pointed out, in Figure 4B and E (non-reducing SDS-PAGEs), Del52-2CA shows multimer bands although there is only one free cysteine in the construct, and in Figure 4B, Del52-3CA still shows dimers and multimer bands. The authors concluded that the intramolecular C105-C137 bridge is disrupted, and those cysteines can also mediate multimer formation, but C163 still plays a deterministic role for the N-domain association to form the ordered dimer. However, in the case of wild-type CRT structures, C163 sidechain seems to be facing to the N-domain core. Can the authors show the mobility of this loop experimentally or by simulation?

Response: Thank you for the question. Available crystal structures of the native CRT from human (5lk5, 6eny, 3pos, 3pow), mouse (3rg0, 3o0v, 3o0w, 3o0x), Trypanosoma cruzi (5hcf) and Entamoeba histolytica (5hca) demonstrate rather similar composition and conformation of the loop 157-170 loop (INKDIRCKDDEFTH). The structure of this loop in aqueous solution is rather stable due to the internal H-bond (between H170 and D166) and hydrophobic interactions between I161, C163 from the loop and the N-domain core residues and between F168 from the loop and the C-domain α-helix. In this conformation, C163 is shielded from water by side chains of neighboring residues (K142, R162, D165) and cannot form an intermolecular S-S bond. We suggest that, under destabilizing conditions (such as heat shock, calcium depletion (Rizvi et al., 2004), low pH (Jorgensen et al., 2003), specific mutations such as H170A ((Jeffery et al., 2011) and this study Figure 6A/B), hydrophobic or hydrogen-bonding interactions may loosen, the loop may change conformation, and water-exposed C163 can form intermolecular C163-C163 bonds stabilizing the CRT dimer. Such S-S bonds may be formed with both WT CRT and CRT_{Del52} mutant (see Fig. 4E, lanes 1-2), but the propensity of this dimerization is higher in the mutant due to additional criss-cross “N-C” interactions between N- and C-domains of two CRT_{Del52} molecules (Fig.5D). Intermolecular S-S bonds between C400 and C404 also can stabilize dimers of CRT_{Del52} mutants both in water and in SDS. Besides, diverse types of non-functional multimers of CRT_{Del52} may by stabilized by many possible combinations of intermolecular disulfides between all free cysteines (C163, C400, C404), as well as via S-S bonded cysteines (C105-C137) upon thiol-disulfide exchange (Fig. 4 B, E).
Here we propose that the Mpl-activating dimer of CRT_{Del52} has a unique conformation, stabilized by intermolecular ionic interactions between charged residues from N-domain loops (160-170) and intermolecular disulfides between C-tail cysteines (C400 and C404), but not by C163-C163 disulfide. Therefore, we did not model the non-native dimer with C163-C163 disulfide that can be formed at destabilizing conditions (heat shock, SDS, Ca-depletion, H170A mutation). These points are indicated in the revised results (page 12, In the crystal structure of the “N-N” dimer, two C163 are not in direct contact**)** and clarified in the revised discussion (page 17).

We don't believe that C163 plays a deterministic role in formation of the productive Mpl-activating dimer, clarified in the discussion (page 17). Ultimately, the structures of purified CRT_{Del52} dimers will be needed to understand the exact conformation of the loop, which is beyond the scope of this study.

The study initially looked at the ability of the MPN-specific CALR mutations to form higher order structures by forming disulfide-stabilized multimers. They showed that their anti-CRT(Cmut) that was raised against the CALR-C-term domain was highly specific to the mutants and convincingly show the multimerization of CALR under non-reducing conditions and native-page, revealing the role of disulfide-bonds in forming multimers. It is interesting that they could not detect CALR with the N-term. commercial Ab (anti-CRT(N)) in the sera of MPN patients, but only with the anti-CRT(Cmut) for the IP experiment in Figure D.

Response: Thank you for the comments about the mutant-specific antibody-we were pleased with the specificity, and indeed this was important for many of the analyses. The only experiments with patient sera are in Figure 1F. The finding that the anti-CRT(Thermo) antibody (that used for the IP in Figure 1F with patient sera) does not detect mutant CRT suggests that the mutant CRT have conformational alterations not recognized by some antibodies raised against the wild type protein.

They also note that some platelet preparations show a possible Hb contamination band in the native page, and unfortunately it is around the size we may see other oligomers; perhaps a further sample clean-up can help clear that up.

Response: As noted above, we have included 2 new patients without Hb contamination in the revised Figure 1G. The predominant species migrate at the top of the gel, close to the stacking lanes, similar to the other platelet lysate native blots. This is also seen in transfected HEK cells when CRT_{Del52} and Mpl are co-expressed (new Figure S3A, native blot, lane 3). We thus believe that the bulk of CRT_{Del52} in platelets (which co-express Mpl) migrates as a slow mobility species.

In Figure 2, by making several CALR truncations and testing their specificity and ability to bind to MPL, the authors claim that as the C-term. domain is progressively shortened (truncated) compared to the Del52, you get a progressive loss of binding to MPL. Given, and as they noted, that each of the truncations had varying levels of the actual protein being expressed (possibly less stability/proteasomal degradation), it is difficult to conclude (from Figure 2B) that there is a difference in the amount of CALR protein associated with MPL between the mutants. Though it is clear that compared to WT, all mutants (except for Del52-36), show increased binding to MPL. In Figure 2C, they show compellingly that this dimerization is due to the cysteine residues by mutating them. The protein normalization in Figure 3A, is what I would have liked to have seen in 2B.

Response: Thank you for the note about the compelling result in Figure 2C. Relating to 2B, since the proteins are co-expressed with Mpl for Figure 2B, it is more difficult to achieve protein normalization similarly to 3A. As noted by the reviewer, the key point we

make based on this figure is that “binding interactions were observed between Mpl and the isolated C-domains of all the CRT_{Del52} constructs, except the poorly expressed $CRT_{Del52\Delta36}$, whereas binding between Mpl and the C-domain of CRT_{WT} was not observed (Figure 2B, lanes 7-12) (page 7).

From the native page in Figure 3D, the authors claim that the $CRT_{Del52\Delta28}$ truncation is needed to partially destabilize CRT_{Del52} multimers, but the formation/detection of monomers shown is very minimal. Though it is clear that the $CRT_{Del52\Delta36}$ truncation is indeed needed to fully destabilize the CRT_{Del52} multimers

Response: We agree with the reviewer that the $CRTDel52\Delta28$ truncation construct shows reduced monomers in the native PAGE relative to $CRT_{Del52\Delta36.}$ This is clarified in the revised text: “In native gels, only multimers were detectable for $CRT_{Del52\Delta12}$ and $CRT_{Del52\Delta19,}$ monomers begin to appear for $CRT_{Del52\Delta28}$, whereas $CRT_{Del52\Delta36}$ migrated largely as monomers “(page 9).

In Figure 4B, they claim that the 'Ala- substitution of this residue in the CRTDel52-CA (CRTDel52(C163A)) mutant resulted in a dimer and multimer pattern similar to CRTDel52', but it seems the pattern more closely resembles the Del52-2CA mutant with the multimer band pattern, monomer and dimer intensity.

Response: The $CRT_{Del52\text{-}CA}$ mutant’s disulfide linked species pattern is similar to CRT_{Del52} in that the dimer bands have similar mobilities, which is clarified on page 10 of the revised manuscript. However, in case of $CRT_{Del52\text{-}2CA}$ mutant, the mobility of the dimer band is slower as compared to CRT_{Del52} and $CRT_{Del52\text{-}CA}$, suggesting differences in oxidation. Both CRT_{Del52} and $CRT_{Del52\text{-}CA}$ would be able to form the C-terminal disulfides (Figure 5C), whereas $CRT_{Del52\text{-}2CA}$ would not.

Based on the dimer model (from Figure 5), the D165K mutation in combination with the CRTDel52-3CA mutations was claimed to induce more monomeric species in non-reducing blots (Figures 6A and 6B), but its visualization in 6A seems rather minimal. Though I would agree that there is more multimerization with the D166K mutations, which allowed for enhanced levels of disulfide-linked species.

Response: The original Figure 6A and 6B correspond to revised Figures 6C and 6D. Figure 6C, left panel corresponds to native blots, where there is a clear induction of monomer bands migrating between the 50 kDa and 64 KDa markers (Figure 6C, lanes 1-3 compared with 4-6), particularly based on the corresponding lower total protein load (Figure 6C, lower left panel, reducing SDS-PAGE, lanes 1-3 compared with 4-6). The most relevant comparison of non-monomer species would be the proteins migrating below the stacking gel, the position of which is now indicated in the figure.

In Figure 7, S8, it seems the only mutant constructs expressed are Del52 and Del52-12, and at much lower levels than WT. So assuming that the untagged constructs

expressed in Ba/F3 will have similar expression, it is not surprising that you get no cell growth from the other truncated proteins. It may be the lack of cell proliferation does not have to with the actual biology, per se, but the lack of protein even in the system. However, the Del52-19 does not show any protein in Sup 8, yet cell growth in the proliferation assay. Still, they showed convincing that a larger truncation is needed to completely abolish CRTDel52 mediated proliferation. Additionally, based on the IPs in Figure 7G, that CRTdel52 has a significantly reduced ability to bind to MPL and induce proliferation of Ba/F3 cells when the cysteine-residues and H170 site is mutated, strongly supports the necessity for both disulfide bonds and ionic interactions at both N-domain and C-domain dimerization interfaces to mediate the cell proliferation and oncogenic transformation previously noted with CALR-del52 in MPN.

Response: Thank you for pointing out that the data are convincing and provide strong support of the model. In revised figure S5A (original figure S8), we detected the expression of CRT Del52Δ12, 28 and 36 but not Del52Δ19 (using nucleofection of pcDNA vectors; stronger promoter). However, based on the proliferation assay, it is clear that the Del52Δ19 construct is active, and the expression is just not at the detectable level in the lysates. In Figure 7A, we used retroviral transductions (MSCV vectors; weaker promoter) to express the truncation mutants. Based on both figure S5 and Figure 7A, we concluded that the large novel C-terminal domain truncations (Del52Δ28 or Del52Δ36) are needed to inhibit the cytokine independent proliferation.

Minor points

(1)

As authors cited, Elf et al. Blood 2018 reported that CRTdel52 Δ10, Δ18, and Δ28 are as active as full-length CRTdel52, which is in contrast to the observation in this manuscript and against the hypothesis that the newly derived C-terminal cysteines are important for the abnormal Mpl activation. A more explicit discussion about this deviation is encouraged to be added.

Response: The study differences are stated on page 14 "These results deviate from those of Elf et al (Elf et al., 2018), where the transforming capacity of CRT mutant was abolished only after the most severe truncation of its C-terminus in $CRT_{Del52\Delta36}$".

Precisely for this reason, we tested the proliferation efficiency of full-length truncation constructs using two different expression vector systems, retroviral transduction and electroporation into Ba/F3-Mpl cells (Figure 7A and revised Figure S5A). Both results indicate impaired proliferation of large novel C-terminal constructs Δ28 and Δ36. However, it should be noted that the Elf et al. paper did not directly show expression of their truncation constructs in the Ba/F3 Mpl cells. Nonetheless, this note is added to page 14 "The basis for differences in results with $CRT_{Del52\Delta28}$ between our studies and those of Elf et al remain unclear, but could related to protein expression levels achieved, although we were unable to measure functional activities of $CRT_{Del52\Delta28}$ with two different expression systems."

(2)
...indeed the mutants but not wild type CRT are detectable in patient serum by coimmunoprecipitation analyses (Figure 1D).

Is it possible to identify if the secreted form includes dimer/multimer species? Although CRT's affinity for surface Mpl proteins would be lower with mature glycans, are the secreted CRT mutants able to activate the Mpl signaling on the neighboring cells?

Response: Del52 purified from HEK cell media also multimerizes (new Figure S3A). Previous studies showed that the secreted CRT cannot activate the Mpl containing neighboring cells (Araki et al., 2016; Han et al., 2016). This is likely because mature cell-surface would not be expected to contain monoglucosylated glycans important for CRT binding. This point is indicated on page 4, "Secreted calreticulin does not mediate paracrine activation of Mpl (Han et al., 2016). This is possibly explained by the absence of immature monoglucosylated N117-linked glycan on cell surface Mpl".

(3)
Do the authors suggest the abnormal signaling is mainly from the ordered dimer unit *(proposed in Figure 5A and 8 or multimers of this dimer unit exampled in in Figure S6C), against random cross-linking of CRT/Mpl that would produce many different forms of dimer units with a spectrum of signaling strengths? Or, is the dimer model one of the signaling unit compatible with Mpl activation?*

Response: We predict that the abnormal signaling is mediated by dimers, based on the observation of CRT_{Del52} dimers as the major multimeric species observed with purified CRT_{Del52} (New Figure 8A) and the findings of Pecquet et al, which indicate a 2:2 CRT_{Del52} complex (Pecquet et al., 2019a). This point is further discussed below.

It is encouraged to report more details and state material availability of the anti-CRT mutant C-terminal antibody generated in this work, if possible.

Response: The antibody will be made available upon request.

Reviewer #3 (Comments to the Authors (Required)):

Myeloproliferative neoplasms (MPNs) involve the amplification of myeloid cells and platelets. Two mutations in the CALR gene are associated with MPN that result in a frameshift caused by the deletion of 52 bp (Del52) or the insertion of 5 bp (Ins5). Calreticulin is a major calcium binding protein of the endoplasmic reticulum that also acts as a carbohydrate-dependent molecular chaperone. These frameshift mutations change the C-termini of calreticulin resulting in a loss of its C-terminal ER retention sequence (KDEL) and calcium binding sites but maintain its N-glycan binding site. MPL is activated by these CALR mutations, leading to the overproduction of myeloid lineage lines. Previous studies have demonstrated that the binding of the mutant calreticulin to Mpl is at least in part mediated by an N-glycan of Mpl (Asn117) and these truncated forms of calreticulin oligomerize or aggregate. As calreticulin binding to Mpl and its activation appears to be central to MPN, the current study explores the nature of

oligomeric mutant calreticulin binding to Mpl in cells. Three different cell-based assays are used to query protein interactions and activity: (1) oligomerization of calreticulin on denaturing (non-reducing and reducing) and native gel electrophoresis; (2) co-immunoprecipitations of calreticulin and Mpl; and (3) cell proliferation after the expression of Mpl with various calreticulin constructs. The main discovery was that the two novel Cys in the C-terminus of calreticulin created by shift in the reading-frame along with a natural lone Cys, support disulfide mediate interactions between calreticulin monomers to form dimeric and higher order oligomeric species. Available structures of full length oligomeric calreticulin were used to model a predicted dimeric structure for the truncated Del52 mutant. This structural model that predicts C- and N-termini dimerization interfaces was tested using additional site mutations to disrupt dimerization, as well as there ability to support cell proliferation. This model potentially helps to explain the nature of the calreticulin and Mpl interactions that leads to Mpl activation associated with MPN.

Overall the manuscript is well written with a strong introduction that sets up an important biological problem that addresses the etiology of a subset of MPNs.
My main issue is with the over reliance on the calreticulin dimer structural model of Del52 (Figure 5A) that is used to explain how MPNs exploits a natural dimerization interface of calreticulin. This model is based on extrapolating a dimeric structure of the full-length protein that has a different C-terminus and appears to be a result of crystal packing.

Is there any evidence that full length calreticulin exists as a homodimer?

Response: We thank the reviewer for the positive comments. Wild type CRT forms dimers and multimers following heat shock, exposure to low calcium, low pH conditions truncation of acidic C-domain, or specific mutations including H170A (Jeffery et al., 2011; Jorgensen et al., 2003; Rizvi et al., 2004). This information is included on page 12.

Furthermore, extending this structure to the dimerization and activation of Mpl (Figure 8), while an attractive model, is highly speculative at this stage. Is there evidence for a calreticulin dimer-Mpl dimer heterotetrameric structure? The deletion or addition of Cys frequently creates protein aggregates that are not expected to be uniform but rather heterogenous poorly behaved complexes.

Response: Based on size exclusion chromatography of purified complexes of soluble Mpl and CRT_{Del52}, Pecquet et al observed heterotetrameric $(Mpl\text{-}CRT_{Del52})_2$ complexes with soluble Mpl and some 1:1 $Mpl\text{-}CRT_{Del52}$ complexes (which could result from partial dissociation of heterotetramers) (Pecquet et al., 2019a). Taking together this finding along with the result that purified CRT_{Del52} forms disulfide-linked homodimers (new Figure 8A and discussion below), and the findings that disruption of dimer-stabilizing interactions abrogates cell proliferation (Figure 7), we suggest, as a working model (Figure 8B), that the structure of a heterotetramer would include a dimer of Mpl in the activated state and a dimer of CRT_{Del52}, stabilized by ionic interactions between N-

domains and covalent interactions (two C400-C404 disulfides) between novel C-tails of two molecules. The related points are discussed in a new results section entitled “Stable dimers are observable with purified CRT_{Del52} ” (page 16) and in a revised discussion (Page 18) “The formation of soluble Mpl_2-$(CRT_{Del52})_2$ heterotetramers (~200 kDa) was previously suggested by size-exclusion chromatography (Pecquet et al., 2019b)”.

While the bands designated as calreticulin homodimers throughout the study are likely homodimers of calreticulin, this has not been directly tested or demonstrated.

Response: To further assess the presence of disulfide-linked CRT_{Del52} dimers, a FLAG-his-tagged version of CRT_{Del52} was expressed and purified from HEK cells using anti-FLAG beads. Analyses of the protein by Coomassie staining revealed the presence of bands consistent with the size of CRT_{Del52} monomers and dimers, the identities of which were verified by mass spectrometric analyses (Figure 8A). Higher-order bands were also visualized, although less distinct or at lower abundance compared with monomers and dimers, based on the relative staining intensities. Within the excised dimer band, the number of peptide-to-spectrum match (PSM) values derived for CRT_{Del52} was 64 from the band indicated as “dimer”. The only other protein within that band with a PSM value >10 was keratin, a common contaminant in mass spectrometry samples. These findings demonstrate the prevalence of CRT_{Del52} homodimers with purified CRT_{Del52}.

A strength of the study is the use human MPN patient platelets to examine calreticulin oligomerization in diseased samples. In Figure 1, patient donor platelets expressing mutant calreticulin show higher oligomeric species but these species do not appear to be dimeric. How does this inform or support the model depicted in Figure 8?

Response: In platelets and transfected HEK cells that express both Mpl and CRT_{Del52}, native PAGE blots show predominantly low mobility species at the top of the gel, but dimers and heterogenous multimers are detectable by non-reducing SDS-PAGE (Figure 1 and new Figure S4A/B). In presence of Mpl, in immunoblots of native gels, the CRT_{Del52} becomes upshifted to lower mobility species in cells expressing Mpl+CRT_{Del52} compared with cells expressing CRT_{Del52} alone (new Figure S4A, lower panel). The slow mobility of CRT_{Del52} in native gels in the presence of Mpl resembles that in platelet lysates (Figure 1), which also contain Mpl. These points are discussed on page 11 of the revised manuscript.

Mobility in native gels is determined by both protein size and charge. Based on Figure 6, in native gels, CRT_{Del52} monomer migrate between the 50 and 64 kDa markers, and the second band observed, presumably corresponding to dimers, migrates between the 64 and 98 kDa markers. The band marked as dimer in Figure S4A (between 75 and 100 kDa) becomes depleted in Mpl+CRT_{Del52} complexes compared to CRT_{Del52} alone indicating that they are incorporated into complexes with Mpl. While monomeric or high order multimeric forms of CRT_{Del52} may also bind Mpl in cell lysates, our working model is that dimer is the essential activating unit as discussed on page 18.

Figure 2A would be aided by highlighting the novel Cys and designating where the sequence is altered in the MPN associated calreticulin mutants. How does a 52bp deletion and a 5bp insertion produce apparently a similar reading frame as the C-termini for both these mutants have similar sequences?

Response: Both mutations induce a parallel frameshift, and sequences are based on the originally-described mutations (Klampfl et al., 2013; Nangalia et al., 2013). We have updated figure 2A in the revised manuscript.

In Figure 2B and C, some of the truncated protein bands do not appear to be sufficiently separated from the endogenous calreticulin band to accurately quantify the monomeric fraction.
Response: In Figure 2B, only the truncated CRTs are used, and endogenous CRT is not detected with the anti-His antibody. We thus believe the comment refers to 3B and 3C, which show endogenous calreticulin. Lower exposure blots are shown in the revised Figure 3.

Delta36 is not expressed and delta 28 is poorly expressed so it is difficult to draw any conclusions about their binding to MPL in the IP/IB experiment in Figure 2B
Response: We agree with the reviewer that the delta 28 and 36 constructs are not expressed well, possibly due to enhanced proteasomal degradation, and thus our conclusion as stated in the manuscript is “Using co-IP assays, binding interactions were observed between Mpl and the isolated C-domains of all the CRT_{Del52} constructs, except the poorly expressed $CRT_{Del52\Delta36}$, whereas binding between Mpl and the C-domain of CRT_{WT} was not observed”. The main point is that of preferential Mpl binding to the mutant C-domain.

Summary
We have addressed all reviewer comments. New data are shown in Figures 1G, 8A, S3A, S4A/B and S5B/C and text changes in response to reviewer comments are highlighted in yellow. Some supplementary Figures are
condensed and reorganized into main Figures 2, 5+6 to conform with the JCB maximum of five figures. Table 1 is moved into the main manuscript.

We are grateful to the reviewers for the comments and suggestions and hope the manuscript is now acceptable for publication

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March 5, 2021

RE: JCB Manuscript #202009179R

Dr. Malini Raghavan
University of Michigan-Ann Arbor
6706A Medical Science Building II
Ann Arbor, Michigan 48109

Dear Dr. Raghavan,

Thank you for submitting your revised manuscript entitled "Mpl activation by dimers of MPN calreticulin mutants stabilized by disulfides and ionic interactions". You will see that the reviewers praise your thorough revision efforts and recommend publication. We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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Mechanism of calreticulin-mediated activation of the myeloproliferative leukemia protein

Running title (50 characters max, including spaces):
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suggestion:

In myeloproliferative neoplasms, oncogenic transformation involves mutations in the ER chaperone calreticulin (CRT) and the myeloproliferative leukemia protein (Mpl). Venkatesan et al. here describe the molecular mechanism underlying CRT-mediated constitutive activation of Mpl.

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- Please include species and source for all antibodies, including secondary, as well as catalog numbers/vendor identifiers if available.
- Sequences should be provided for all oligos: primers, si/shRNA, gRNAs, etc.

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Sincerely,

Ira Mellman, Ph.D.
Editor, Journal of Cell Biology

Melina Casadio, Ph.D.
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors have addressed all of my concerns from the first version through additional experimental data and careful textual revision.
This kind of thorough analysis of protein structure and multimer formation of mutated calreticulin is refreshing and of potential clinical significance.

Reviewer #2 (Comments to the Authors (Required)):

the authors have done a good job addressing comments and I recommend publication

Reviewer #3 (Comments to the Authors (Required)):

The revisions fully address all my concerns with the initial submission. This Ms provides an exciting advance in the molecular understanding how a mutant ER chaperone (calreticulin) activates Mpl and is associated with MPN.