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LETTER TO THE EDITOR

Mutated calreticulin retains structurally disordered C terminus that cannot bind Ca^{2+}: some mechanistic and therapeutic implications

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Two very recent reports[1,2] made a tremendous breakthrough in our understanding of the molecular basis of myeloproliferative neoplasms (MPNs) without mutations in *Janus kinase 2* (*JAK2*) and myeloproliferative leukemia (*MPL*) virus oncogene genes. Klampfl *et al.*[1] and Nangalia *et al.*[2] independently identified recurrent somatic mutation in the calreticulin (*CALR*) gene exclusively in patients with *JAK2* and *MPL* mutation-negative MPNs. Strikingly, all these mutations were small deletions and insertions in exon 9 of the gene leading to a shift in the open reading frame and expression of peptides in which the wild-type C terminus was substituted for a novel identical in all mutants C terminus and a small mutant-specific portion of variable length. This specific feature of CALR mutations necessitates further elucidation of the mechanisms through which they promote neoplastic transformation and might be particularly challenging to dissect as CALR is involved in a plethora of intra- and extra-endoplasmic reticulum (ER) cellular processes.[3]

However, as pointed by the two reports, the C terminus of the wild-type CALR has three important features: (i) its secondary structure is disordered; (ii) it is acidic and binds calcium ions at low affinity; (iii) it has an ER retention signal. Both Klampfl *et al.*[1] and Nangalia *et al.*[2] reported the loss of C-terminal ER retention signal (KDEL) in the mutant proteins. However, both groups did not observe significant changes in the subcellular distribution of mutant CALR protein as compared with the wild type. A possible explanation for their observation would be that as reported previously,[4] CALR retro-translocation from ER to the cytoplasm is also dependent on the C-terminal domain.

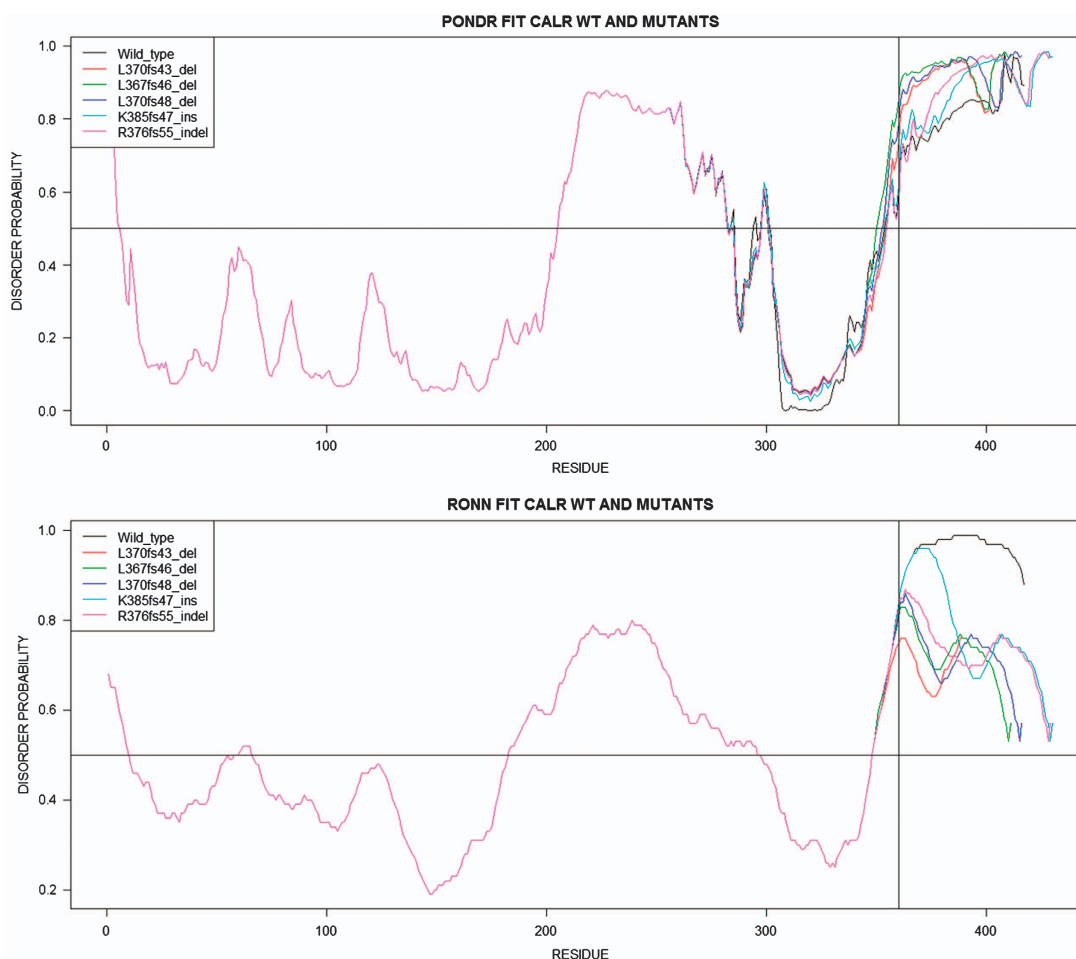

Figure 1. Graphical representation of the probability for structural disorder of full-length wild-type and selected mutant CALR proteins. The horizontal line designates the generally accepted cut-off for structural disorder of 0.5, whereas the vertical line designates the position of the common Q361 amino-acid residue. The plots were generated using the basic graphical functions of R statistical environment (http://www.r-project.org/).

Here, we used a straightforward bioinformatic approach to address the question of whether the mutant peptides share the first two of the abovementioned features, which may then help point to the possible molecular processes that these mutant proteins are involved in. We applied two commonly used computational prediction tools to verify the presence of structurally disordered domains in proteins. These were PONDR-FIT (http://www.disprot.org/pondr-fit.php)[5] and RONN (http://www.app.strubi.ox.ac.uk/RONN/?p=RONN&HTTP_VARS).[6] As shown in Figure 1, both predictors confirmed the structurally disordered C terminus of the wild-type CALR. Furthermore, the C-terminal domains of all mutant proteins showed significant probability of disordered sequence as well. Therefore, we concluded that it is unlikely that changes in the structural disorder of the mutant C terminus are the cause of its altered cellular functions, as the loss of chaperone function of CALR is not responsible for the embryonic lethality of CALR knockout mice (see below). We then used a peptide charge calculator to assess the isoelectric points (pI) of the C termini of the wild-type and mutant proteins. As shown in Table 1, the highly acidic nature of the wild-type C terminus was substituted for the highly basic mutant C termini ($P = 0.0001$). This was because of the loss of aspartic and glutamic acid residues and the presence of a 12 arginine residues in the common mutant C-terminal sequence. Another important observation is the higher pI values of deletion mutants compared with those with insertions and complex rearrangements (Table 1). One can speculate that this difference might account for the higher relative frequency of deletion mutations because of the strong positive selection of mutants with greater loss of calcium binding activity (that is, higher pI) (see below).

The highly basic C termini of the mutant CALR proteins obviously have reduced binding activity toward the Ca^{2+} cation. This is expected to lead to structural and functional consequences for several reasons. First, CALR C-terminal domain was experimentally verified to have high-capacity and low-affinity calcium-binding activity.[7] Furthermore, deletion of the C-terminal domain or calcium depletion can enhance the ability of the CALR to exert its function as a chaperone in the ER.[8] Related to the latter observation is a more recent study by Villamil Giraldo *et al.*[9] showing that Ca^{2+} in concentrations higher than its average ER concentration causes a switch in the C-terminal structure from disordered peptide to a more rigid and compact conformation. A more ordered structure of the C terminus was reported based on modeling of small-angle X-ray scattering data but the effect of Ca^{2+} concentration on this model is unclear.[10]

CALR ablation in mice is embryonically lethal.[11] A series of studies (reviewed by Coe *et al.*[12]) showed that Ca^{2+} binding activity of CALR is more important for this phenotype than the loss of chaperone function. In the absence of CALR, the nuclear localization of nuclear factor of activated T cells (NFAT) is abrogated. Notably, this phenotype can be rescued by overactivation of calcineurin.[13] Furthermore, a recent study[14] showed that the calcineurin–NFAT axis acts as a negative regulator of myeloid development at the level of hematopoietic progenitors. Another study showed that *in vivo* suppression of NFATc leads to increased megakaryopoiesis,[15] which was in accord with an earlier report that an endogenous inhibitor of calcineurin called FKBP51 was 2–8 times overexpressed in megakaryocytes from primary myelofibrosis (PMF) patients.[16] Calcineurin is also associated with downstream signaling of erythropoietin (Epo) receptor during erythropoiesis. However, it is having a role in Epo-induced differentiation through *c-myb* suppression and not proliferation.[17] In other words the lower activity of calcineurin–NFAT signaling in erythroid progenitors is not providing a proliferative advantage. Therefore, it is tempting to speculate that in hematopoietic stem/progenitor cells, mutated

Table 1. Isoelectric points (pI) for wild-type and mutant CALR peptides (reported by Nangalia *et al.*[2]) derived from the C terminus (from the common Q361 residue onwards). Calculations were performed using the Scripps Institute's on-line Protein Calculator v.3.3 (http://www.scripps.edu/~cdputnam/protcalc.html)

Designation	*Sequence*	*pI*
Wild-type	QDEEQRLKEEEEDKKRKEEEEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL	3.98[a]
Deletions		
L367fs*46	QDEEQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	12
E370fs*43	QDEEQRLKEVMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	11.76
E370fs*48	QDEEQRLKEQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	11.96
L367fs*48	QDEEQRQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	12.05
L367fs*44	QDEERRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	11.95
K368fs*51	QDEEQRLRRRQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	12.18
L367fs*52	QDEEQRRRRRQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	12.21
R366fs*53	QDEEQKRRRRQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	12.8
E371fs*49	QDEEQRLKEERQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	11.91
K368fs*43	QDEEQRLMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	11.88
E370fs*37	QDEEQRLKERMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	11.66
D373fs*47	QDEEQRLKEEEERTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	11.63
K374fs*53	QDEEQRLKEEEEDKRRRRQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	11.79
E371fs*49	QDEEQRLKEERTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	11.85
Mean		11.97[b,c]
Insertions		
K385fs*47	QDEEQRLKEEEEDKKRKEEEEAEDNCRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	10.09
K385fs*47	QDEEQRLKEEEEDKKRKEEEEAEDLCRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	10.09
Mean		10.09[d]
Complex		
R376fs*55	QDEEQRLKEEEEDKKLCKRRRRQRTRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	11.71
K385fs*47	QDEEQRLKEEEEDKKRKEEEEAEDSCRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	10.09
E381fs*48	QDEEQRLKEEEEDKKRKEEEDPCRRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA	10.54
Mean		10.78

[a] $P = 0.0001$ vs all mutants. [b] $P = 0.03$ vs mutants with insertions. [c] $P = 0.01$ vs mutants with complex rearrangements. [d] $P = 0.33$ vs mutants with complex rearrangements.

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CALR may not mediate the Ca^{2+} export from the ER thus keeping the calcineurin–NFAT signaling pathway significantly less active, which in turn favors myeloid/megakaryocyte lineage commitment and not erythroid lineage proliferation. This may explain the observation that among the classical MPNs, *CALR* mutations are found only in PMF and essential thrombocythemia and are not associated with erythrocytosis. Furthermore, Klampfl *et al.*[1] showed that in two PMF patients with multiple mutations, the CALR mutations were early events, that is, they had already been present in the proliferative phase of PMF. Finally, if the hypothesis for the role of calcineurin signaling in *CALR*-mutated MPN cases is true, it may open the way toward a specific treatment. For instance, it was shown that compounds such as chlorogenic acid can cause calcineurin activation in a calmodulin-dependent manner.[18]

In conclusion, based on a bioinformatic analysis of structural and electrochemical properties of the recently identified mutated C termini of CALR, we propose that the loss of Ca^{2+} binding activity may contribute significantly to the pathogenetic mechanisms of these mutations. However, as CALR is a protein with versatile cellular functions, this might be just one of several mechanisms. This will hopefully lead to innovative therapies for this subset of MPN patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1 Klampfl T, Gisslinger H, Harutyunyan AS, Nivarthi H, Rumi E, Milosevic JD *et al.* Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med* 2013; **369**: 2379–2390.
2 Nangalia J, Massie CE, Baxter EJ, Nice FL, Gundem G, Wedge DC *et al.* Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. *N Engl J Med* 2013; **369**: 2391–2405.
3 Wang WA, Groenendyk J, Michalak M. Calreticulin signaling in health and disease. *Int J Biochem Cell Biol* 2012; **44**: 842–846.
4 Afshar N, Black BE, Paschal BM. Retrotranslocation of the chaperone calreticulin from the endoplasmic reticulum lumen to the cytosol. *Mol Cell Biol* 2005; **25**: 8844–8853.
5 Xue B, Dunbrack RL, Williams RW, Dunker AK, Uversky VN. PONDR-FIT: a meta-predictor of intrinsically disordered amino acids. *Biochim Biophys Acta* 2010; **1804**: 996–1010.
6 Yang ZR, Thomson R, McNeil P, Esnouf RM. RONN: the bio-basis function neural network technique applied to the detection of natively disordered regions in proteins. *Bioinformatics* 2005; **21**: 3369–3376.
7 Baksh S, Michalak M. Expression of calreticulin in *Escherichia coli* and identification of its Ca2+ binding domains. *J Biol Chem* 1991; **266**: 21458–21465.
8 Rizvi SM, Mancino L, Thammavongsa V, Cantley RL, Raghavan M. A polypeptide binding conformation of calreticulin is induced by heat shock, calcium depletion, or by deletion of the C-terminal acidic region. *Mol Cell* 2004; **15**: 913–923.
9 Villamil Giraldo AM, Lopez Medus M, Gonzalez Lebrero M, Pagano RS, Labriola CA, Landolfo L *et al.* The structure of calreticulin C-terminal domain is modulated by physiological variations of calcium concentration. *J Biol Chem* 2010; **285**: 4544–4553.
10 Norgaard Toft K, Larsen N, Steen Jorgensen F, Hojrup P, Houen G, Vestergaard B. Small angle X-ray scattering study of calreticulin reveals conformational plasticity. *Biochim Biophys Acta* 2008; **1784**: 1265–1270.
11 Mesaeli N, Nakamura K, Zvaritch E, Dickie P, Dziak E, Krause KH *et al.* Calreticulin is essential for cardiac development. *J Cell Biol* 1999; **144**: 857–868.
12 Coe H, Michalak M. Calcium binding chaperones of the endoplasmic reticulum. *Gen Physiol Biophys* 2009; **28**: Spec No Focus F96–F103.
13 Guo L, Nakamura K, Lynch J, Opas M, Olson EN, Agellon LB *et al.* Cardiac-specific expression of calcineurin reverses embryonic lethality in calreticulin-deficient mouse. *J Biol Chem* 2002; **277**: 50776–50779.
14 Fric J, Lim CX, Koh EG, Hofmann B, Chen J, Tay HS *et al.* Calcineurin/NFAT signalling inhibits myeloid haematopoiesis. *EMBO Mol Med* 2012; **4**: 269–282.
15 Zaslavsky A, Chou ST, Schadler K, Lieberman A, Pimkin M, Kim YJ *et al.* The calcineurin-NFAT pathway negatively regulates megakaryopoiesis. *Blood* 2013; **121**: 3205–3215.
16 Giraudier S, Chagraoui H, Komura E, Barnache S, Blanchet B, LeCouedic JP *et al.* Overexpression of FKBP51 in idiopathic myelofibrosis regulates the growth factor independence of megakaryocyte progenitors. *Blood* 2002; **100**: 2932–2940.
17 Schaefer A, Magocsi M, Stocker U, Fandrich A, Marquardt H. Ca2+/calmodulin-dependent and -independent down-regulation of c-myb mRNA levels in erythropoietin-responsive murine erythroleukemia cells. The role of calcineurin. *J Biol Chem* 1996; **271**: 13484–13490.
18 Tong L, Song Y, Jia Z, Zhang W, Wei Q. Calmodulin-dependent activation of calcineurin by chlorogenic acid. *IUBMB Life* 2007; **59**: 402–407.

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