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Role of rare calreticulin mutants and of the endoplasmic reticulum stress in the pathogenesis of Myeloproliferative Neoplasms

Par M. KATTE RAO TOPPALDODDI

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Dirigée par Dr. William Vainchenker

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Président du jury : AJZENBERG Nadine, Professeur, Université Paris-Diderot
Rapporteurs : JAMES Chloe, Professeur, Université de Bordeaux
CONSTANTINESCU Stefan, Professeur, Université catholique de Louvain, Belgique
Examinateurs : GARÇON Loïc, Professeur, Université d'Amiens
Directeur de thèse: VAINCHENKER William, Directeur de recherche, émérite, INSERM
Co-directeurs de thèse: ROSS Cecil, Professeur, St. John's Medical College, Indes

Titre:

Rôle de mutants rares de la calréticuline et du stress du réticulum endoplasmique dans la pathogenèse des néoplasmes myéloprolifératifs

Résumé:

Après la découverte des mutations de la calréticuline dans les néoplasmes classiques myéloproliferatifs négatifs pour le Ph1, les travaux se sont focalisés sur les deux mutations les plus fréquentes, c'est-à-dire la calréticuline del52 et l'ins5, mais il existe environ 20% de mutants rares de la calréticuline (une cinquantaine), qui ont été classés en type-1 « like » et type-2 « like », classification basée sur leur structure. Cependant il reste à déterminer si cette classification est pertinente du point de vue fonctionnel, ce qui pourrait avoir des conséquences pour la prise en charge des patients et leur traitement. Ici, nous démontrons que deux mutants rares de type-1 (del34 et del46) et un de type-2 (del19) se comportent de manière similaire aux deux mutations fondatrices de cette classification, del52 et ins5, respectivement. Ces résultats ont été validés par des expériences *in vivo* chez la souris. Tous les mutants de la calréticuline (del19, del34 et del46) nécessitent absolument le récepteur de la thrombopoïétine, appelé MPL, pour induire une transformation cellulaire en provoquant une activation indépendante de la thrombopoïétine de la voie MPL / JAK2-STAT, comme les mutants del52 et ins5.

Dans les expériences de transplantation de moelle osseuse de souris, les mutants rares de type-1 sont associés à une progression fréquente de la maladie d'un tableau proche d'une thrombocytémie essentielle à une myélofibrose, tandis que le mutant rare de type 2 est associé à une légère thrombocytose. Du point de vue hématopoïétique, les mutants rares de type-1 provoquent une amplification au niveau des cellules souches hématopoïétiques donc à un stade précoce tandis que les mutants rares de type-2 provoquent une amplification tardive de la mégacaryopoïèse.

Grâce à une modélisation protéique basée sur l'homologie des mutants de calréticuline, nous avons identifié des domaines oncogènes qui seraient potentiellement responsables de l'interaction pathologique de la calréticuline et de MPL pour conduire à une activation indépendante de la thrombopoïétine. Maintenant, ces résultats *in silico* doivent être absolument validés par des études structure-fonction.

Enfin, nous avons modélisé un nouveau mécanisme de signalisation dans la leucémie myéloïde chronique comprenant IRE-1alpha, un bras de la voie de réponse des protéines mal repliées (UPR), qui pourrait être responsable de la perte de la fonction de la p53 pendant la progression de la leucémie myéloïde chronique vers une leucémie aiguë. Un tel mécanisme pourrait être impliqué dans les autres MPN.

Mots clefs:

La calréticuline, les néoplasmes myéloprolifératifs, les mutants de type-1, les mutants de type-2, la thrombocytémie essentielle, la myélofibrose, IRE-1alpha, EPU, la leucémie myéloïde chronique, p53

Title:

Role of rare calreticulin mutants and of the endoplasmic stress in the pathogenesis of myeloproliferative neoplasms

Abstract:

After the discovery of calreticulin mutations in classical Ph1$^-$ Myeloproliferative Neoplasms, extensive investigation is underway on the two most frequent mutations, i.e., del52 and ins5, but it remains that the rare calreticulin mutants, which include both type-1 like and type-2 like require a similar investigation for ascertaining whether the classification of type-1 and type-2 has a functional relevance as well as for therapeutic intervention and patient management. Here we demonstrate that type-1 like (del34 and del46) and type-2 like (del19) mutants behave similarly as del52 and ins5 mutants, respectively. Moreover, we validate our findings with *in vivo* experiments. All the calreticulin mutants (del19, del34 and del46) absolutely require the thrombopoietin receptor, MPL, to induce cell transformation by causing ligand independent activation of the MPL/JAK2-STAT pathway.

In mouse bone marrow transplantation experiments, type-1 like mutants are associated with frequent progression from an essential thrombocythemia-like phenotype to myelofibrosis whereas type-2 like mutant is associated with mild thrombocytosis. Type-1 like mutants cause clonal amplification of early hematopoetic stem cells whereas the type-2 like mutant causes late platelet amplification.

Further, by homology based protein modeling of calreticulin mutants, we have identified possible oncogenic domains responsible for pathologic interaction of CALR and MPL leading to ligand independent activation of MPL. Now they must be validated by structural-functional studies

Finally, we have modelled a novel signaling mechanism in chronic myeloid leukemia comprising of IRE-1alpha, an unfolded protein response (UPR) pathway arm, which may be responsible for loss of the WT p53 function during leukemic development and progression. Such a mechanism may be involved in the other MPNs

Keywords:

calreticulin, myeloproliferative neoplasms, type-1 like mutants, type-2 like mutants, essential thrombocythemia, myelofibrosis, IRE-1alpha, UPR, chronic myeloid leukemia, p53

I dedicate my thesis to cancer patients.

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INTRODUCTION

1.0: Classical Bcr-Abl1 negative myeloproliferative neoplasms

Classical Ph1 or Bcr-Abl1 negative myeloproliferative neoplasms (MPNs) (Ph1$^-$ MPN) are clonal disorders, which mainly include polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF).[1] They occur due to acquisition of somatic mutations in hematopoietic stem cells (HSCs).[1] These diseases are characterized based on which mature blood cells according to the different cell lineages that are excessively produced.[2,3,4] The diagnosis is based on clinical, laboratory and histopathological/cytogenetic/molecular genetic features.[1] These disorders lead to erythrocytosis and/or thrombocytosis and a variable degree of bone marrow fibrosis.[1] These three disorders are characterized by common features: increased production of mature blood cells with the absence or mild features of myelodysplasia.[1] Their principal complications are mainly thrombosis and sometimes hemorrages, which can reveal the disease and their possible progression to secondary acute myeloid leukemia (AML) with different rates according to the type of MPN.[1] Splenomegaly is frequent but mainly in PV and PMF and very rarely in ET. This is due to extramedullary hematopoiesis, which is constant in PMF and frequent in other MPNs.[2,3,4]

These three disorders have been considered as belonging to a same pathogenic entity called myeloproliferative diseases by W Dameshek.[5]

If in their classical forms the three disorders are quite different, there are many forms of transition, which render the classification between these three disorders not so easy explaining the permanent changes in the WHO classification and new criteria of diagnostic.[1] This is because the boundaries between some ET and PV are very tight and that some ET can progress to PV.[1] In addition ET and PV can progress to myelofibrosis (post-ET and post-PV MF or secondary MF).[1] More particularly, a fourth entity has been recently described called pre-fbrosis, or pre-fibrosis PMF or early PMF, which clinically has many features of ET, but with the presence of an increased myeloid proliferation and megakaryocyte myelodysplastic features with frequently a splenomegaly but without myelofibrosis.[1] This pre-fibrosis can progress to a true PMF in 30% of the cases and has a more severe prognosis than clasical ET, but has a better prognosis compared to that of overt PMF.[6, 1]

PV is a clonal proliferation leading to abnormally high production of red blood cells.[1] It harbors dysregulated erythropoiesis.[1] In PV, trilineage proliferation is found.[1] In erythrocytotic or proliferative phase of PV, an increased production of white blood cells, red blood cells and platelets is found.[1] The bone marrow is hypercellular.[1] PV progresses to myelofibrosis wherein the peripheral blood changes are similar to primary myelofibrosis with change in leukoerythroblastic features, splenomegaly and bone marrow fibrosis.[1] Up to 10% of PV patients will develop acute myeloid leukemia (AML) or myelodysplasia (MDS) in 15 years[6,1]. The rate of transformation after 20 years is controversial, but may reach up to 50%.[1] The treatments play an important role in transformation such as P^{32}, chlorambucil and pipobroman.[1] The role of Hydoxyurea in the transformation is controversial[6,1]. The strategy of performing

phlebotomy in PV patients is seen as one of the extensively followed procedures to relieve the symptoms and avoid disease induced complications.[1]

Essential thrombocythemia (ET) is a clonal proliferation leading to increased production of platelets.[1] In many cases, ET is only associated with an isolated thrombocytosis and gives a clinical disease close to secondary thrombocytosis or hereditary thrombocytosis.[1] The most frequent complications of ET are thrombosis and hemorrhage.[1] Progression of ET to acute myeloid leukemia is the rarest among MPNs and is considered to be around 5% at 15 years.[1] Progression to myelofibrosis is much more frequent but considered by some authors as resulting from a confusion between ET and pre-fibrosis.[1] Furthermore, ET associated with the JAK2V617F mutation are frequently associated with an increased level of red blood cells and hemoglobin and are frequently considered as a "fruste" PV.[1] Bone marrow biopsy observations play a very important role in permitting to differentiate ET from pre-fibrosis PMF.[1] It has been suggested that pertaining to WHO based classification, some clinicians may encounter that some patients may present thrombocytosis with bone marrow biopsy showing pre-fibrosis PMF phenotype but these clinical findings may lack the minor criteria of WHO classification and in these kind of cases, according to the current WHO classification these disorders may be diagnosed as unclassifiable MPN.[1]

Primary myelofibrosis (PMF) is characterized by striking splenomegaly, occurrence of dry tap from the bone marrow aspiration due to massive bone marrow fibrosis and change in leukoerythroblastic features in the peripheral blood.[1] The bone marrow fibrosis is variable and has been classified in several types.[1] Type 1 myelofibrosis (mild reticulin fibrosis) can be present in classical PV or ET.[1] However type 2 and more myelofibrosis are characterized by a collagen fibrosis that defines true MF.[1] An important MF can be associated with osteosclerosis.[1] The frequent causes of death are complication of bone marrow failure occurring in around 22% of the patients, including anemia, infection and hemorrhage; transformation to acute leukemia occurs in up to 30% patients and complications linked to massive splenomegaly in around 11% patients.[1] PMF is a very heterogeneous disease and has been classified according to International Prognostic Scoring System (IPSS) in 4 risk groups.[1] Type 1 has a good prognosis (more than 15 years average survival) whereas in type 4 the average survival is less than 3 years.[1] The current effective therapeutic options for PMF are limited and allogenic stem cell transplantation provides a chance for cure, but the morbidity and mortality remain significant although important progresses have been made. [5,6,1,7]

Table 1: WHO criteria for diagnosis of classical Bcr-Abl1 negative MPNs. Adapted from DA Arber et al.[6]

Major criteria	PV	ET	PMF (overt)	Pre-MF
	1.Hemoglobin >16.5g/dL (men) >16g/dL (women) or Hematocrit	1.Platelet count >450*10^9/L	1.MK proliferation and atypia and > grade 2 reticulin/collagen fibrosis	MK proliferation and atypia Increased cellularity, Granulocytic proliferation

	>49% (men) >48% (women) or red cell mass >25% above mean			and decreased erythropoesis
	2. Bone marrow trilineage proliferation with pleomorphic mature megakaryoctes (MKs)	2. BM MK proliferation with large and mature and morphology and hyper lobulated nuclei Reticulin fibrosis grade should be <1	2.Not meeting WHO criteria for other myeloid neoplasms	2.Not meeting WHO criteria for other myeloid neoplasms
	3. Presence of JAK2 mutation	3.Not meeting WHO for other myeloid neoplasms 4. Presence of JAK2, MPL or CALR mutation	3.Presence of JAK2, MPL or CALR mutation Or Presence of another clonal marker Or Absence of evidence for reactive bone marrow fibrosis	3.Presence of JAK2, MPL or CALR mutation Or Presence of another clonal marker Or Absence of evidence for reactive bone marrow fibrosis
Minor criteria	Subnormal serum erythropoetin level	Presence of a clonal marker Or Absence of evidence for reactive thrombocytosis	Anemia not otherwise attributed Leukocytosis $11*10^9/L$ Palpable splenomegaly Increased lactate dehydrogenase (LDH) above upper normal limit	Anemia not otherwise attributed Leukocytosis $11*10^9/L$ Palpable splenomegaly Increased lactate dehydrogenase (LDH) above upper normal

				limit

2.0: Genetic basis of Bcr-Abl1 negative MPNs (see annexe 1)

Major progress has been made in deciphering the molecular basis of Ph1$^-$ MPNs.[7] Majority of the MPN patients carry mutations in either of *JAK2, MPL* and *CALR* genes, which have been characterized as driver mutations that can induce MPN phenotype.[7] Hyperactive JAK/STAT pathway is found to be the hallmark of MPN even in patient group called "triple negative" MPN where the driver mutations are still unknown.[8] Thus classical MPNs are more precisely characterized by a deregulated activation of JAK2 and of the signaling pathways downstream cytokine receptors.[9]
Other associated mutations in epigenetic regulators, splicing regulators, transcription factors and signaling molecules modify the course of the disease in addition to contributing to the disease initiation and/or progression.[7] The central role of JAK-STAT pathway in Ph1$^-$ MPNs has led to the development of a small molecular JAK2/JAK1 inhibitor that is FDA approved.[9]

2.1: Somatic mutations in Ph$^-$ MPNs

Somatic mutations are responsible for clonal expansion of the HSC compartment not only in MPNs, but also in most types of myeloid malignancies.[7] Sanger sequencing and now a high speed sequencing by next-generation sequencing (NGS) have resulted in the discovery of several gene mutations across myeloid malignancies including MPNs.[7]

2.1.1: JAK2 gene mutations

Before 2005, the genetic basis for Ph1$^-$ MPNs was not known. In 2005, several teams reported a landmark discovery of a G to T somatic mutation at nucleotide 1849, in exon 14 of JAK2 gene, resulting in the substitution of valine to phenylalanine at codon 617 in the pseudokinase domain of JAK2 protein (JAK2V617F).[10,11,12,13] This mutation is found in approximately 70% of MPNs: 95% of PV and 50-60% of ET and PMF. JAK2V617F can undergo a transition from heterozygosity to homozygosity due to mitotic recombination resulting in loss of heterozygosity on the short arm of chromosome 9 (9pLOH).[11,14] This mutation hits a HSC and is present in all myeloid lineages. It can also be detected in lymphoid cells, mainly B and NK cells and rarely and later during disease progression in T cells.[7] It is not present in cells other than blood lineages though it has been detected in endothelial cells of the spleen in case of Budd-Chiari syndrome and other mesenteric thromboses.[15] The mutant allele burden in granulocytes shows large variations, providing a continuum between the threshold of detection of around 1% to 100%.[7] The mutant allele burden is usually low in ET, which is around 25%, is higher in PV, frequently over 50% and close to 100% in post-PV or post-ET MF.[7] Homozygosity is particularly associated with PV and

secondary MF.[7] In PMF, the mutant allele burden varies but is frequently high.[16] JAK2V617F mainly occurs in classical MPNs.[7] Refractory anemia with ring sideroblasts and thrombocytosis (RARS-T) stands as an exception as it is found in more than 50% of the cases associated with the SF3B1 mutation.[17] It is rarely found in other hematological malignancies.[18] However, JAK2V617F has been found to be present at threshold levels ranging between 0.2% and 0.8% in the normal population including in a neonate.[19,20] It is one of the most common mutations associated with clonal hematopoiesis linked to aging.[21,22,23]

JAK2 exon 12 mutations are also found in MPNs, and are mainly present in the JAK2V617F negative PV.[24] They are not associated with ET and PMF, but JAK2 exon 12 mutation can lead to the progression of PV to secondary MF.[25] There are several different mutations in the exon 12, in their majority in-frame small deletions and insertions type of mutations.[7] Exon 12 mutations in JAK2 are all located in the linker between the Src homology 2 (SH2) and the pseudokinase domains, a region between amino acids 536 and 547.[7] The most frequent mutations are N542-E543 deletion (23%), E543-D544 deletion (11%) ,F537-K539 deletion insertion and K539L mutation (10%).[7]

2.1.2: MPL (Thrombopoietin receptor gene) mutations

Two types of myeloproliferative leukemia virus (MPL; thrombopoietin (TPO) receptor (TPOR)) mutations in exon 10 have been found in MPNs.[7] The most common mutations are on the tryptophan W515 located at the boundary of the transmembrane and the cytosolic domains of MPL and the most prominent mutations are MPLW515L and K.[26] Several other mutations have also been found, which are W515R, W515A and W515G.[27] Mutations occur as heterozygous, but may become homozygous during disease progression.[28] Mutations on MPLW515 are confined to ET (around 3%) and PMF (around 5%) and can be also found in RARS-T.[29,30,31] The other mutation, MPLS505N is rarer and is located in the transmembrane domain, stabilizing receptors in an active dimeric orientation.[32] Originally, this mutation was identified in hereditary form of thrombocytosis as a germline mutation.[33] Further on, acquired MPLS505N somatic mutations were found in ET in less than 1% of the cases.[29,30] This underscores the similarities in the mechanisms of thrombocytosis in hereditary thrombocytosis and ET.[7] Very recently, a number of non-canonical mutations in MPL gene were found in triple negative ET (ET negative for *JAK2*V617F, *CALR* and *MPL*W515L/K/*MPL*S505N mutations).[32] These mutations are found to occur at a very low frequency leading to amino acid changes either in the extracellular (S204 or E230) or in the intracellular (Y591) domain.[34,35] These reported non-canonical mutations are more often acquired and induces a true MPN.[7] In addition, germ line non-canonical mutations of MPL have also been found strongly supporting that some triple negative ET cases are not MPN, but hereditary thrombocytosis.[7]

2.1.3: Mutation in calreticulin (*CALR*) gene

Discussed later under the section “CALR mutated MPNs”.

2.1.4: *LNK (SH2B3)* mutations

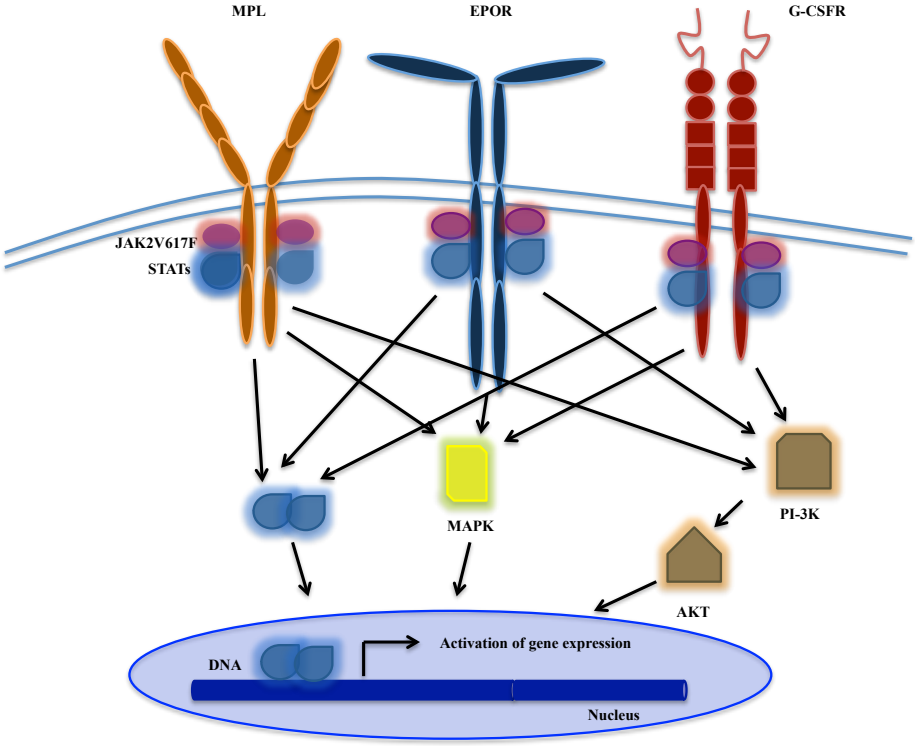
During 2010, acquired mutations in exon 2 of LNK (SH2B3), an adaptor protein involved in negative regulation of JAK2 signaling were found in two patients (PMF and ET).[36] These mutations were thought to be the driver mutations since they had an impact on proliferation.[7] Recent studies have elucidated that LNK mutations may not be truly the driver mutations.[37] LNK mutations have been found in several hematological pathologies, more particularly in erythrocytosis.[38] A few of these mutations can be germline, including the initially discovered LNKE208Q and some of the other mutations are secondary acquired mutations associated with JAK2V617F or CALR mutations, more specifically during disease progression.[39] Therefore, rather than primary drivers, LNK mutations may act more as predisposition mutations when germline or secondary mutations, increasing the pathogenecity of *JAK2*V617F and *CALR* when present.[37]

2.1.5: How do driver mutations in MPNs induce constitutive activation of JAK2 signaling ?

JAK2, a member of the JAK family is characterized by two kinase domains: one catalytically active at the C-terminus and the other catalytically inactive (or quite weak) pseudokinase that prevents self-activation of the kinase domain.[40] At the N-terminus, JAKs harbor a four-point-one ezrin radixin moesin (FERM)-like domain and a SH2-like domain.[7] The non-covalent association of JAKs to cytokine receptors is due to the FERM domain.[7] The JAK family kinases can be regarded as the catalytic domains of the hematopoietic cytokine receptor family as they are constitutively associated with the receptors intracellularly.[7] In addition, the association of JAKs with receptors is important for their normal trafficking to the cell surface.[32] Homodimeric receptors such as erythropoietin (EPO) receptor (EPOR), MPL and granulocyte colony-stimulating factor receptor (G-CSFR) are associated with JAK2, whereas heteromeric receptors are associated with JAK1 and JAK2/tyrosine kinase 2 (TYK2) or JAK3 for receptor mediated signal transduction.[7] Cytokine binding causes conformational changes or hetero/homodimerization of the receptors resulting in activation of JAKs by trans-phosphorylation.[32] The activated JAK2 will induce phosphorylation of the receptors, which subsequently act as docking sites for the downstream targets such as STATs.[7] In the canonical pathway, STATs are phosphorylated by the JAKs, which causes their homodimerization or heterodimerization and subsequent translocation to the nucleus where they activate expression of their target genes.[7] The pseudokinase domain of JAK2 has mainly two roles: one is to negatively regulate the kinase domain and the other is to positively regulate cytokine dependent signaling.[7] Activation of JAK2V617F is not completely elucidated, but both structural and functional data have established that the first conformational change induced by V617F is due to helix C of the pseudokinase domain.[41,42] JAK2 exon 12 mutations have an impact on domain upstream of pseudokinase domain and the mechanism of activation appears to be different from JAK2V617F.[43] JAK2V617F or JAK2 exon 12 mutations when expressed in

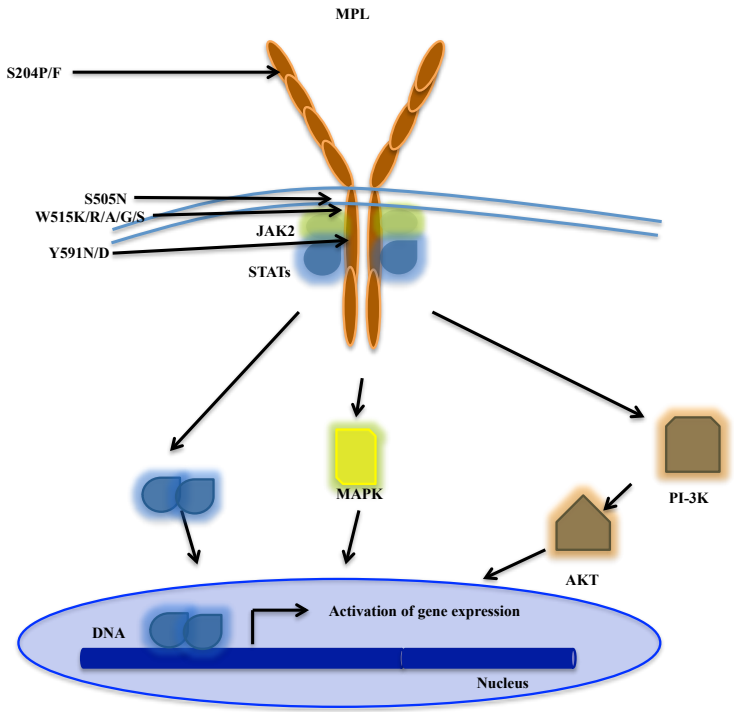
interleukin-3 (IL-3) dependent cell lines cause cytokine hypersensitivity or cytokine independence.[7] The presence of homodimeric receptors favours these phenotypic effects.[44] Homodimeric cytokine receptors are indispensable for induction of constitutive signaling by JAK2V617F.[7] JAK2V617F induces constitutive activation of STATs, phosphatidyl-inositol 3-kinase (PI-3K) and MAPK pathways.[10] The biological effects observed in cell lines mimic the results found in human patient samples.[10,45] In addition to causing constitutive signaling through type 1 cytokine receptors, two reports have described the effects of JAK2V617F on methylation status of histones.[46,47] The role of these effects of JAK2V617F in the pathogenesis of MPN are unknown.[7]

Figure 1: Association of JAK2V617F with type 1 homodimeric cytokine receptors leading to constitutive activation of JAK2 signaling.



TPO induced activation of MPL/JAK2 pathway is absolutely required for MPL mediated cell proliferation, but it has been found that TPO also induces activation of TYK2 whose precise role is unknown.[32] MPLW515 is present in the amphipathic domain of MPL, which inhibits ligand independent activation of MPL.[48] All the known substitutions of W515 except W515C and W515P result in ligand independent activation of MPL and cause activation of JAK2.[32] The S505N mutation brings about a conformational change in MPL leading to its activation.[32] However, both types of mutations cause constitutive activation of MPL signaling through modulation of conformation of transmembrane helix of MPL.[27,33,49]

Figure 2: Mutations in MPL leading to constitutive activation of MPL/JAK2 signaling.



CALR mutants also cause constitutive activation of JAK2 signaling via MPL[50] (discussed later under the section "CALR mutated MPNs").

Overall, the main MPN-associated driver mutations, *JAK2*V617F, *JAK2* exon 12 mutations, *MPL*W515L/K and *CALR* mutants, activate the cytokine receptor pathways and their downstream signaling, through the three homodimeric cytokine receptors (EPOR[51], MPL and G-CSFR) for JAK2V617F and through MPL for CALR and MPL mutants.[7] This may be useful in explaining why these different mutations occur in a mutually exclusive manner.[7] However rarely, two driver mutations can be found in a single patient, and even if they are found in a single patient, they may be present in different subclonal populations.[52,53]

2.1.6: MPN driver mutations and mouse models

All the three mutation types have been expressed in mice to investigate whether they are true MPN drivers and subsequently to make disease models for preclinical studies and drug testing.[7] *JAK2*V617F has been expressed in mouse hematopoietic cells by various methods: transplantation of virally transduced bone marrow cells, transgenic mice and knock-in (KI) mice.[7] Irrespective of the model, *JAK2*V617F induces a myeloproliferative phenotype in mice.[7] The frequently observed phenotype is a PV-like disorder progressing to MF.[54,55] A strong correlation between the cellular level of expression of JAK2V617F and disease phenotype has been observed: low expression

in comparison to wild type JAK2 is associated with an ET-like phenotype and a higher relative expression over 30% of WT or more is linked to a PV-like phenotype.[56] Hence, in mouse models *JAK2*V617F is a driver mutation of MPNs and the level of *JAK2*V617F expression determines the MPN phenotype, a condition close to human setting.[7] Recently, *JAK2* exon 12 mutations transgenic mice have been found to develop an isolated erythrocytosis.[57]

Retroviral mouse models for *MPL*W515K/L/A develop an ET-like phenotype rapidly progressing to MF.[26] These models are not ideal situation since they lead to the expression of MPLW515 mutants in all hematopoetic cells.

Retroviral mouse models for *CALR* mutants also develop ET-like phenotype, which in case of *CALR* del52 progresses to MF[58] (discussed later under the section "CALR mutated MPNs").

Ultimately, all three MPN driver oncogenes discovered by genetic approaches are the true drivers of MPN phenotype with *JAK2*V617F giving rise to ET, PV and MF, *JAK2* exon 12 mutations giving only an erythroid hyperplasia phenotype (PV), whereas *CALR* and *MPL*W515 mutations are associated with ET and MF phenotypes.[4] Current literature strongly supports the hypothesis that phenotype of MPNs is mostly connected to the types of receptors activated.[10,58] However, in none of the mouse models there was a development of PMF, all MF being secondary (post-ET or post-PV like diseases) and no leukemic transformation was observed suggesting the requirement of other genetic or epigenetic events to develop such disorders.[7]

2.1.7: Other acquired mutations and their roles in MPN disease progression

The three main driver mutations do not completely explain the heterogeneity of MPNs.[59] Other somatic mutations can be associated with the three MPN restricted mutations. In contrast these other mutations are not confined to MPNs and have frequent association with MDSs and AML.[7] This shows that there exists a continuum between the different myeloid malignancies and most of these mutations are more specifically associated with events involved in disease progression in MPNs.[7]

Secondary mutations identified in myeloid malignancies target DNA methylation regulators *(TET2*, *DNMT3*A, *IDH1/2*), histone modifiers (Polycomb repressor complex 1 and 2 members, *IDH1/2*), transcription factors (*TP53*, *CUX1*, *IKZF1*, FOXP1, *ETV6*, *RUNX1*, *NF-E2*), signaling molecules (*NF1*, *NRAS*, *KRAS*, *LNK*, *CBL*, *FLT3*) and splicing factors (*SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*).[9,60,61]
PMF does not appear to be a pure MPN, but a mixed myeloproliferative/myelodysplastic syndrome.[1] The presence of mutations in genes other than the three MPN driver mutations increases the myelodysplastic features of the disease and the severity of the disease, hinting strongly at continuity between MPN, MPN/MDS and MDS.[1] The presence of myelodysplastic features of MKs in PMF may be an important process in the development of a myelofibrosis, which seems to results from the induction of myofibroblasts from a Gli+ Mesenchymal Stromal Cell (MSC) by cytokines released by MKs.[62,63]

A large part of the heterogeneity of PMF is related to the type and the number of associated mutations, a high number of mutations being associated with a poor prognosis and a single mutation with a good prognosis.[1] Mutations in *ASXL1*, *SRSF2* and *EZH2* are associated with a poor prognosis and ASXL1 mutated MF patients do not come under the IPSS classification of PMF.[7]

Mutations in epigenetic regulators can be found in the three MPNs.[7] Mutation in splicing genes are nearly absent from PV and *CALR* ET.[7] Furthermore in *JAK2*V617F MPNs mutations in *TET2*, *DNMT3A*, *EZH2* and more rarely *ASXL1* can be the initiating events but can be also secondary events whereas for CALR MPNs these mutations are almost always secondary events.[7]

Finally acquisition of secondary mutations in epigenetic regulators and even more in *TP53* increase the risk of leukemic transformation.[7] Mutations in transcription factors such as *RUNX*1 are associated with leukemic development.[4]

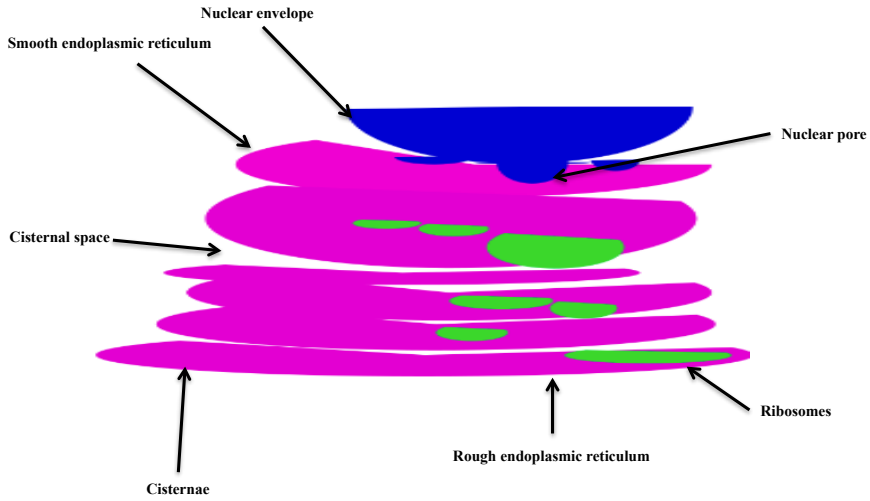
2.1.8: Predisposing mutations in MPNs

Various susceptibility mutant alleles corresponding to common polymorphisms in genes such as *JAK2* 46/1 haplotype, *TERT*, *MECOM*, *SH2B3* (*LNK*), *CHEK2*, *PINT* and *GFI-IB* may increase the development of the disease by two to six fold.[64,65] Currently, the mechanism of these predisposition variants is not known. These predisposition variants are the genes associated with either DNA damage response (*CHEK2*, *TERT*, *JAK2* 46/1 haplotype) or the JAK2/STAT pathway (*SH2B3*, *JAK2* 46/1 haplotype).[7]
Recently, predisposition factors leading to a true mendelian transmisson have been discovered in true MPN families: a duplication leading to the overexpression of two genes in hematopoietic cells, *ATG2B* and *GSKIP,* of which ATG2B is involved in autophagy and mutations in *RBBP6*.[66,67] In these families the MPNs were associated with the same acquired mutations than sporadic MPNs.

2.2: Calreticulin

The endoplasmic reticulum (ER) is one of the largest membrane organelle present in most of the types of eukaryotic cells, except red blood cells and spermatozoa.[68] ER is a type of organelle that forms an interconnected network of flattened, membrane enclosed sacs or tube like structures known as cisternae.[68] ER maintains a continuum with the outer nuclear membrane.[68] There are two types of ER: rough ER and smooth ER. The outer or cytosolic face of rough ER contains ribosomes acting as the sites for protein synthesis, whereas the smooth ER lacks ribosomes and functions mainly in lipid manufacture and metabolism.[68]

Figure 3: Structural features of endoplasmic reticulum.



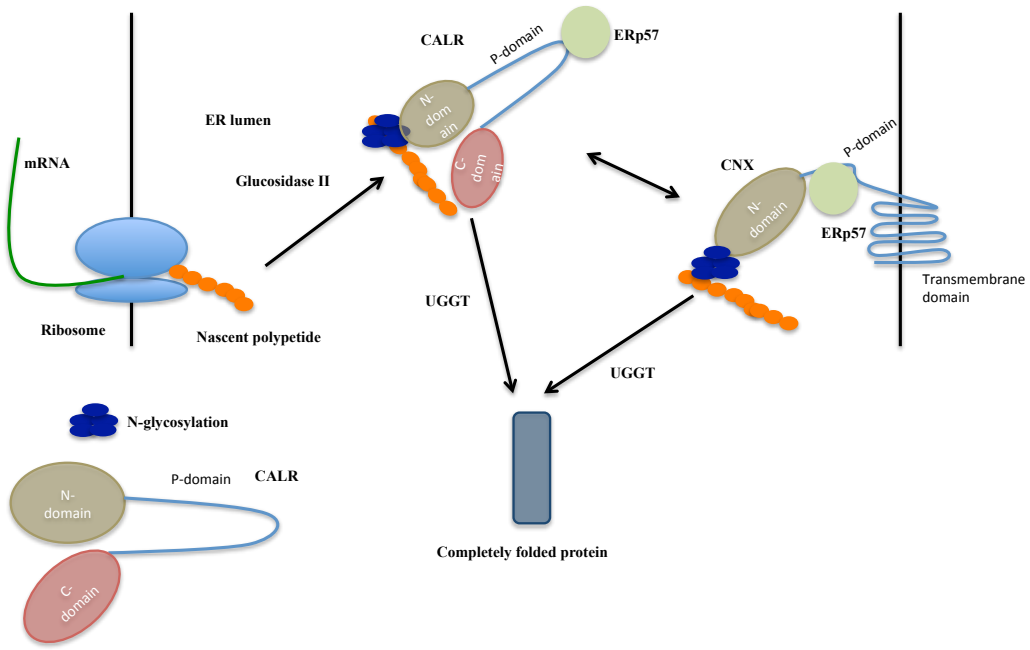
Further, ER plays an important role in calcium storage and release, lipid synthesis, protein synthesis and folding and post-translational modifications.[68] ER induces cellular signaling including organelle communication during stress conditions, transcriptional activation, calcium signaling, modulation of plasma membrane Ca^{2+} channels and ERAD (ER associated degradation).[68] Continous modulation of ER calcium concentration may result in induction of several ER signaling networks including protein and lipid synthesis.[69] Thus, in response to environmental changes, ER plays a role as a multifunctional organelle to generate output signals from the incoming signals.[69] To confer these diverse cellular functions, ER possesses many luminal and integral membrane proteins.[68]

ER being the protein synthesis site for integral membrane proteins and secretory proteins, it contains a range of folding enzymes and molecular chaperones to assist the folding of newly synthesized proteins.[68] Peptidyl cis-trans-isomerase and protein disulfide isomerase family enzymes catalyze the rate-limiting steps in the folding pathway of polypeptides and molecular chaperones like BiP act to inhibit aggregation via cycles of binding and release of unfolded proteins.[70] The other set of molecular chaperones, calreticulin (CALR) and calnexin (CNX) interact with glycoproteins, which possess N-linked oligosaccharides to assist their folding and subunit assembly.[71,72,73] The binding of CALR and CNX to N-linked oligosaccharide possesing polypeptides require lectin site present in CALR and CNX.[74] The binding of CALR and CNX to polypeptides with N-oligosaccharides is specific to oligosaccharide-processing intermediate, $Glc_1Man_9GlcNAc_2$.[75,76,74,77] Both CALR and CNX can also bind to non-glycosylated proteins, peptides and glycoproteins, which

do not posses $Glc_1Man_9GlcNAc_2$ oligosaccharide.[68] CNX is a type 1 transmembrane protein and CALR is a soluble paralog of CNX.[68] Both CNX and CALR bind Ca^{2+} and they share sequence similarity mostly in a central segment, which contains two proline-rich sequence motifs repeated in tandem.[78,79,80,81] In *in vitro* setting, CNX and CALR transiently interact with newly synthesized glycoproteins and dissociate at or near the acquisition of a native conformation.[68,70] Enhanced interaction of CNX and CALR with client proteins is observed in case of misfolded or incompletely folded glycoproteins.[68] Such prolonged interactions result in either increased or inhibition of degradation of incompletely folded glycoproteins and the incompletely folded glycoproteins remain in the ER.[68] The mechanism for these diverse folding results are controversial[68]:

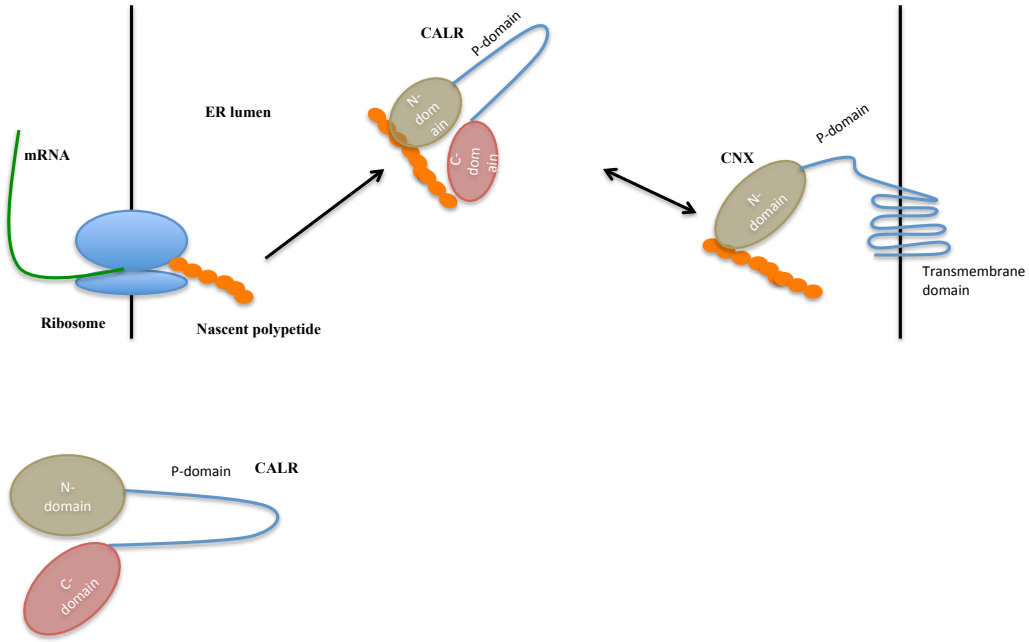
- In one model, CALR and CNX interact with glycoproteins solely based on the presence of $Glc_1Man_9GlcNAc_2$ oligosaccharide.[75,82] The mechanism of binding and release are regulated by glucose removal and readdition, which are catalyzed by the action of ER resident enzymes, glucosidase II and UDP-glucose:glycoprotein glucosyltransferase, respectively.[68] In such a model, CNX and CALR do not function as classical molecular chaperones, which are thought to retain incompletely folded glycoproteins and regulate protein folding in coordination with the activities of other ER chaperones and folding enzymes.[68] For example, such a coordination exists between CALR and CNX with the ER resident thiol oxidoreductase, ERp57.[83]

Figure 4: Pictorial representation of role of CALR and CNX as chaperone machinery for folding of glycoproteins.



- Another model has been proposed wherein unfolded glycoproteins interact with both lectin and polypeptide binding sites in CALR and CNX.[77,84] In this model, the polypeptide based interaction of CNX and CALR with client polypeptides suppresses aggregation of nascent peptides.[70] This model is concluded from the demonstration that in *in vitro* systems, both CNX and CALR can discriminate between folded and unfolded non-glycosylated proteins and they suppress the agregation of nascent proteins as potent as HSP60, HSP70, HSP90 and small heat shock families of chaperones.[85,84,86]

Figure 5: Pictorial representation of CALR and CNX as inhibitors of protein aggregration of nascent polypeptides during protein folding process.

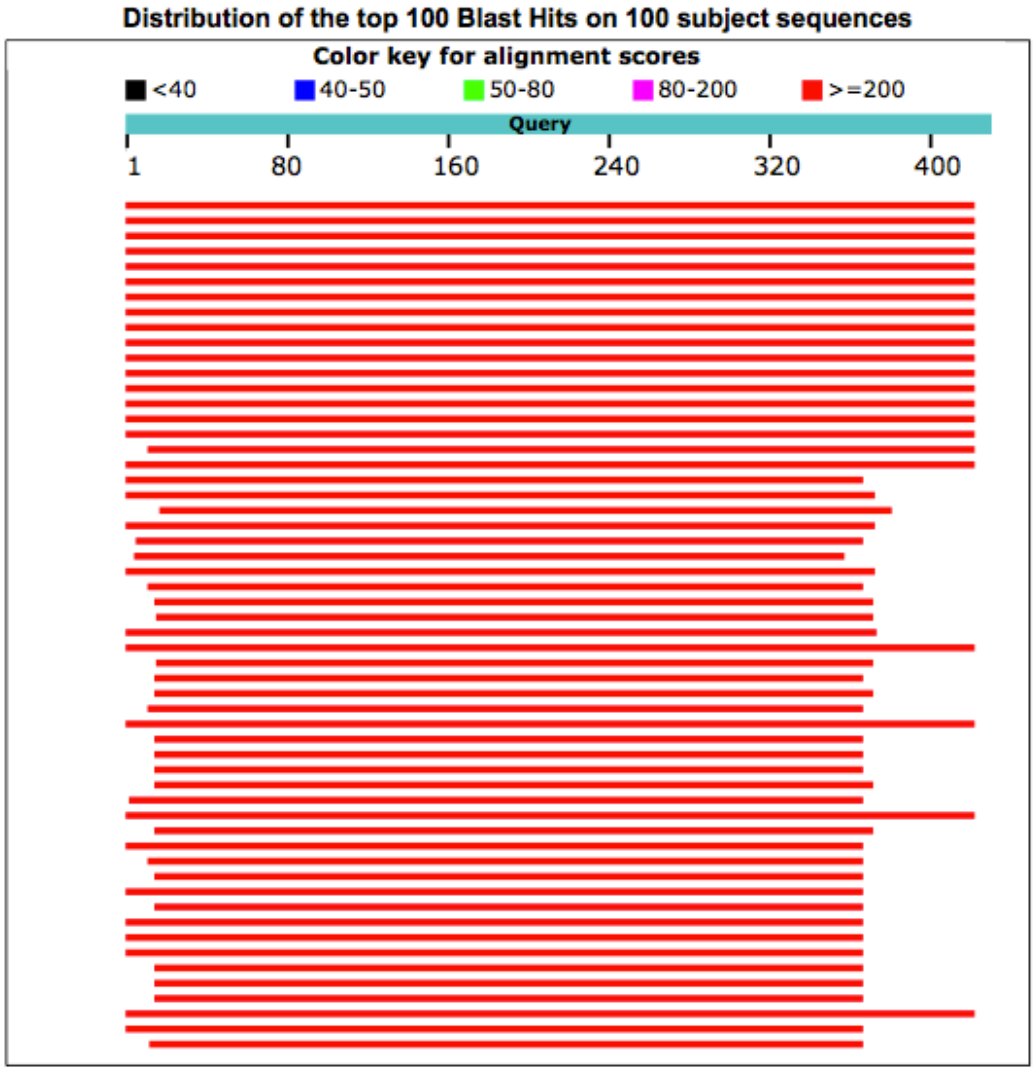


2.3: A brief overview of *CALR* gene and protein

CALR was first identified as a strong calcium binding ER protein.[69] Since, it has been extensively studied by several teams. *CALR* gene is evolutionarily conserved.[68] In humans, *CALR* gene is 3.6 kb and contains nine exons and is localized on chromosome 19.[68,70] The promoter of *CALR* gene contains several regulatory elements, which get activated in response during cellular proliferation.[68,70] *CALR* gene expression is also strongly activated in response to ER stress and ER calcium depletion.[68]

CALR protein is a 46 kD protein, which contains N-terminal cleavable ER signal and KDEL motif in the C-terminus, which is an ER retention signal.[68] N-domain contains three cysteine residues and two of them play an important role in protein folding of CALR by forming di-sulfide bonds.[68,70]

Figure 6: Results of NCBI blast of CALR protein. *The different horizontal red bars represent the CALR protein from different organisms. And further, it indicates the similarity is high when human CALR amino acid sequence is compared with amino acid sequence from different organisms including vertebrates, invertebrates etc. Obtained from NCBI protein blast results for human CALR protein*

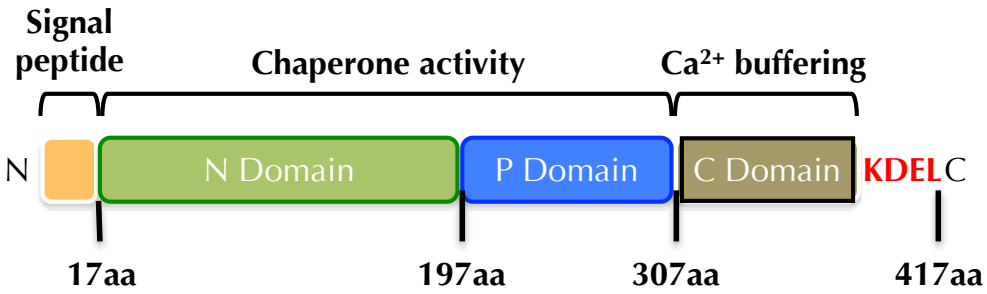


2.4: Domains in Calreticulin protein

Structural studies predict that CALR protein has at least three domains.[74,77,87] The N-terminal region of CALR protein contains N and P domains and possesses a highly conserved amino acid sequence.[68] A detailed examination of the exon-intron organization of the CALR gene suggests that the N-domain is encoded by first four

exons, P-domain is encoded by exons 5, 6 and 7 and C-domain is encoded by exons 8 and 9.[68,70]

Figure 7: Protein domains in CALR.



2.4.1: N – domain

In vitro studies provide hints that oligosaccharide and polypeptide binding sites are present in N-domain.[74] In the presence of calcium, CALR forms a stable core, which is resistant to proteolysis.[68] Oligosaccharide binding induces a conformational change influencing polypeptide binding.[68] Both oligosaccharide and polypeptide binding is required for complete chaperone function of CALR.[70] It was found that two amino acid residues in the globular domain present in the N-domain in CALR (Tyr105 and Asp135) abolish protein interaction with oligosaccharides.[68,70] Other residues, which might be involved in sugar moeity binding to proteins include Lys111, Tyr128 and Asp317.[68] The di-sulfide binding between Cys88 and Cys120 and Trp244 and Trp302 , which are present in globular domain, are important for chaperone function of CALR.[70] Mutation of His153 leads to loss of chaperone function in CALR.[68] Functional studies show that the N-domain in synergy with the P-domain may form a functionally crucial folding unit responsible for CALR chaperone function.[68,70]

2.4.2: P-domain

Central portion of CALR is termed as the P-domain, which has potential flexibility. It contains repetitive amino acid sequences (repeat 1, IXDPXA/ DXKPEDWDX, and repeat 2, GXWXPPXIXNPXYX).[68,70] It contains three replicates of repeats 1 and 2 and these amino acid sequences repeats form the structural backbone of P-domain which could be crucial for lectin-like function of CALR.[68,70] NMR studies have shown that the P-domain in CALR possesses an extended region, which is stabilized by antiparallel beta sheets, which play an important role in the interaction of CALR with ERp57.[68,70] It has been shown that ERp57 interaction of CALR gets disrupted by mutations of Glu239, Asp241, Glu234 and Trp244 at the tip of the P-domain.[68] P – domain binds to calcium with high affinity and low capacity (1 mol of calcium per mol of protein).[68,70]

2.4.3: C-domain

C-domain of CALR protein contains large number of negatively charged residues,

which show an interesting property of calcium buffering by binding to calcium with high capacity (25 mol of calcium/mol of protein) and low affinity and it binds nearly 50% of ER luminal calcium.[68,70]

2.5: *CALR* knockout mice

CALR deficient embryonic stem cells show impaired integrin mediated adhesion, as demonstrated by quantitative cell attachment assays on fibronectin and laminin, completely consistent with previous results that changes in CALR expression affect cell adhesion.[88,89,90] *CALR* deficient ES cells have significantly decreased integrin mediated calcium influx from extracellular region, which could be due to affect on integrin mediated cell adhesion.[90] The roles of CALR as chaperone or in modulation of certain receptor functions have not been investigated in *CALR* knockout mouse models.

Since CALR confers important functions, it was thought that the *CALR* knockout mice would not be viable. Indeed, the constitutive induction of *CALR* knockout in mice by homologous recombination, is embryonically lethal at 14.5-16.5 days.[91] *CALR* deficient mice embryos die due to an impaired cardiac development.[70] The *CALR* gene expression is activated during cardiac development at embryonic developmental stages and sharply decreases in the newborn heart.[91] *CALR* may belong to the family of cardiac embryonic genes and could play an important role during cardiogenesis.[70] Grp94, another ER luminal calcium binding chaperone is also expressed at higher levels during cardiomyogenesis.[70] These insights suggest that ER chaperones involved in calcium homeostasis/signaling may be playing an important role during developmental stages of cardiogenesis.[70]

2.6: Functional roles of CALR

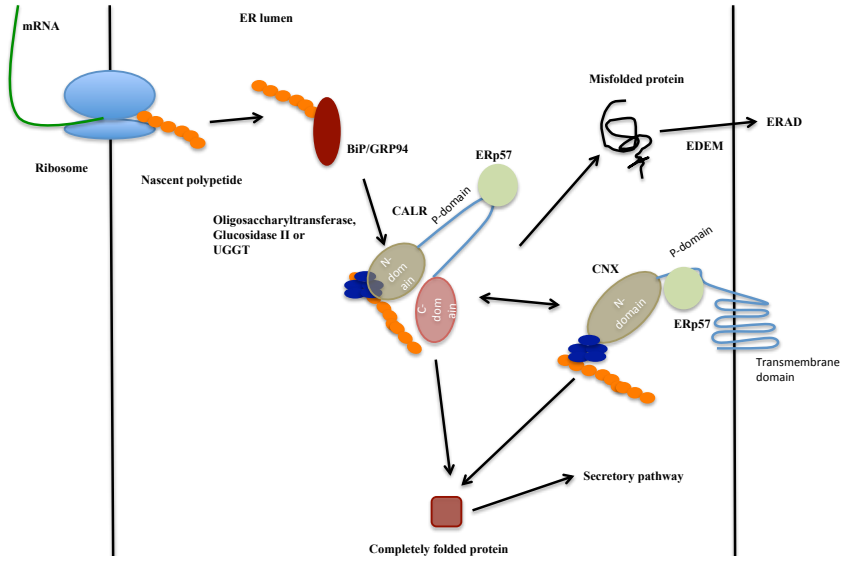
CALR is a multifunctional protein majorly playing a role in calcium homeostasis and as a molecular chaperone, it has been implicated in numerous pathways and biological systems.[68,70] Many of these functions remain difficult to be understood, more particularly if they are related to calcium homeostasis and/or the chaperone function of CALR.[68,70] However, many demonstrations indicate that CALR has the ability to influence several processes at the cellular, organ and organism level, thus CALR may be considered as a multifunctional protein.[68,70]

2.6.1: CALR as a molecular chaperone

Approximately 30% of cellular proteins are synthesized in the ER, wherein they interact with molecular chaperones followed by transportation in cargo vesicles to various cellular compartments or to the extracellular region.[70] Each of the chaperones or folding factors has their own peculiar mechanism for preventing the unfolded proteins from being transported out of the ER.[70] BiP/Grp78 and Grp94 recognize the exposed hydrophobic regions in the nascent polypeptides to assist their folding and assembly whereas CALR and CNX interact with nascent glycoproteins through polypeptide and lectin binding.[70] PDI and ERp57, both thiol oxidoreductase folding factors with the help of oxidizing environment in the ER induce disulfide linkages resulting in the formation of intra- and inter-disulfide bonds, which is an important factor for maturation of secretory and integral membrane proteins.[70] If the protein is

unable to fold, then it is targeted for degradation via the proteosomal pathway.[92] If the misfolded protein build-up in the ER, then several pathways are activated, which are downstream targets of ER stress in order to re-establish ER homeostasis.
N-linked oligosaccharides are synthesized on the cytoplasmic side of the ER by their addition to a lipid anchor, dolichyl phosphate.[70] Initially, two N-acetylglucosamine and five mannose residues are added to dolichyl phosphate, which is flipped into the ER lumen.[70] Then, four mannose and three glucose residues are added in the ER lumen.[70] This type of oligosaccharide modification is composed of $Glc_3Man_9GlcNAc_2$.[70] This process of N-linked oligosaccharide formation occurs as the nascent protein traverses the translocon as an extended chain and emerges into the ER lumen.[70] An enzyme called oligosaccharyltransferase, which closely associates with the translocon recognizes a specific sequence in the protein, NXS/T (Asn-any amino acid-Ser/Thr) and attaches the oligosaccharide to the asparagine residue via formation of amide bonds.[70] This is the process by which proteins are co- and post-translationally modified by N-linked glycosylation.[70] Then, two ER luminal enzymes modify the oligosaccharide by cleaving the terminal glucose residues.[70] Glucosidase I removes an initial glucose residue, whereas glucosidase II cleaves two more glucose residues.[70] Before the glucosidase cleaves the third glucose residue, the glycoprotein is recognized by the CALR and CNX folding cycle.[93] When the protein attains functionally competent folding state, the third glucose moiety is removed by glucosidase II, then the protein is released from the quality control cycle followed by transportation of the protein out of the ER.[70] Unfolded proteins, with the third glucose removed are recognized by UDP-glucose:glycoprotein glucosyltransferase (UGGT) and this enzyme re-glucosylates to create a folding substrate to be recognized by CALR-CNX cycle.[70] This mechanism helps in maintaining the unfolded proteins in the CALR-CNX cycle until they are folded properly and no longer recognizable by UGGT.[70] Abnormally increased interaction with CALR-CNX targets the proteins for degradation through interaction with the EDEM (ER degradation enhancing 1,2-mannosidase like protein).[92] The unfolded proteins are recognized by alpha 1,2-mannosidase, which specifically cleaves mannose residues allowing recognition by ER associated degradation (ERAD) process.[92]

Figure 8: Classical model for CALR-CNX protein folding cycle.



MHC class I assembly has provided an important model for studying the chaperone function of CALR and CNX.[70] In *CALR* deficient fibroblasts, MHC class I molecules undergo abnormally rapid export from the ER, improper peptide loading and impaired T-cell recognition at the cell surface.[94] Expression of the ER luminal chaperone domains of CNX does not rescue these protein folding abnormalities, which indicates that MHC class I assembly in a unique manner is dependent on CALR.[70] In addition, *CNX* deficient cells show formation of complex with the heavy chains of MHC class I molecule.[95] These demonstrations show that CALR employs peptide based interactions to confer folding assistance to the assembly of MHC class I.[70]

Upon induction of protein misfolding in the ER due to heat shock or chemical agents, CALR forms dimers and gains enhanced polypeptide binding to target polypeptides.[96] EGTA treatment of CALR disrupts its ability to bind to monoglucosylated oligosaccharides whereas EGTA treatment enhances CALR binding to unstable polypeptides, coincident with CALR oligomerization.[96] Hence, calcium binding regulates transitions between different conformational states of CALR and influences the mode of substrate interaction.[96] A *CALR* mutant with a deleted C-domain is under a conformational state in which both polypeptide and oligosaccharide binding properties are preserved.[96] In full length CALR, a C-domain deletion like conformation may be attained at low calcium concentrations.[96]

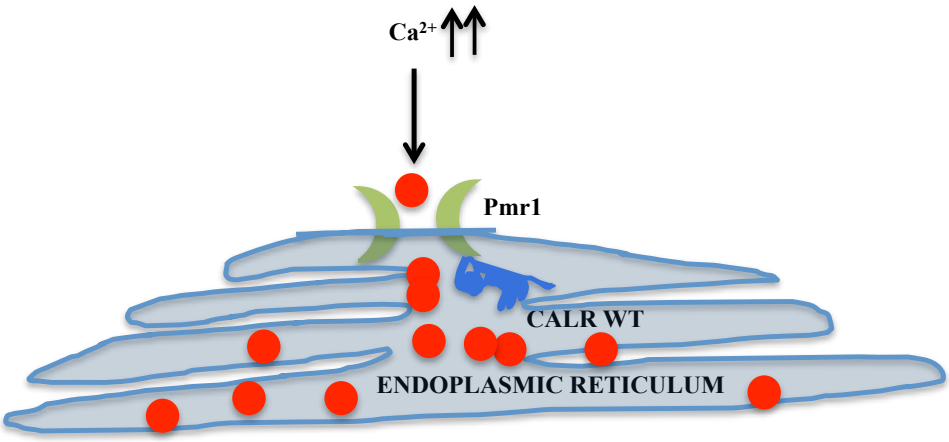
2.6.2: Role of CALR in calcium homeostasis

Studies involving animal models and cells overexpressing CALR prove that calcium buffering property may be one of the important physiological functions of CALR.[68,70] *CALR* deficient or overexpressing cells do not show remarkable defects in protein folding, but show dysregulation of calcium homeostasis.[68] These studies hint that the role of CALR as a regulator of calcium homeostasis could be more important than its role as molecular chaperone.[70]

Differential expression of CALR affects calcium homeostasis of multiple cellular compartments.[97] Calcium signals are affected in the ER, cytosol, at the plasma membrane and mitochondria; but the most predominant affects are observed in the ER.[97] CALR overexpression induces increased total amount of calcium stored in the ER.[97] Increased CALR expression has significant effects on the free intraluminal ER calcium as it is found to be doubled.[97] However, the increased calcium pumping activity is not due to increased activity of SERCA isoforms.[97] Thapsigargin, an inhibitor of calcium uptake into the ER by SERCA, showed an identical passive ER calcium permeability in control and CALR overexpressing cells.[97] Since, at steady state the calcium pumping activity is equal to the ER calcium leak, the activity of SERCA is not altered by CALR overexpression.[97] Hence, this indicates that increased calcium uptake into the ER could be due to thapsigargin insensitive pumps.[97] A likely candidate would be Pmr1 family of calcium transport ATPases.[97] Pmr1 is mainly localized in the Golgi complex, but a significant fraction is found in a functional state in the ER.[97] Pmr1 calcium store is associated with a reduced calcium leak and weak $InsP_3$ responses.[97] Cells overexpressing Pmr1 pump show delayed calcium influx.[98] It

has been speculated that CALR may interact with Pmr1 pump to promote its retention in the ER, thereby inducing increased calcium pumping activity in the ER.[97] The experimental evidences strongly suggest that CALR interacts differentially with distinct calcium pumps to modulate the rate of calcium uptake into the ER, thus directly influencing the calcium concentration in the ER.[97] In addition to increasing the total and free calcium concentration in the ER, CALR also increases the rate of agonist inducing calcium release.[97] This increased release was observed over a broad range of ER calcium concentrations, which indicates that it not only reflects increased driving force for calcium, but also increased fluxes through $InsP_3$ gated calcium channels.[97] In CALR overexpressing cells, the $InsP_3$ channels are exposed to higher amounts of calcium ions both on the ER and cytosolic sides, thus the increased calcium release may be due to a direct action of CALR on $InsP_3$ gated calcium channels, which are localized in the ER.[97] Due to the increased calcium load and driving force, enhanced calcium gets released into cytosol in response to treatment of cells with agonists or thapsigargin, but store operated calcium influx was reduced.[97] However, CALR expression levels confer no direct effects on store operated calcium influx.[97]

Figure 9: Schematic representation for increased ER calcium influx due to overexpression of CALR.



The effects of CALR expression on calcium homeostasis were present on mitochondria also.[97] The larger release of calcium from the ER was not associated with equal calcium accumulation in the mitochondria, but with a reduced signal as calcium concentration in the mitochondria returned to basal levels despite ER calcium efflux.[97] The abnormal calcium concentration in the mitochondria did not show structural damage of mitochondria but was associated with mitochondrial depolarization.[97] The depolarization by reducing the driving force for calcium is expected to reduce mitochondrial calcium uptake and may account for a weak mitochondrial calcium response.[97] In addition, the activity of the mitochondrial calcium uniporter might be inhibited by the high calcium concentrations found at the ER/mitochondria microdomain.[97] Increased exposures to high levels of calcium might desensitize the uniporter since low levels of calcium are required for rapid uptake of

calcium uptake by mitochondria.[97] Several mechanisms may account for the abnormal mitochondrial calcium response observed in CALR overexpressing cells, including depolarization of mitochondria together with an increased calcium uptake and release by ER.[97]

The effects of CALR on calcium homeostasis are not due to chaperone function of CALR, since impaired ER and mitochondrial calcium responses were observed in cells expressing CALR with deleted N-domain.[97] Moreover, overexpression of CNX did not affect cytosolic or ER calcium homeostasis which indicates that effects are not due to N or P domain, but are dependent on C-domain of CALR.[97] Hence, alteration in calcium sensing rather than chaperone activity is responsible for increased calcium pumping and release activities, which causes higher calcium turnover between the ER and mitochondria.[97]

By allowing the ER to take up, store and release increased calcium, an increase in CALR expression may induce the cellular calcium signaling machinery, which may allow non-calcium signaling cells to generate calcium signals.[97] However, when CALR expression is increased for long period, it brings about apoptotic features in cells.[97] The high levels of calcium microdomain around mitochondria, would induce short term increase in mitochondrial metabolism but during the long term may negatively affect the function of mitochondria.[97] Thus, this defective signaling mechanism may account for the cells expressing increased levels of CALR to undergo enhanced apoptotic stimuli.[97]

2.6.3: CALR functional roles outside the ER

CALR has also been implicated in various cellular processes occuring outside the ER including at the cell surface, in the cytoplasm and within the nucleus.[68,70]

It has been demonstrated that cell surface CALR plays an important role in both antigen presentation and complement activation[94,99,100], phagocytosis,[93] immunogenic cancer cell death,[68] wound healing[101,102] and thrombospondin signaling.[103,104,105,106] CALR acts as a second general recognition ligand at the cell surface during phagocytosis, by stimulating LRP (low density lipoprotein receptor-related protein) on the surface of engulfing cells.[107,104] CALR is expressed on the extracellular surface of platelets and it interacts with integrin alpha2beta1 and glycoprotein VI, which demonstrates a role for CALR in platelet-collagen interaction.[108] CALR is expressed on the cell surface in many cell types where it may play a role in antigen processing events[109] in addition to playing a role in adhesion.[110] CALR also localizes to the extracellular environment in the teeth mediating a role in mineralization.[111] Yet, the molecular mechanisms involved in targeting CALR to the plasma membrane remain poorly understood.

Other functions of CALR in the cytoplasm remain poorly understood. For example, CALR binds to the sequence KXGFKKR found in the cytoplasmic domain of alpha-integrins.[88] Through this mode of binding CALR plays a role as a cytoplasmic activator of integrins and a signal transducer between integrins and calcium channels in the plasma membrane wherein CALR modulates calcium release and influx[112] and

that CALR may also directly interact with hormone receptors such as glucocorticoid and androgen receptors to inhibit steroid sensitive gene transcription.[113,114]

2.7: CALR in cancer

Cancer is characterized by uncontrolled proliferation of cells. Abnormal modulation of CALR protein levels has been found to be associated in conferring strong effects on cancer cell proliferation in various cancer types.[115] In pancreatic cells, CALR overexpression positively regulates cell growth whereas genetic knockdown of *CALR* inhibits cell growth.[116] In oral cancer, genetic knockdown of *CALR* causes cell cycle arrest at G_0/G_1 phase resulting in inhibition of cell growth, colony forming ability and anchorage independent growth of cancerous cells.[117] In gastric cancer cells, enhanced CALR expression promotes cell proliferation and upregulates the proangiogenic factor vascular endothelial growth factor (VEGF) expression.[115] In bladder cancer cells, knockdown of *CALR* inhibits VEGF protein expression and secretion and further, *CALR* knockdown inhibits cell growth.[118] Recently, it has been demonstrated that increased CALR levels in prostate cancer cells results in suppression of tumor growth.[119] In addition, in neuroblastoma cells CALR inhibits cell prolferation and promotes cell differentiation implying that increased CALR levels in neuroblastoma cells show better prognosis.[120] Therefore, these insights show that the effects of CALR on cancer cell proliferation may depend on the cell type.[115]

Metastasis is mainly a lethal event in the timecourse of cancer progression. It involves cell adhesion, migration and invasion. Many studies have suggested that overexpression of CALR contributes to metastasis in ovarian, pancreatic, prostate and gastric cancers.[116,121,122] One of the suggested mechanisms is that CALR interacts with alpha subunits of integrins.[90] It has been demonstrated that interaction of CALR and integrins is stimulated by phosphorylation of integrins.[123] Cells with increased expression of CALR exhibit enhanced adhesiveness linked to calmodulin/calmodulin – dependent kinase II pathway.[124] Further, cell surface CALR affects thrombospondin mediated focal adhesion assembly through PI-3K pathway pathway.[125] These demonstrations show that CALR plays an important role in affecting cell adhesion and migration during metastasis.

2.8: CALR as a pro-phagocytic signal in cancer

Cell surface expression of CALR is found to be a hallmark induced by intrinsic cell death mechanisms.[126] In addition, cells harbouring DNA damage also show expression of cell surface CALR.[126] During pre-apoptotic stage and in malignant cells increased cell surface expression of CALR is observed.[126] Macrophages induce phagocytosis of dying cells by recognising CALR on the cell surface of apoptotic cells whereas in normal cells, CALR on the cell surface is not found except in activated T cells and circulating neutrophils and platelets.[126] The malignant cells are not phagocytized due to increased cell surface expression of CD47.[126] Cell surface CD47 serves as a "don't eat me signal" and it is found that in malignant cells, cell

surface expression of CD47 is dominant over cell surface expression of CALR.[126] CD47 blocking by a monoclonal antibody induces CALR dependent phagocytosis whereas in normal cells this phagocytosis is not induced due to the absence of cell surface CALR.[126] It is demonstrated that CALR-CD47 axis plays an important role in immunogenic cell death of malignant cells.[126]

Two hypotheses have been speculated to explain the role of cell surface expression of CALR in immunomodulation. First, cell surface expression of both CALR and CD47 are highly correlated in malignant cells.[126] A sub-population of cells may evade CALR dependent phagocytosis due to overexpression of CD47.[126] Second, cell surface CALR may confer pro-tumorigenic functions independent of phagocytic effects as it is observed that in human patients increased cell surface expression of CALR is strongly associated with poor prognosis.[126] So, cell surface CALR may interact with its ligand, LRP, to induce angiogenesis and cell migration which is supported by the experimental results that overexpression of CALR or CALR fragments induces enhanced invasion and cell migration in vitro.[126] Other pro-tumorigenic effects may include cell adhesion and evasion of immune surveillance due to reduced MHC class I antigen presentation.[126]

Interestingly, in malignant cells cell surface expression of CALR is also induced by chemokines particularly CXCL8/CXCR1-2 axis and in general other soluble factors operating in an autocrine/paracrine manner.[127] Several soluble factors acting on GPCRs have been found to induce cell surface expression of CALR.[127] These chemokines and GPCRs are found to be constitutively active in cancer.[127] Thus, it may be possible that induction of cell surface CALR expression by chemokines and GPCRs may confer pro-tumorigenic effects leading to poor prognosis.[127,126]

Figure 10: Cell surface CALR mediated phagocytosis. *Adapted*[127,126]

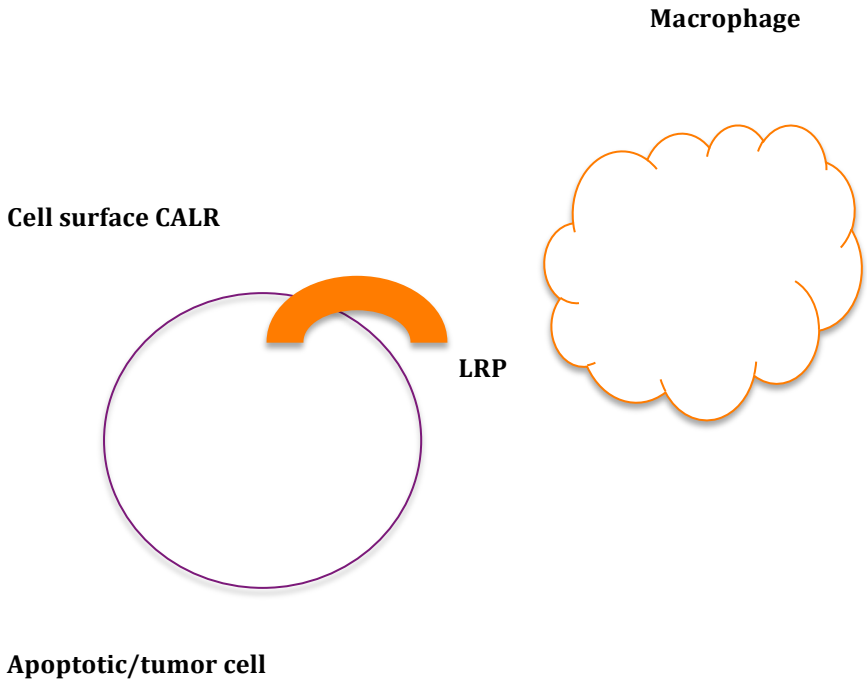


Table 2: CALR/CRT expression pattern in different cancers. Adapted from Lu YC et al. 2015.[115]

Cancer type	CALR expression	Clinical outcomes
Oral	Increased	-
Esophagus	Increased	Poor prognosis
Breast	Increased	Poor prognosis
Pancreas	Increased	Poor prognosis
Gastric	Increased	Poor prognosis
Colon	Increased	-
Bladder	Increased	-
Prostate	Increased	-
Cervical	Increased	-
Ovarian	Increased	Better prognosis
Neuroblastoma	Increased	Better prognosis

Table 3: Summary of CALR structural features and function. Adapted from Michalak et al.[70,68]

(a) Structural features and function		
N-domain Proceeded by N-terminal signal sequence targeting the protein to the ER lumen Highly conserved amino acid sequence Potential phosphorylation site Potential glycosylation site (bovine proteins) Putative autokinase activity Inhibits PDI activity Supresses tumours Inhibits angiogenesis	P-domain Proline rich domain Amino acid sequence similarity to calnexin, calmegin and CANLUC Putative glycosylation site (Leishmania protein)	C-domain Rich in acidic amino acids ER retrieval signal Putative glycosylation site Anti-thrombotic activity Prevents restenosis Ca^{2+} sensor of calreticulin-protein interactions
(b) Ion binding N-domain Binds Zn^{2+}	P-domain High affinity Ca^{2+} binding with low capacity	C-domain High capacity Ca^{2+} binding site with low

		affinity
(c) Molecules binding N-domain Binds to the DNA binding domain of steroid receptor Binds to alpha-subunit of integrin Binds rubella RNA Interacts with PDI Interacts with ERp57 Weak interactions with perforin	P-domain Binds to a set of ER proteins Strong interactions with PDI Strong interactions with perforin Lectin like chaperone site	C-domain Binds to a set of ER proteins Binds Factor IX and Factor X Binds to cell surface

2.9: *CALR* mutated MPNs

In 2013, frameshift mutations in the *CALR* gene were discovered in majority of *JAK2* and *MPL* non-mutated ET and PMF (50-60% ET and 75% PMF).[128,129] More than 30 types of mutations have been described and all are located in exon 9 inducing a +1 frameshift.[128,129] Only the mutations causing this +1 frameshift are found to be pathogenic.[128] The other nonpathogenic mutations are usually germ line variants of *CALR*.[130] Frameshift reading leads to a novel C-terminus with loss of KDEL motif.[128,129] The mutated CALR protein harbors a common new amino acid sequence that possesses positively charged amino acid residues.[128,129] In contrast to wild-type (WT) CALR, the negative charges, which play an important role in calcium binding are lost at different extents, depending on the mutant.[128,129] The two most frequent mutations are 52-bp deletion also called type 1 and 5-bp insertion also called type 2.[128,129] They represent the two types of modifications observed in exon 9, del52 having lost most of the WT exon 9 sequence and calcium binding sites whereas ins5 being closer to the WT sequence possessing around 50% of negative charges.[128,129]

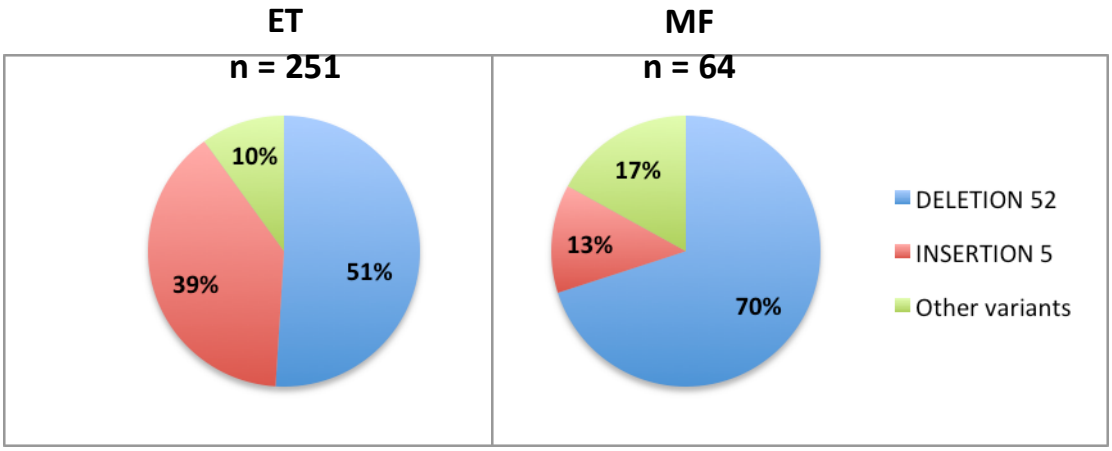
Figure 11: Novel C – terminal found in all types of CALR mutant proteins in ET and PMF patients[128,129]

RRMMRTKMRMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA

In *CALR* mutated MPN patients, type 2 mutations are mainly associated with ET and are less frequent in MF whereas type 1 mutations represent about 50% of the mutations in ET and as much as 70% in MF (70%).[131] It has also been found that the

mutant allele burden is in average higher in type 1 mutations compared to type 2 mutations .[128,129]

Figure 12: Distribution of CALR mutants in MPNs. *Adapted from X Cabagnols et al.*[131]



In mouse model, bone marrow transplantation experiments have demonstrated that CALR 52bp deletion and insertion 5 alone are sufficient to cause an MPN phenotype in vivo and supports the findings that CALR mutations are alone sufficient to drive MPN phenotypes.[58] This suggests that oncogenic CALR mutants play an important pathogenic role in MPN. In addition, CALR mutations are detectable in the long term hematopoietic stem cell compartment.[128,129]

CALR mutants are found to induce cytokine independence of factor dependent cell lines such as Ba/F3 or UT-7, only when MPL is expressed.[58,50,132] No other cytokine receptor was capable to mediate this factor independent growth.[58] Cytokine independence is associated with a constitutive activation of JAK-STAT signaling and these cells are susceptible to pharmacological inhibition of JAK2 signaling.[58,50,32,133,134,135,132] These findings align with result from microarray gene expression analysis which show an increased JAK-STAT pathway activation in CALR mutant expressing MPN granulocytes[8] and with the observation that CALR mutant MPN patients have shown response to JAK2 inhibitors.[128]

CALR mutants del52 and ins5 induce cell transformation only in the presence of MPL by inducing a TPO independent activation of MPL/JAK-STAT pathway.[58,50,132,136] A weak activation of G-CSFR is found, but it needs further detailed investigation to elucidate the precise role of CALR mutant induced mild activation of G-CSFR in the pathogenesis of the disease.[50] It has been demonstrated that the requirements for CALR mutant to induce MPL/JAK-STAT pathway activation are: N-linked glycosylation in extracellular domain of MPL, glycan binding site in CALR mutants, MPL-JAK2 protein complex and common cytokine receptor domain in MPL.[50,132] G-CSFR and EPOR both possess one repeat of common cytokine receptor domain whereas MPL possesses two repeats of this domain.[132] It is unknown if CALR mutants favor homodimerization of MPL receptors or only causes conformational

change in MPL-JAK2 protein complex resulting in ligand independent activation of MPL/JAK-STAT signaling.
CALR mutants harboring deletion of entire C-domain do not induce ligand independent activation of MPL/JAK-STAT signaling, which indicates that the novel C-terminus in mutant CALR leads either to a novel conformational change in mutant CALR protein or modify the interaction of CALR with MPL leading to its activation.[58]
The difference in retention of calcium binding sites in del52 and ins5 may be leading to differential induction of orientation of MPL-JAK2 protein complex out of several possible active orientations leading to different disease phenotypes and it remains to be investigated whether P-domain in CALR mutants also interacts with MPL.[58,50,136]
The finding that all types of CALR mutations lead to generation of the same novel C-terminus peptide tail strongly suggests that CALR mutations are gain of function or that they confer a new function on mutant CALR, with the mutant C-terminus playing a crucial oncogenic role.[136] Mutagenesis based structure-function analysis has revealed that oncogenic activity of CALR mutant is not encoded within a specific sequence of novel C-terminus tail in mutant CALR, but rather the positive electrostatic charge of the mutant C-terminus influences the ability of mutant CALR to physically associate with MPL to facilitate transformation.[136]
Both type 1 and 2 mutants cause different effects on calcium release in cultured megakaryocytes. More specifically, the largest cytosolic calcium mobilization and store operated calcium entry (SOCE) were found in megakaryocytes from type 1 mutant carrying patients.[137] It has been shown that type 1 mutant induces increased calcium release in comparison to type 2 mutant.[137] The result is increased cytoplasmic concentration through both ER calcium release and calcium uptake from extracellular environment, which could be one of the reasons for the difference in phenotypes induced by different CALR mutants.[137]

3.0: Unfolded protein response pathway

Protein folding efficiency is continuously regulated via the dynamic integration of several cellular signals.[138] Various feedback mechanisms ensure fine tuning to adapt to fluctuations in protein folding requirements by regulating the events in the secretory pathway.[139] Pioneer evidence for the presence of a regulatory pathway to overcome perturbations in protein folding at the ER came from an experimental demonstration in which the pharmacological inhibition of protein folding resulted in transcriptional upregulation of several key ER chaperones.[140] This demonstration revealed that a signaling pathway acts as a feedback loop to reprogram gene expression during perturbations in ER function.[138]

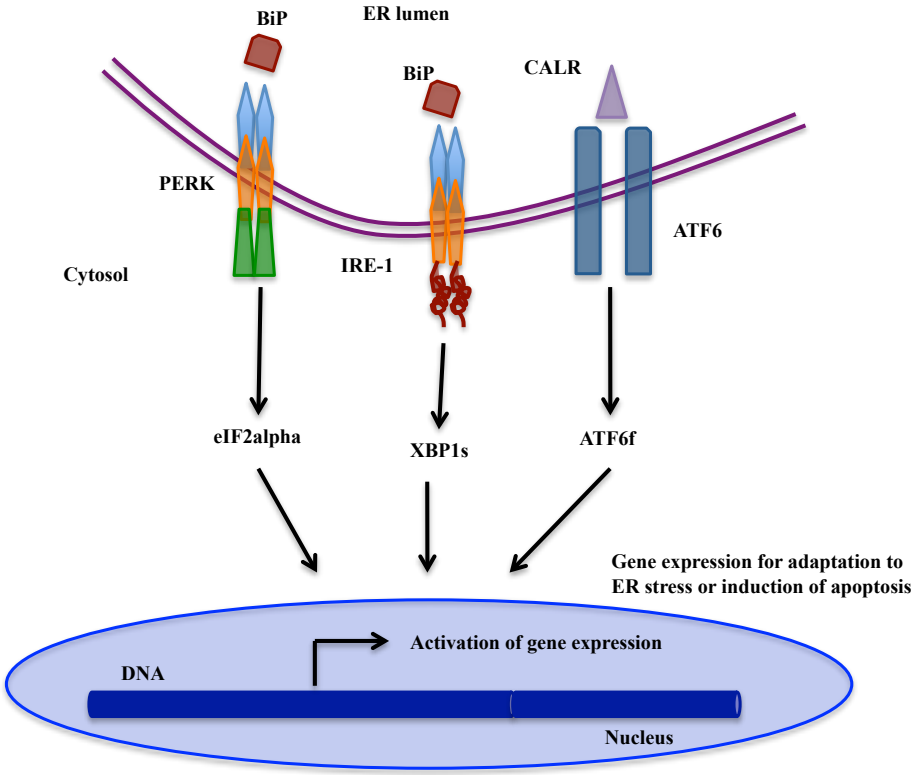
It is now known that upon ER stress, cells activate a cascade of adaptive mechanisms to cope with protein folding alterations, which together are known as unfolded protein response (UPR).[138] UPR transduces signals about the protein folding status in the ER lumen to the nucleus and cytosol to regulate unfolded protein load.[141,142] When cells harbor irreversible ER stress,[143] this pathway eliminates damaged cells by apoptosis, which reveals that a mechanism exists to integrate information about the duration and intensity of stress response.[138]

Though UPR is majorly connected to protein folding stress under both physiological and pathological conditions, it may confer other additional important functions.[138] Components of UPR regulate multiple cellular processes including lipid and cholesterol metabolism, energy homeostasis, inflammation and cell differentiation.[144] At the molecular level, a dynamic signaling framework is integrated by the UPR to ensure organelle homeostasis in a continuously fluctuating environment.[138]

3.1: UPR as an adaptive mechanism

ER stress signaling in vertebrates has evolved into a sophisticated signaling cascade that integrates various cellular responses and the mechanism involves activation of at least three major stress sensors: Inositol requiring enzyme1 (IRE1), activating transcription factor 6 (ATF6) and protein kinase RNA-like ER kinase (PERK).[138]
At the same time, two different waves of cellular responses are observed in mammalian cells harboring ER stress.[138] As a quick reaction, the activation of PERK inhibits general protein synthesis through the phosphorylation of eukaryotic translation initiation factor 2alpha (eIF2alpha).[145] In addition, mRNAs coding for proteins destined to ER are rapidly degraded through regulated IRE1-dependent decay.[146,147,148] Lysosomal degradation pathway is activated by ER stress to eliminate damaged ER and protein aggregrates.[149] Ultimately, pre-emptive quality control[150] and co-translational degradation[151] inhibit the entry of ER destined proteins. Overall, these mechanisms drastically reduce the influx of proteins into the ER to re-establish ER homeostasis.[138]

Figure 13: UPR signaling pathway as an adaptive mechanism to ER stress or inducer of apoptosis during severe ER stress.



A second line of signaling cascade occurs to activate gene expression response through the regulation of three major UPR transcription factors.[138] Each stress sensor uses a particular mechanism to induce activation of a unique transcription factor leading to upregulation of a subset of UPR associated genes.[139] IRE1 has both kinase and endoribonuclease activities under ER stress conditions.[138] Upon induction of ER stress, IRE1 undergoes a conformational change leading to activation of its cytosolic RNase domain.[152] Activated IRE1 processes the mRNA encoding the transcription factor X box binding protein 1 (XBP1), leading to frameshift.[153,154,155] This leads to the expression of an active and stable transcription factor, called as spliced XBP1 (XBP1s), which is responsible for the gene expression related to ER associated degradation, the entry of proteins in the ER and protein folding in addition to other functions.[156,157] XBP1s also regulates expansion of ER under ER stress by inducing phospholipid synthesis.[142]

ATF6 belongs to a family of ER related basic leucine zipper transcription factors. Under ER stress conditions, ATF6 translocates to the Golgi, where it is processed by site-1 proteases and site-2 proteases, leading to generation of a cytosolic fragment (ATF6f) that directly regulates genes encoding ERAD components and XBP1.[154,158,159] Phosphorylation of eIF2alpha by PERK allows mRNA translation of transcription factor ATF4, which regulates the levels of pro-survival genes that are involved in redox balance, amino acid metabolism, protein folding and autophagy.[141,160] PERK also regulates protein synthesis or protein translation via inducing expression of a range of microRNAs.[161]

Together, ATF4, XBP1s and ATF6f regulate the gene expression of a broad range of genes with some common targets, which are majorly involved in adaptation to stress or apoptosis during a state of chronic ER stress.[138] The target genes of these UPR transcription factors may be dependent on other transcription factors during various cellular processes by formation of protein complexes to regulate a particular cellular outcome.[138]

Questions asked

CALR mutants discovered so far are mainly found in ET and MF patients. Among the CALR mutants, many of them occur at a very low frequency i.e., in 1% of patients, which include deletion 19, deletion 34 and deletion 46. So due to their occurrence at a very low frequency, clinical and biological data pertaining to these mutations is limited in the literature since it is difficult to study these mutations from patient derived cells. They have been classified into type-1 and type-2 like mutations based on their structure, however it is not known whether these rare mutations behave similarly or differentially to high frequency mutations, i.e., deletion 52 and insertion 5.
In addition deletion 9 in *CALR* gene is an in-frame deletion mutation found in a few ET patients, which do not possess novel C-terminus, but it has not been demonstrated whether this mutation is oncogenic or a polymorphism. Thus, to study the oncogenic effects of these rare mutants, we have tried to answer these different questions:

- Are their effects on cell lines dependent of MPL? To answer this question we have used the Ba/F3 cell line that do not endogenously express type-1 homodimeric cytokine receptors and that can be engineered to express one of these receptors.
- Do all these mutants activate the JAK-STAT pathway and the other pathways downstream of MPL activation? Are there some differences among mutants?
- In the retroviral mouse model, we have previously shown that del52 and ins5 do not give a similar phenotype: del52 giving a very significant thrombocytosis, an ET-like phenotype, which progresses to MF, ins5 a mild thrombocytosis. Will the other mutants classify in type-1 like or type 2 like which will also give two types of disorder.
- Since mutations in CALR may induce aberrant chaperone function, do CALR mutant expressing cells harbor ER stress induced abnormal activation of UPR?
- Finally we have tried to make an *in silico* structural modeling of CALR mutants

By searching to answer some of these questions, I aim to characterize the rare CALR mutants found in ET and MF patients.

PART1
(results)

Functional characterization of rare calreticulin mutants (Annex 2, Draft of the first version of a paper to be submitted.)

INTRODUCTION

Classical Bcr-Abl myeloproliferative neoplasms include PV, ET and PMF. During the last decade tremendous progress has been made in elucidating the genetic basis of these MPNs.[7] Mutations in *JAK2* and *MPL* genes are found to be driver mutations as observed by mouse modelling and *in vitro* cell transformation assessment in an in vitro/cytokine dependent cell line models.[7] Since JAK2 is associated with type 1 cytokine receptors as an intracellular signaling part of this class of cytokine receptors, JAK2V617F is found to cause aberrant signaling via IL-3R, EPOR, G-CSFR and MPL.[7] JAK2V617F is associated with all the three MPN phenotypes whereas MPL mutants are associated with only ET and MF.[7]

Recent discovery of mutations in *CALR* gene in ET and MF has paved the way for the advancement of understanding the genetic basis of *JAK2*V617F and *MPL*515 negative MPNs.[128,129] Most of the characterization carried out in the literature is focused on the high frequency mutations i.e., *CALR* del52 and ins5 mutations.[58,132,136] So, yet it remains to be understood the pathogenesis and nature of low frequency mutations.

Similar to del52 and ins5 mutations, the other low frequency mutations occur in the last exon (exon 9) of *CALR* and lead to the same novel C-terminus as del52 and ins5. The only variable among the different *CALR* mutations is the interface sequence, i.e., the sequence between the common novel C-terminus and the WT CALR sequence.

*CALR*del52 and ins5 are found to absolutely require MPL both in *in vitro* cell transformation and in *in vivo* phenotypic development of ET and MF in mouse models.[58,132,136] It has been also observed that the expression of TPO (MPL ligand) causes disease penetrance during the disease phenotype caused by *CALR* del52.[58]

CALR protein is mainly an ER resident protein and confers calcium homeostasis and chaperone function during the folding process of N-linked oligosaccharides glycoproteins.[70] Some studies hint that calcium buffering function of CALR could be more prominent than its chaperone function.[70,69] Other various non-ER functions of CALR have been found which remain as a challenge for a detailed characterization, except cell surface CALR since less than 10% of ER CALR pool is involved in non-

ER functions.[70] Cell surface CALR acts as a pro-phagocytic signal for clearance of apoptotic cells by the macrophages.[126,127]

Here we demonstrate that the low frequency CALR mutants also absolutely require MPL for cell transformation or in other words to induce cytokine independent growth of factor dependent cell line whereas the other type 1 cytokine receptors IL-3R, EPOR, and G-CSFR do not mediate this mutant CALR induced cell transformation. We found that the low frequency mutants also cause constitutive activation of JAK-STAT pathway via MPL.

RESULTS

CALR mutants were modelled in Ba/F3 cell line. Ba/F3 is a murine pro-B cell line which proliferates in response to IL-3. It is a very suitable cell line to model CALR mutants since it does not endogenously express type 1 homodimeric cytokine receptors. So the CALR mutants were expressed in Ba/F3 cell line with either EPOR, G-CSFR or MPL being co-expressed. GFP reporter gene was used for CALR mutant expression. 48 hours after transduction of Ba/F3, Ba/F3 – EPOR or Ba/F3 – G-CSFR with CALR mutants, the cell were sorted at equal GFP intensity. For clonogenecity assay one cell/well was sorted on a 96-well plate and for other analyses the cells were sorted at equal GFP intensity as a bulk population.

Expression of *CALR* WT, *CALR*del 9, type 2-like mutant *CALR*del19 and type -1 like mutants *CALR*del34 or *CALR*del46 in parental Ba/F3 cell line did not induce cytokine independent growth of these cells. Similarly, expression of these CALR mutants did not induce cytokine independent growth of Ba/F3 cell line expressing either EPOR or G-CSFR. Only CALR mutants with novel C-terminus i.e., del19, del34 and del46, but not CALR WT and del9 induced cytokine independent growth of Ba/F3 cells only when MPL was expressed.

Ba/F3-MPL expressing different CALR mutants were serum and cytokine starved for 6 hours. Next, the cell lines were stimulated with or without TPO (10ng/mL) for 10 minutes. Signaling proteins of JAK-STAT pathway analysed by western blotting showed that Ba/F3-MPL cell lines expressing only CALR mutants with novel C-terminus induced ligand independent/constitutive activation of JAK-STAT pathway. But Ba/F3-MPL cells expressing either CALR WT or del9 did not show ligand independent/constitutive activation of JAK-STAT pathway.

In mouse models, type-1 like mutants del34, del46 induced a penetrating disease phenotype progressing to MF similar to del52 whereas type-2 like mutant del19 did not induce a penetrating disease but mild thrombocytosis which was not associated with bone marrow fibrosis. The type -1 like mutants were associated with early clonal advantage whereas type-2 like mutant was associated with late expansion of megakaryocytes.

CONCLUSION

The main conclusion from this work are type-1 like mutants del34, del46 behave like del52 and type-2 like mutant del19 behaves like ins5. All the CALR mutants with novel C-terminus induce constitutive activation of MPL/JAK-STAT pathway but not CALR WT and CALR del9 (data not show for CALRdel9).

All the CALR mutants with novel C-terminus induce MPL mediated cytokine independent growth of Ba/F3 cells. But the other cytokine receptors do not mediate long term cytokine independent growth of Ba/F3 cells.

The differences observed among the pathogenesis induced by type-1 mutants and type-2 mutants could be due to activation of additional signaling pathways such as calcium signalling and UPR pathway since defective protein maturation is present during CALR mutant expression and especially type-1 *CALR* mutant shows increased cytosolic calcium concentration which may activate both calcium signaling and UPR.

Also, the differences observed between type-1 like and type-2 like mutants could be due to different dimeric inerfaces of MPL activation or the difference in intensity of activation of MPL/JAK-STAT pathway. The differences may also be attributed to the aberrant interactions of N and P-domains in mutant CALR.

UPR activation was found to be evident in Ba/F3-MPL cells expressing type-1 mutant but not with type-2 mutant (data not shown). Future studies pertaining to activation of UPR in cells expressing CALR mutants may investigate whether there is differential activation of PERK, IRE-1 and ATF6 signaling arms over the course of time.

Ultimately, studies aiming to better understand the differences between type-1 and type-2 mutants induced diseases hold a great promise to understand biology of CALR mutant MPNs, therapeutic intervention and patient management.

Part 2
(Results)

Homology modeling based protein structure and function prediction of del19, del34, del46, del52 and ins5 CALR mutants found in ET and MF

INTRODUCTION

Mutations in *CALR* gene in ET and MF patients were reported during the end of 2013.[128,129] The mutations were of insertion and deletion types occurring in the exon 9 of *CALR*.[128,129] All these mutations lead to frameshift in the protein sequence leading to generation of novel C-terminus.[128,129] This novel C-terminus is rich in positively charged amino acids in contrast to wild-type sequence, which is rich in negatively charged residues.[128,129] It is known that these negatively charged residues in WT CALR play a crucial role in calcium homeostasis.[70] The only difference between all the CALR mutants (derived from a +1 frameshift) is a small variable region preceding the common novel sequence, which is at the interface between the WT sequence and novel C-terminus.[128,129] Thus, it is important to investigate whether specific differences in this variable region of protein sequences among the CALR mutants lead to difference in the pathogenesis and disease progression.

Out of several clinical studies one of the evidences revealed that del52 and ins5, the two most frequent CALR mutants are equally associated with ET, but in MF del52 is associated with 70% of patients and ins5 is associated with only around 15% of MF *CALR* mutated patients.[131] Furthermore ins5 in ET is associated with a higher thrombocytosis than del52.[131] In addition, *CALR* del52 PMF or post-ET MF exhibit a very good prognosis and even better than the other mutations in the presence of additional mutations such as ASXL1, which is not the case for ins5 MF since ins5 MF harboring secondary mutations show poor prognosis.[1,7] This difference between *CALR*del52 and ins5 is also illustrated in the retroviral mouse model. It was shown that *CALR* del52 and ins5 induce a thrombocytosis progressing to MF and a mild thrombocytosis, respectively. These results indicate that although both CALR del52 and ins5 possess the same novel C-terminus found in all the CALR mutants, the variable/interface sequence shows extreme differences, which may be responsible for different disease phenotypes induced by del52 and ins5. Ins5 retains approximately 50% of negatively charged residues in the C-terminus (9 out of 22) whereas del52 has almost lost all the negatively charged residues.
However, it has been shown by several teams including ours, that CALR mutants (del52 and in5 and in this thesis other type 1- and type 2-like mutants) absolutely require MPL to induce constitutive activation of the JAK-STAT pathway and other downstream signaling induced by MPL/JAK2. This induces TPO independent growth of cell lines and of MK progenitors. Such a biological effect is not observed with

other cytokine receptors including IL-3R, EPOR or G-CSFR although CALR mutants more particularly del52 can activate the G-CSFR. Then, it was found that del52 and ins5 strongly interact with MPL, but not with the other receptors.[51] This strong interaction is related to the binding of the lectin (N-terminal globular) CALR domain to MPL extracellular domain, around the first N-glycosylation site (N117) this binding being reinforced by the C-terminus explaining the differences with the CALR WT.[50] It has been demonstrated that the positive electrostatic charge in the novel C-terminus of CALR mutant play a crucial role in this interaction of CALR mutant with MPL, but it is not related to a specific sequence.[58,50,136]

The C-terminus plays an essential role in MPL activation by a mechanism, which is not completely elucidated. CALR mutants by increasing the binding to MPL may favor the dimerization of MPL leading to change in conformation on the model of TPO activation of MPL. This can be related directly to the electrostatic charges of CALR and its 3-D conformation and/or to loss of calcium-binding residues in CALR mutants that may drastically prolong the interaction leading to an abnormal stabilization of a novel CALR mutant-MPL-JAK2 protein complex.[49] However the loss of calcium-binding residues cannot explain the entire activation of MPL by CALR mutants because CALR deleted in the whole exon 9 does not induce MPL activation.[58,136]

One of the major challenges faced in the field of protein structure determination is the dependency on X-ray crystallography for elucidation of protein structure conformation of newly discovered proteins. This challenge has created a wide gap between the low number of known protein structures and the availability of amino acid sequences of many known proteins. To overcome this challenge, we sought to model the 3D structures of CALR mutants using Phyre2 (Protein homology/analogY recognition Engine 2) web-based tools (http://www.sbg.bio.ic.ac.uk/phyre2). We used Phyre2 in intensive mode for our protein structure predictions.

Phyre2 web-based platform for protein modeling is a very useful tool for the 3D structure prediction of proteins whose structures have not been solved by classical methods like X-ray crystallography or NMR spectroscopy.[162] There is a method in the Phyre2 web tool called as intensive mode of protein modeling, this method uses uses the alignment of hidden Markov models and a combination of multiple template based modeling. It also incorporates a new ab-initio to model proteins with no homology to known structures and to assess folding simulation.[162] Phyre2 performs protein modeling in four technical stages.[162]

Here we seek to understand whether abnormal interactions of CALR mutants (specifically N-domain and P-domain) are responsible for the MPL/JAK-STAT pathway activation.

RESULTS

CALR mutants were modeled using web based Phyre2 server.[162] Phyre2 uses homology based modeling to predict 3D protein structures from the input amino acid sequences.[162] Figure 14 shows the representation of the colored protein structures,

blue color structures represent mainly the N-domain, similarly green and yellow color structures represent P-domain whereas the terminal red color structures represent novel C-terminus in CALR mutants and WT C-domain in CALR WT.

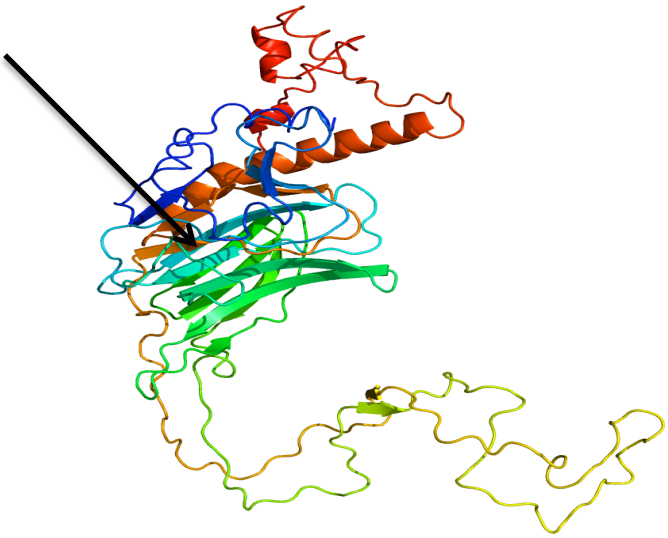
We found that the results of Phyre2 based structure prediction of CALR mutant proteins held high confidence, as observed in the description of results output.[162] The confidence interval for protein structure prediction by Phyre2 was found to be 90% in intensive mode.[162] The predicted structures were then viewed and analysed using 3D view in JSmol and are shown in figure 14. The analysis of structural changes especially in N- and P- domains was compared to CALR WT structure. The structural changes found from the analysis was further compared to the existing literature on the X-ray crystallography and NMR based studies of CALR WT 3D structural models and solved structures of segments of CALR.

Normally V191 is present in a beta strand in parallel to the strand containing V184 and K185 with an evident bonding between the two beta strands. Also, S300-Y306 is present as a beta strand. In our analysis, we found that the results showed that there is a possibility of occurrence of pi configuration based interactions in CALR mutants in the region containing V184, K185 and V191 and may behave as a binding site for hydrophobic patches in client proteins. Thus we thought that V191 in combination with V184 and K185 could be making a hydrophobic interaction with MPL. Similarly we thought that other hydrophobic interactions also could be taking place between CALR mutants and MPL. Thus, we propose that in addition to V191, V184 and K185, S300-Y306 containing beta strand could also be making a hydrophobic interaction. V191 could be involved in bond formation with V184 and K185 in CALR WT, but in CALR mutants this interaction may have been lost by V191 and in CALR mutants V191 may itself behave as a binding residue to hydrophobic residue/site in client proteins. F/Phe at 74 is predicted to be involved in binding to hydrophobic region in client proteins. This residue is pointed as adjoining residue to S69-R73, which is a segment involved in forming compact packing upon binding to client proteins. F74 is an evolutionary conserved residue for CALR WT except for some plants. F74 is one important residue, which creates a specific conformation in CALR WT when comparing with Calnexin.
In the modeling by Phyre2, it is found that F74 is in loop in CALR WT along with S69-R73 but in case of CALR mutants, F74 is found to be in beta strand along with S69-R73. This complete patch in mutant CALR S69-F74 could also be involved in peptide based binding to client proteins leading to folding of client proteins with exposed hydrophobic regions even after completion of folding.

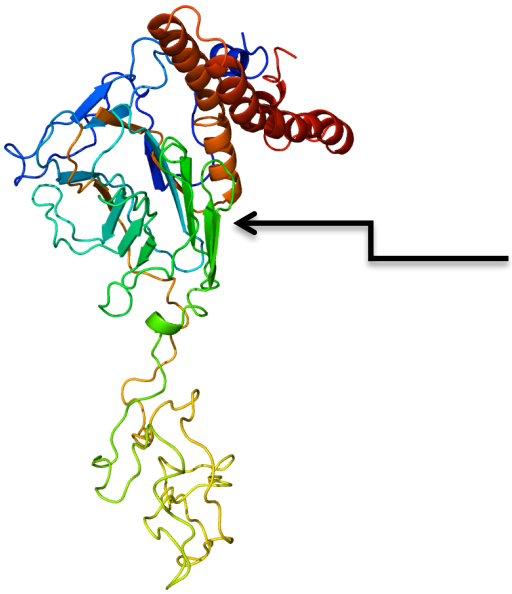
Figure 14: The representative outputs from the results of Phyre2 based modelling of CALR mutants are shown. *Each structure is nomenclatured on the above of the structure with its respective type of CALR mutant or WT CALR. The results were downloaded from interactive phase of the respective results. A confidence key is shown below which represents the folding efficiencies of a particular region. The colors represent both the domains and the efficiencies of folding. More than 85% of each sequence was modelled at more than 90% confidence. In the proteins models, amino acid (aa) 1-180 is represented by blue color loops, beta strands and alpha helices aa 181-290 is represented by yellow*

color loops, beta strands and alpha helices an 291-417 or more is represented by green and red color loops, beta strands and alpha helices.

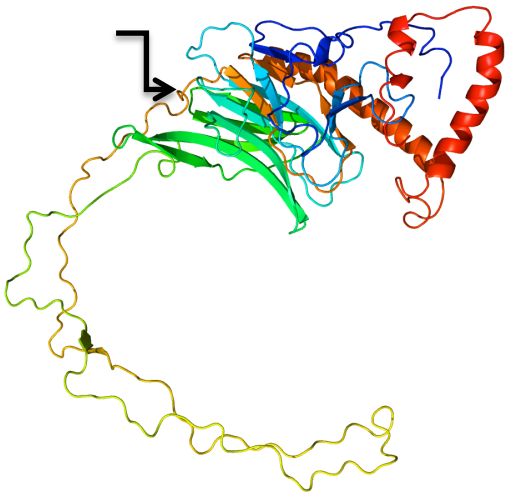
CALR WT



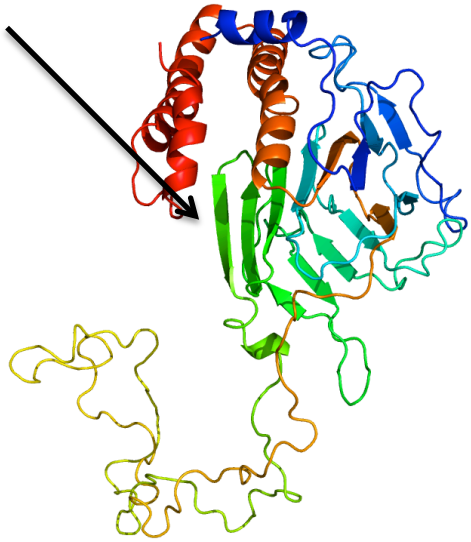
DEL 19



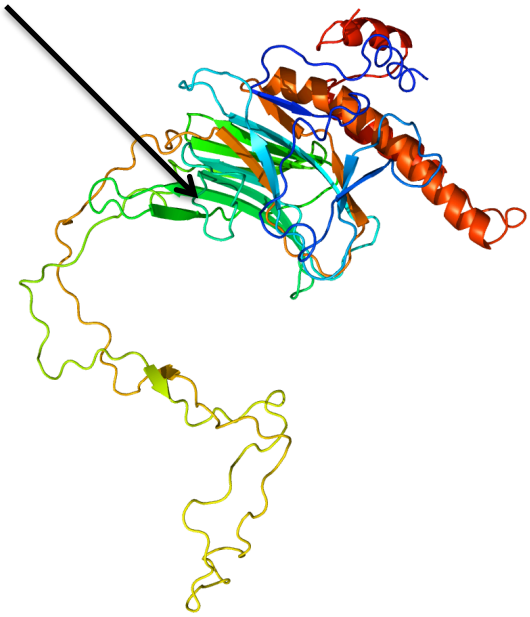
DEL 34



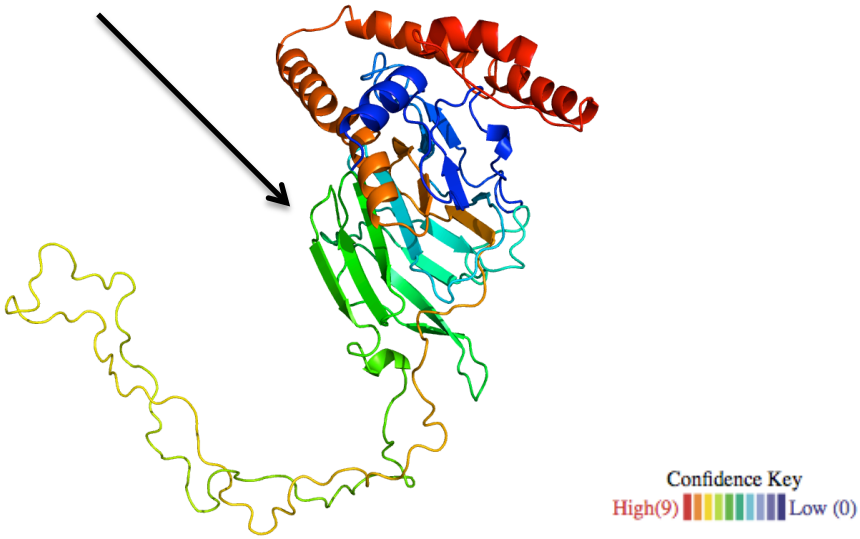
DEL 46



DEL 52



INS 5



EXPERIMENTAL VALIDATION

In collaboration with Ilyas Chachoua and Gaelle Vertenoeil in the group of S. Constantinescu at Ludwig Institute for Cancer Research in Brussels the mutants V191W, V184E, K185E were introduced by site directed mutagenesis. For the

V191W the mutation was introduced both in *CALR* del exon 9 and in *CALR* wild type. For the V184E and K185E, the mutations were introduced in *CALR* del52.

The major result was that the V191W mutation in the context of the WT *CALR* is able to induce MPL/JAK-STAT activation, like CALR mutants in MPN. The same mutation did not activate the CALR mutant, which had a deletion of exon 9. Thus, disorganizing the potential interactions with surrounding residues by introduction of the bulky W residue, the N-terminus becomes able to activate MPL signaling. These results mean that an anchor C-terminus is necessary for the V191W mutation to induce stable binding of CALR N-terminus lectin domain to MPL. Current experiments examine whether the CALR WT V191W also requires N117 of MPL for activation, and what effects are induced on other cytokine receptors. These data argue that the pathologic interaction between CALR mutants and MPL requires a specific conformation of the CALR lectin domain, which can be induced by several mutations.

DISCUSSION

In the literature, till now there have been limited approaches to understand polypeptide based binding of CALR mutants to MPL, which then latter would induce activation of MPL/JAK-STAT pathway. It is now known that CALR mutants i.e., del52 and ins5 bind to N-linked oligosaccharide MPL and this oligosaccharide modification of MPL is indispensable for CALR mutant mediated cell transformation of MPL expressing cells.[50] But, it has not been deciphered whether CALR mutants interact with MPL based on polypeptide binding also, or whether simply the surrounding peptide sequence could be masked by the CALR binding. So we have tried to understand here if the polypeptide based binding of CALR mutants can be predicted based on protein modeling. There could be at least 2 possibilities leading to activation of MPL by CALR WT V191W:

1. From our observations on protein modelling it is possible that CALR mutants may be making hydrophobic interactions with the client polypeptides. Thus, we propose the experimental validation of our observations to infer whether polypeptide based binding of CALR mutants to MPL is also responsible for ligand independent activation of MPL/JAK-STAT pathway.

It could be possible that the binding site of the predicted structure in CALR mutants may be interacting with PP residues in the extracellular region of MPL since pi configuration based interaction leads to interaction of aromatic amino acid side chains with P residues in general and particularly nitrogen containing side chains in amino acids, so this could be tested to determine if mutated PP residues (at position 135 and 136) in extracellular region in MPL still leads to CALR mutants dependent activation of MPL/JAK-STAT pathway.

2. Mutation of V191W in CALR WT may affect the folding mechanism of CALR WT leading to abnormal interaction of N-domain, C-domain or both in CALR WT V191W with MPL resulting in activation of MPL/JAK-STAT pathway.

Initially it was predicted that V191W in CALR WT del exon9 can lead to activation of MPL/JAK-STAT pathway, but it is clear from experimental validation that CALR

WT del exon9 V191W does not activate MPL/JAK-STAT pathway. This could be due to again 2 possibilities:

1. Calcium bound exon 9 which would be positively charged may be required for stabilization of pi configuration based interactions around the V191W region in CALR WT V191W.
2. Another possibility could be that the positively charged calcium bound exon 9 may itself bind to MPL during activation of MPL/JAK-STAT pathway by CALR WT V191W.

Finally, we suggest to explore the importance of polypeptide based binding of CALR mutants to MPL by proposing the experimental investigation based on our observations as follows:

1. Does a CALR mutant containing novel C-terminus along with all the negatively charged residues also causes ligand independent activation of MPL/JAK-STAT pathway?

2. Does F74E mutation in CALR mutants with novel C-terminus cause inhibition of CALR mutants induced MPL/JAK-STAT pathway activation?

3. Does deletion of the amino acid stretch S69-F74 in CALR mutants with novel C-terminus causes inhibition of CALR mutants induced MPL/JAK-STAT pathway activation ?

Part 3
(Results)

IRE – 1α may be causing abnormal loss of p53 at post-transcriptional level in chronic myeloid leukemia (manuscript accepted in arXiv. Reference - Toppalddodi KR: arXiv:1701.05338 [q-bio.SC])

INTRODUCTION

Chronic myeloid leukemia (CML) is a malignant disorder arising from a translocation occurring in a hematopoietic stem cell, which leads to the Bcr-Abl fusion gene[163] encoding the protein $p210^{BCR/ABL}$, a constitutively active form of ABL tyrosine kinase[164]. CML phenotype is very strongly related to the dramatic expansion of hematopoietic progenitors[165] leading to a chronic form chracterized by a marked increase in mature myeloid cells. However the disease always progresses to acute leukemia in a few years in the absence of an efficient treatment.
Concerning to the current therapeutic outcomes, majority of the patients require life – long therapeutic intervention with ABL kinase inhibitors, which permits a clinical, hematological and molecular remission avoiding the progression to acute leukemia. However long term treatment with tyrosine kinase inhibitors may induce non-hematological toxicities although toxcity with Imatinib is overall extremely moderate. In addition, in around 5% of patients, progression may occur under tyrosine kinase inhibitor treatment. In these cases, allogeneic bone marrow transplantation is the only curative alternative, but requires an HLA matching donors and is associated with an important morbidity. It remains that the life expectancy of CML patients treated with tyrosine kinase inhibitors is close to a control population of the same age.

CML leukemic clones are associated with abnormally low levels of *p53* mRNA.[166] It is not known that when the *p53* allele is wild type with intact gene regulatory elements, how low levels of *p53* mRNA arise during CML disease progression.[166,167,168,169]

It has been suggested that downregulated *p53* mRNA levels in CML could be due to either destabilization of its mRNA or epigenetic modulation.[167] Further, it has been demonstrated that down-regulated *p53* mRNA levels in CML are not due to defective epigenetic modulation or defective transcriptional factor function responsible for activation of *p53* gene expression.[169] These results suggest that down–regulation of *p53* mRNA levels in CML is possible through destabilization of its mRNA.

Previously it has been demonstrated that, unfolded protein response (UPR) pathway is a downstream target of BCR-ABL and that UPR confers an anti-apoptotic response,[170] which indicates that UPR may be aberrantly activated in CML leukemic clonal cells. IRE-1α is an evolutionarily conserved member of the UPR pathway and is found to be over expressed in BCR-ABL expressing cells.[170] From the known findings, IRE-1α has mainly two activities , endoribonuclease and serine/threonine

kinase. IRE-1α can be activated by either ER stress or an over-expression.[171] IRE-1α is capable of degrading the target mRNAs by a mechanism called Regulated Ire-1 Dependent Decay (RIDD).[171] IRE-1α induces mRNA degradation by recognising the CUGCAG consensus element accompanied by stem – loop structure in target mRNAs.[172]

RESULTS

In the analysis, we found that the IRE-1α recognition consensus element CUGCAG in the *p53* mRNA is accompanied by a stem-loop structure. This stem- loop structure is formed from the residue 2218 to 2231 and the recognition consensus element exists from residue 2224 to 2229 in the *p53* mRNA. The difference in Gibbs free energy before and after formation of this stem-loop structure is -3.5 units of Gibbs free energy for the 14 nucleotide chain calculated by Mfold web server[173] indicating that the formation of this stem-loop structure in the *p53* mRNA is spontaneous. Thus its formation is highly favourable thermodynamically. Similar results were obtained with other largely used RNA secondary structure prediction web tools.

Figure 15 : Nucleotide alignment of *p53* mRNA and IRE – 1α recognition consensus element (CUGCAG) using EMBOSS Needle[174,175,176]

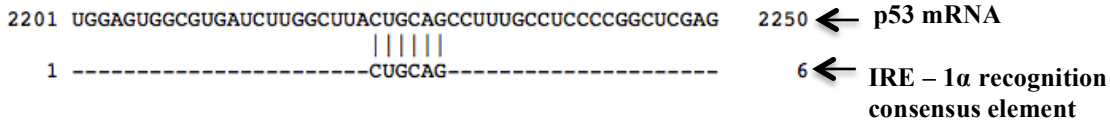
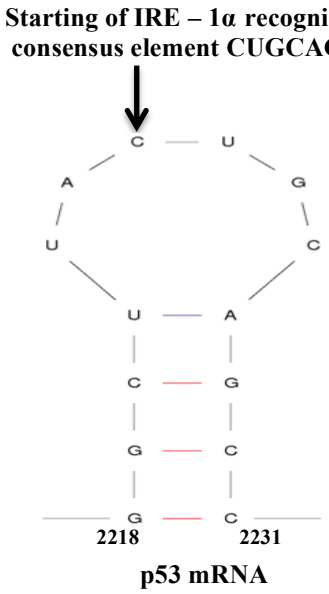


Figure 16 : IRE – 1α recognition consensus element CUGCAG accompanied by stem – loop structure in the *p53* mRNA determined by using Mfold Web Server[173]



CONCLUSION

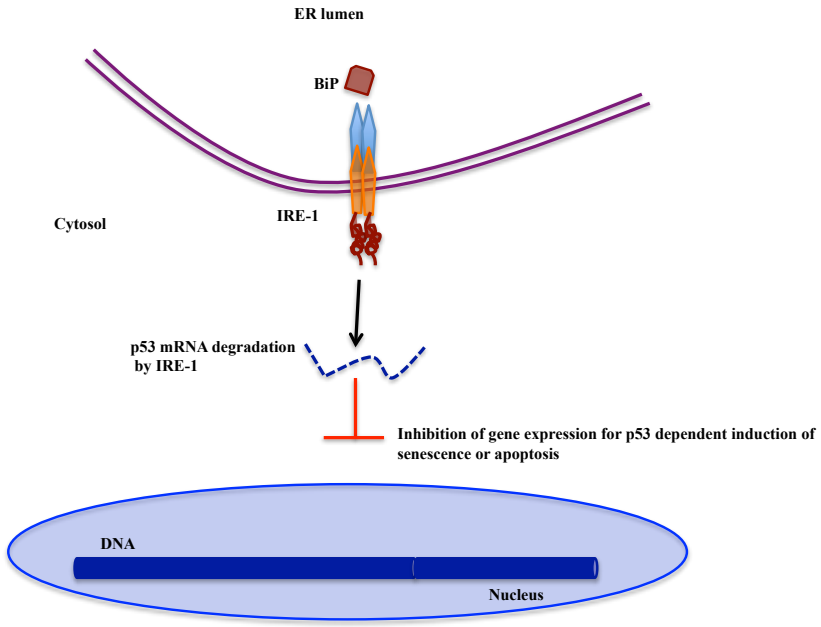
The evidence for the role of p53 as a classical tumor suppressor came from several studies during the 20th century on genetic landscape of cancer.[177] The mutations associated with *p53* in cancers were later found to be loss of function mutations. The gene targets of p53 are mainly associated with apoptosis and senescence.

It has been a keen interest for cancer biologists to characterize the mechanisms for loss of WT p53 function during cancer initiation and progression. From this interest came the evidence that MDM2 is activated by kinase signaling and causes proteasomal degradation of WT p53 contributing to cancer development.[177]

Here we uncover an unexpected mechanism which is a downstream target of both ER stress and kinase signalling in CML which may cause loss of WT p53 at post-transcriptional level. This mechanism shows that when cancer cells harbour pathological ER stress it may lead to aberrant activation of IRE-1alpha causing WT *p53* mRNA degradation.

In CML, it is now known if pathological activation of UPR plays an important role in leukemic clones proliferation by inhibiting apoptosis. But the mechanism downstream of UPR activation was not elucidated to determine how pathological UPR activationl leads to cytoprotection in CML clonal population. Thus we have determined this mechanism for future experimental investigation to identify potential UPR members as therapeutic targets to treat TKI resistant CML patients and patients who show aggressive disease progression. Moreover, detailed investigation may lead to introduction of small molecule inhibitors against endoribonuclease activity of IRE-1alpha as a first line of therapy to treat CML patients.

Figure 17 : Schematic diagram of the model for loss of p53 protein expression in CML



DISCUSSION

Discovery of mutations in *CALR* gene in a large fraction of MPNs[128,129] was totally unexpected because all the driver mutations strictly associated with MPNs were affecting genes involved in the cytokine receptor/JAK2 pathway.[10] They all lead to an abnormal activation of JAK2 and induce downstream signaling.[7] CALR is a mutifunctional protein but its main function is a chaperone of the ER and not a signaling molecule of the MPL/JAK2 pathway.[68]

Thus the transforming activity of CALR could be due to an unxepected involvement of other pathways than activation of JAK2 such as calcium signaling or ER stress. However recent studies have underscored that the CALR mutants, more particularly del52 (type 1 mutation) and ins5 (type 2 mutation) induce a switch from factor dependency to factor independency in cell lines through binding to MPL and activation of JAK2.[58,132,136] They are really phenotypic drivers as they induce a MPN-like disorder in mouse models, more particularly an "ET" by the same signaling pathway as *in vitro*, i.e. the MPL/JAK2 pathway.[58] Thus cell transformation by CALR type 1 and type 2 mutants appears to be related to a major change in its function from a chaperone protein to a new MPL-like ligand capable to bind and activate this receptor.[50,136]

In my thesis, we wanted to investigate whether this is true for other mutants and to study if their transforming activity was related to similar mechanisms with that of the classical type 1 and type 2 mutants. In addition we wanted to model the structure of the different mutants to develop in the future new structure/phenotype studies, which may allow to more precisely understand how CALR mutants bind to MPL and in long term inhibit this interaction.

Activation of MPL by CALR mutants mediates cell transformation

Thus our first goal was to extend our previous results to three other rare mutants (*CALR* del19, del 34, del46), which were associated in our cohorts with MPNs. These 3 mutants give a +1 frameshift. In contrast, the del9 mutant has been described in some patients, but in contrast to the other mutants it is an in frame mutation of the C-terminus, which does not induce an important change in the structure of the protein. There is some evidence that this mutant is a rare polymophism of *CALR* and not an acquired mutation.[130]

It was also not known whether these low frequency CALR mutants behave similarly to high frequency mutants i.e., del52 and ins5 and whether they activate MPL/JAK2-STAT pathway since it has important therapeutic implications for MPN patients, carrying these low frequency CALR mutants.

All these CALR mutants (except del9) contain the same novel C-terminus as del52 and ins5, but the interface sequence i.e., the sequence between CALR WT region and the novel C-terminus is variable in the protein sequences. We thought that similar to differences in disease phenotypes induced by del52 and ins5, these low frequency CALR mutants may have a specific behavior, different among them and from the two highly frequent mutants.

Thus we followed the same experimental strategy, as we have previously performed for *CALR* del52 and ins5.[58]

Our first hypothesis was that type 1 homodimeric/heterodimeric cytokine receptors such as IL-3R, G-CSFR, EPOR and MPL undergo maturation in the ER by interacting with CALR and CNX for protein folding,[70] so we studied whether they play an important role in CALR mutant mediated cellular transformation. We tested this experimentally in Ba/F3 cells by exogenously expressing CALR mutants and either of the type 1 cytokine receptors; we noted that there was no cytokine independent growth of Ba/F3 cells when CALR mutants were expressed with either EPOR or G-CSFR or in parental Ba/F3 cells, whereas all the CALR mutants with novel C-terminus induced cytokine independent growth of Ba/F3 cells in the presence of MPL. This was not the case for *CALR* del9. These results provided us with the inputs that these low frequency mutants are similar to *CALR* del52 and ins5, with an absolute requirement of MPL for cell transformation as measured on cytokine independence.

These results suggest that MPL WT itself behaves as an oncogene in these CALR mutated MPNs. Novel C-terminus of CALR mutants contains repeats of amino acid residues homologous to splicing molecules (RRM domains), but the mechanism of *MPL* gene being spliced in an abnormal manner leading to oncogenicity is ruled out since in our system full length *MPL* cDNA was expressed under the control of a viral promoter. So it may mean that CALR mutants specifically interact with MPL WT protein causing cell transformation. MPL mediated signaling network is involved in self-renewal of HSCs and is a central regulator of megakaryocyte differentiation since *Mpl* or *Thpo* knockout mice show approximately 90% reduction in circulating platelets and reduced self-renewal of HSC compartment.[178]

In the mouse models for *CALR* del52 and ins5, our team has demonstrated even in an *in vivo* setting, that CALR mutants are dependent on MPL to give rise to ET and MF-like phenotypes.[58] At the same time, TPO mediated activation of MPL is found to be an important event in the development of penetrating disease phenotype,[58] which is not unexpected as similar results have been reported for *JAK2*V617F.[54] This can be explained that in mouse models as in the human disease, all MPL on the cell surface are not activated by JAK2V617F or CALR mutants. In Ba/F3-MPL cells, the endogenous level expression of CALR mutants is sufficient to cause cell transformation as demonstrated by knock in experiments,[136,179,180] but this model is artificial as MPL is over expressed in this cell line. Thus it remains to be determined whether endogenous level expression of both CALR and MPL is sufficient to give rise to cytokine independent growth of a factor dependent cell line, which needs a different cell line model than Ba/F3 or UT-7 cell lines.

Results obtained by inducing a +1 framseshift in the CALR gene in Ba/F3-MPL renders Ba/F3 independent of cytokines demonstrating that an endogenous level of CALR mutant is sufficent to induce transfromation.[179] Futhermore in the team, preliminary experiments show that knock in mice for *CALR*del52 develop a myeloproliferative disorder demonstrationg that at least in mice endogenous level of both CALR mutant and MPL are sufficient to induce a disease. This further sustains that CALR mutants are true drivers of MPN.

We found that *CALR*del9 mutant, which is an in-frame deletion found in a few ET patients[130] may not be an oncogenic mutant form of CALR since it did not show cytokine independent growth of Ba/F3-MPL cells, so *CALR*del9 could be a

polymorphism and the ET patients carrying this *CALR* mutation may be harbouring other driver mutations.

Next, we hypothesized that since CALR mutants induce cytokine independent growth of Ba/F3-MPL cells , so CALR mutants could be conferring TPO independent activation of MPL and its downstream signaling. Indeed, as for *CALR*del52 or ins5, we detected constitutive activation of the MPL/JAK-STAT pathway in Ba/F3-MPL cells expressing CALR mutants with the novel C-terminus whereas Ba/F3-MPL cells overexpressing CALR WT or del9 did not show constitutive activation of this pathway.

However we did not find any significant differences among the mutants either type 1-like (del 34, del46) or type 2-like (del19) in the signaling as well as in the capacities to transform. More particularly by limiting dilution experiments, we did not find any significant difference.

There is now strong evidence from our experimental setting and from previous reports that CALR mutants with the novel C-terminus behave as a new MPL ligand.[58,50] This activity is not really due to precise residues in the C-terminus, but more likely to the positive charges that are both responsible for increasing the binding of CALR mutant to MPL followed by its activation.[136] This has been demonstrated both by constructing artificial mutants or by inducing a +1 frameshift in mouse Calr.[179] This frameshift in mouse *Calr* leads to a C-terminus with different residues than in human CALR, but to similar electrostatic charge changes.[179] This means that these positive charges may interact with some negative charged residues in MPL that are absent in other cytokine receptors with may be the exception of the G-CSFR. These charges may increase the binding of CALR to MPL and its further activation.

However it remains to determine whether the active conformation(s) of MPL induced by CALR mutants are similar to or differ from the TPO induced active conformation. Indeed it is possible that MPL being more flexible than the other cytokine receptors such as EPOR[51] and G-CSFR can be activated by CALR mutants under a different conformation than that induced by TPO.[49] Moreover CALR type-like 1 and type-2 like mutants may activate MPL under different conformations, which could explain the different phenotypes in the mouse models. These insights shed light on ability of MPL to induce JAK-STAT signaling through various homodimeric orientations.[181] This could be one of the explanations why CALR mutants induce long term cytokine independent cell proliferation only in the presence of MPL, but not other type 1 cytokine receptors in addition to a specific interaction with the new C-terminus.

3D protein structure prediction of CALR mutants

Till now there is no protein structure prediction of del19, del34, del46, del52 and ins5 CALR mutants, and thus we aimed to model these protein structures focusing on changes in N and P-domains conformations in CALR mutants.

P-domain is largely thought to be involved in peptide based binding of CALR WT to the client proteins and in the formation CALR dimers,[96] although N-domain is also known to possess the ability to bind to client proteins independently of N-glycosylation.[70] In our *in-silico* analysis based on protein structures predicted by Phyre2, we found that the residues in CALR mutants involved in hydrophobic

interactions have undergone a change in conformation compared to their conformations in CALR WT.
We aimed to decipher that when mutant CALR interacts with MPL, it forms an abnormal protein complex, which may lead to an abnormally stabilized mutant CALR-MPL-JAK2 protein complex. Our analysis predicts that V191 and amino acid patch S300-Y306 may be playing a role in making hydrophobic interactions by acting as binding residues to client proteins.
The main conclusion from our protein modelling of CALR mutants is that the peptide based/hydrophobic interactions may be involved when mutant CALR interacts with MPL-JAK2 complex.

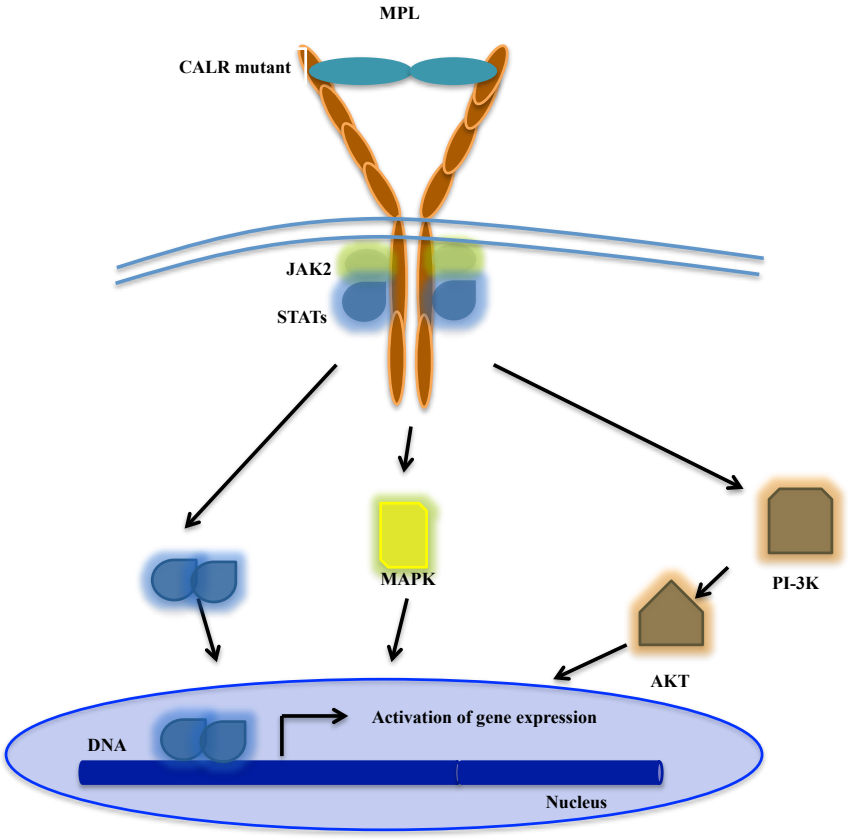
From this elucidation we propose experiments for validation of our protein modelling:

1. Does V191E mutation diminish cytokine independent growth of Ba/F3-MPL or UT-7-MPL induced by mutant CALR ?

2. Does deletion of S300-Y306 diminish cytokine independent growth of Ba/F3-MPL or UT-7-MPL induced by mutant CALR ?

3. Protein-protein docking simulation studies to validate if PP motif in the extracellular domain of MPL interacts with mutant CALR.

Figure 18: Diagramatic representation of mechanistic model for MPL/JAK-STAT pathway activation induced by mutant CALR.



It remains that it is unclear why type 1 and type 2 give different disorders in human. Moreover in this work we show similar differences in the mouse models, on one hand CALR del34 and del46 and on the other hand CALR del19. In both the cases type-1 (del52) and type-1 like (del34 and del46) induce a clear ET like disorder and type 2 (ins5) and type 2 like (del19) a mild thrombocytosis. From our present studies there is no clear evidence that type 1 and type 2 differentially activate MPL although CALRdel52 more clearly activates G-CSFR than Ins5.[50] However the present approach is not enough controlled to see mild differences. Indeed we are using an overexpression system both for CALR mutants and MPL. It will be important to study the MPL/JAK2 activation in del52 and ins5 knock in mice as well as in patients to see if there are some differences in MPL/JAK2 activation.

CALR mutants expressing cells show defects in protein maturation of glycoproteins

In the initial studies it was demonstrated that MPL maturation is inhibited in CALR mutants expressing cells and the CALR mutants are associated with only immature form of MPL at the cell surface.[50] In another important study, it was found that two glycoproteins, eosinophilic peroxidase (EPX) and myeloperoxidase (MPO) are absent from the granules of eosinophil and neutrophil polymorphs granules in patient homozygous for *CALR* ins5.[182] It was demonstrated that the low amounts of CALR mutants in the ER or its major defects in its chaperone function is responsible for a misfolding of MPO leading to its proteosomal degradation.[182] Moreover, it was demonstrated that *Calr* WT knocknout mouse embryonic fibroblasts show defects in protein folding of MPO leading to its proteosomal degradation.[182] Thus it has been concluded that presence of homozygous *CALR* mutations lead to acquired MPO deficiency in patients.[182]
From this important study, it will be crucial to study if such result can be extended to other proteins present in the different blood cells. This will be important for the MK/Platelet lineage where many glycoproteins are packaged in the different granules, more particularly α-granules.[183] Some of them such as TGF-beta and PF4 are negative regulators of HSC inducing their quiescence and also of the platelet production, thus such mechanism may also participte to the induction of an MPN.[183]
In addition it may explain the reduced thrombotic events observed in CALR mutated patients when compared to *JAK2*V617F patients. Thus in the future it will be important to study in details the platelets and platelet function of *CALR* ins5 homozygous patients.
Another factor to be investigated is the MHC class I antigen presentation in CALR mutant expressing cells as CALR WT is extensively involved in MHC class I folding required for antigen presentation for cytotoxic T-cells.[70] These aspects may have important implications in defective antigen presentation by MHC class I and induce a weak immunogenic response mediated by cytotoxic T-cells.[70]

Subcellular localizations of CALR mutants

Several studies have tried to investigate the subcellular localization of CALR mutants. Initially, it was found that there was no marked difference in the localization of CALR mutant with CALR WT in different cell lines.[129] The only difference reported

was a difference in the accumulation in the ER.[128] Further studies have not confirmed this result by showing that CALR mutants are localized in ER (although not prominent), but more particularly in the ER-Golgi intermediate compartment, cell surface, Golgi apparatus and nucleus (occassionally)[128,129,50]. It has been speculated that the increased translocation of the CALR mutants outside the ER could be due to loss of calcium binding residues in the C-domain more than the loss of the KDEL motif.[136]

Furthermore, results in cell lines have shown that CALR mutant proteins are much less present in the cytoplasm than the wild type, one exception might be megakaryocytes.[184] In cell lines the amount of CALR mutants found in cell lysates was lower than CALR WT overexpressing cells.[184] This is not due to a degradation of the proteins by proteosomal or lysozomal degradation, but by high level of secretion with the accumulation at the cell surface and their presence in the culture medium.[184] This result greatly differs from the previous studies that did not show an increase of CALR on the surface of a myeloid cell lines expressing the CALR mutants or on patient leukocytes.[184] These differences could be related to experimental artifacts. Initially results were acquired with an untagged CALR and the best antibodies against CALR recognize its C-terminus and do not bind to the mutants. The other studies have been performed with tagged proteins either in C-terminus or N-terminus, a process, which may change the traffic of the proteins. However recent unpublished results suggest that there is a high level of CALR mutants in the plasma of patients further suggesting that the protein is secreted.[184]

One possibility of the differences between type-1 like and type-2 like mutants could be related to the content of intracytoplasmic mutant CALR. Indeed it has been shown that the level of mutant CALR del52 and ins5 are much lower than the wild type CALR.[184] In our team, using an N-tagged CALR we have found that mutant CALR were secreted at high level and could be easily detected on the cell surface of Ba/F3 either expressing MPL or not. This phenomenon seems to be more marked for ins5 than for del52 and thus the interaction of CALR ins5 with MPL may be decreased in comparison to del52.

The fact that CALR mutants may be expressed at the cell surface has two important consequences[185]:

- The presence of CALR WT on the cell surface is an “eat-me” signal. Thus the fact that there is development of a disease in human with a high clonal dominance may imply that either the CALR mutants have lost this “eat-me” signal or that patients are immunological tolerant to the mutated protein .
- The CALR mutant must be very immunogenic and may induce either the production of auto-antibodies or cytotoxic T cells or both in patients.

  Further studies are required to know if it is the case and if the patients as suggested by the first hypotheis are immunogically tolerant to this mutated protein. It has important therapeutic consequences because it may imply that an immunotherapy either antibodies or induction of cytotoxic T cell or immunecheckpoint could be very efficient in patients. This might be very important in patients with a *CALR* mutated PMF with a poor prognosis or secondary leukemia. Furthermore the effects of IFN therapy in patients could be related to an immunologic effect by allowing the generation of cytotoxic T cells.[186] Another possibility could be CD47 expression levels on the target

cells, which if is more increased than cell surface CALR mutant may inhibit CALR mutant induced phagocytosis.[126]

Studies to ascertain the co-localization of MPL and CALR mutants may also provide insights on where does CALR mutants induce activation of MPL. It would be useful to focus in further studies in determining the translocation of CALR mutants in real time micropscopy. This will allow to accurately determine the traffic through which CALR mutants are secreted into the extracellular regions in an MPL independent way. Furhermore it will be important to determine the CALR mutant traffic in the presence of MPL and if it follows the same pathway.
These insights are crucial to have a lead to discover in which cellular compartment does the activation of MPL by CALR mutants occurs, since it is important for designing therapeutic molecules in order to target JAK2 or MPL. Indeed, if an activation of MPL occurs in the ER it may lead to a more severe phenotype, by increasing the STAT activation and inducing UPR and an aberrant calcium signaling. Also, if the CALR mutants induce activation of MPL in the ER, the vesicles containing activated MPL-CALR mutant complex may traverse through Golgi and cell surface before undergoing degradation. The cell compartment where activation of MPL by CALR mutants occurs may be essential for deciphering the time duration of constitutive MPL/JAK-STAT pathway and also why the activation of the MAPK/ERK and PI3K/mTOR pathway differ from JAK2V617F cells. Finally, it would be also important to determine if the MPL-CALR mutants complex at the cell surface show defects in endocytosis as this defect may facilitate the occurrence of myelofibrosis as recently shown.[187]

Role of CALR mutants in autocrine/paracrine signaling

From these findings the question arises as to what could be the consequences of this mutant CALR secretion in the pathogenesis of MPN ?
An important aspect of CALR mutants especially type 1 mutants has been investigated for its role in autocrine and paracrine signaling leading to activation of JAK-STAT pathway independently of MPL.[184] This idea came in act due to failure of CALR mutants to induce cell intrinsic activation of JAK-STAT pathway of the Ba/F3 WT. Indeed in the first paper published by the team of R. Kralovics,[128] it was shown that the activation of the JAK2/STAT pathway in Ba/F3 could occur. The Ba/F3 cell line does not theoretically express MPL constitutively. However, subsequently several other teams using Ba.F3 could not confirm this result. These controversial results are due to the fact that the Ba/F3 cell lines selected by the team of Kralovics express MPL by an unknown mechanism (selection, induction of MPL by CALR mutant).[180]
Thus several authors have suggested that the mechanism of oncogenic transformation by CALR mutants was not a direct activation of the JAK/STAT pathway, but possibly a paracrine stimulation.
Initially the cell type chosen for the investigation of this hypothesis was monocytes.[184] The cytokine profile was studied for normal monocytes, which were cultured in conditioned media obtained from type-1 mutant overexpressing hematopoietic and non-hematopoietic cells and monocytes from *CALR* type 1 MF patients.[184] The cytokine profile found for the supernatants from type 1 and type 2 mutants were significantly different.[184] In monocytes cultured in the conditioned media from type 1 mutants expressing cells, an hyperactive response of toll like receptor antagonist,

R848 was found.[184] It led to increased secretion of cytokines, but such a response was not found with CALR WT.[184] This indicates that the CALR mutants harbor novel cytokine properties, act as priming factors, cytokine signaling amplifying molecules or induce secretion of cytokine signaling amplyfying factors.[184]

Table 4: List of cytokines, growth factors, chemokines whose secretion is induced at higher levels by CALR mutants[184]

Growth factors, cytokines and chemokines
1. EGF 2. IL-1beta 3. IL-6 4. TNF-alpha 5. VEGF 6. GM-CSF 7. IL-10 8. IL-12 9. MIP-1alpha 10. MIP-1beta 11. MCP1 12. IFN-alpha 13. IL-1Ralpha

Among these cytokines, growth factors and chemokines, a particular interest would be to investigate the effects of IL-1beta, VEGF and IL-1Ralpha since IL-1beta and IL-1Ralpha are important factors involved in pathological reprogramming of bone marrow mesenchymal stem cells (BM-MSCs) leading to fibrosis formation.[183] VEGF would be an important factor in the development of neo-angiogenesis associated with myelofibrosis.[183] Further investigations may be required to assess whether TGF-beta1 is also secreted at abnormally high levels in the type 1 mutant bone marrow microenvironment. Since TGF-beta1 plays a very important role in pathological reprogramming of BM-MSCs to osteoblast differentiation and to myofibroblasts in myelofibrosis.[183] It will be important to also determine if secreted CALR mutants could reprogram monocytes to fibrocytes as it has been suggested that the TPO induced myelofibrosis is related to this mechanism.[62] The role of the high secretion of IFN-alpha is more difficult to assess. On one hand it could induce a clonal evolution during disease progression, on the other hand it may inhibit the *CALR* mutated clone by mechanisms, which remains to be determined.[186] Further, it remains to be determined if similarly the other blood cell types and BM-MSCs show hyperactive response by secreting pro-inflammatory cytokines and collagen, respectively, in the bone marrow environment since they are important factor in the development of fibrosis.[183]

Crucial role of MPL in classical Ph$^-$ MPNs

MPL plays a pivotal role in classical Ph$^-$ MPNs.[188] Dysmegakaryopoiesis has been found to be associated with progression of ET and PV to myelofibrosis.[188] In this disease progression, pathological megakaryocytes are found to play a central role

although recently monocytes and macrophages have been found to be involved through both the formation of fibrocytes and the release of pro-inflammatory cytokines.[62]
JAK2V617F binds to MPL WT causing constitutive activation of JAK-STAT pathway in addition to its association with other type 1 cytokine receptors such as EPOR and G-CSFR, and probably other receptors such as IL-3R.[7] In MPN, MPL WT behaves as a proto-oncogene.[7] MPL mediated cell transformation was first reported from its association with JAK2V617F.[7] The development of *Mpl* knockout *JAK2*V617F transgenic mice has highlighted the crucial role of MPL in MPN development and its severity.[188] It has been demonstrated that *Mpl* KO *JAK2*V617F KI mice show reduced thrombocytosis, neutrophilia, splenomegaly and stem cell pool ultimately leading to inhibition of MPN progression.[188] 50% expression of MPL rescues the hematopoietic stem cell pool and rescues the MPN development.[188] This demonstrates the indispensable role of MPL in MPN development and progression co-operating with JAK2V617F.[188] Ablation of *Tpo* in *JAK2*V617F KI mice, cause less severe MPN, but it is not found to be prominent suggesting that TPO is not indispensable for MPN development.[188]
In retroviral mouse models, *MPL*515 mutants also cause an ET like phenotype in mice rapidly progressing to MF[26] and our team has demonstrated that CALR mutants absolutely require MPL to cause MPN development and progression.[58] In this case, *Tpo* ablation causes much less penetrating disease phenotype but TPO is not indispensable for the development of MPN phenotype.[58]
Thus in the three models of MPNs (JAK2V617F, MPL and CALR mutants) the expression of MPL is indispensable for MPN development.[7] Thus for the future it appears that MPL in addition to JAK2 could be a major therapeutic target by trying to develop MPL antagonists that may inhibit the MPL function and /or activation. This can be performed by small molecules targetting either the extracellular domain by changing MPL conformation or intracellular domain or by dia-bodies changing receptor conformartion as recently demonstrated for EPOR in the JAK2V617F mouse model or patients.[189]

Proposal of hypothetical models to explain effects on cellular processes due to mutant CALR expression

During our experimental work we thought about three possible hypotheses for the induction of constitutive activation of MPL/JAK-STAT pathway by mutant CALR. The hypotheses are as follows:

Hypothesis 1: Each mutant CALR binds to monomer MPL-JAK2 protein complex at the cell surface through N and P-domains and the mutant CALR forms a dimer with another mutant CALR bound to monomer MPL-JAK2 complex causing close proximity of JAK2 leading to ligand independent activation of MPL/JAK-STAT pathway.

Hypothesis 2: Mutant CALR binding to MPL in the ER disorients MPL dimer in an abnormal conformation and when JAK2 associates with MPL, it leads to constitutive activation of MPL/JAK-STAT pathway.

Hypothesis 3: After mRNA translation of MPL, JAK2 associates with MPL and during the N-glycosylation based folding of MPL-JAK2 protein complex, abnormal folding causes constitutive activation of MPL/JAK-STAT pathway.

With the knowledge of current literature and our work, there is evidence that CALR mutants activate MPL/JAK-STAT pathway at the cell surface although it is not sure that the entire activation occurs at the cell surface. Thus the first hypothesis holds promise to putforth the mechanistic model for the activation of MPL/JAK-STAT pathway by mutant CALR. This will mean that CALR mutants nearly behave as a new "MPL ligand" close to a cytokine.

We putforth the mechanistic model with the help of following steps:

1. Immature MPL is expressed at the cell surface via the autophagosome pathway in normal cells or by another pathway in CALR mutated patients.

2. Mutant CALR translocates to the cell surface via an unknown secretory pathway, but probably by the Golgi.

3. At the cell surface, mutant CALR binds to MPL based on N-glycosylation. The nature of interactions involved in binding of mutant CALR to MPL may include lectin binding site in mutant CALR and polypeptide based binding involving P-domain. Alternatively it binds to MPL in the ER and the complex through the golgi traffics to the membrane

4. When each mutant CALR binds individually to MPL monomer in the MPL-JAK2 protein complex, mutant CALR may form a dimer based on their interaction through their novel C-terminus at the cell surface.

5.After the dimer formation between mutant CALR molecules, the mutant CALR-MPL-JAK2 protein complex undergoes a conformational change in which the JAK2 proteins could be positioned at close proximity leading to their autophosphorylation and constitutive activation of MPL/JAK-STAT pathway.

Role of IRE-1 in ER stress induced cytoprotection in CML

Since, the role of ER stress induced activation of UPR and its pathological role have started emerging recently in case of Ph^- MPNs, we thought of modelling this UPR signaling mechanism in case of CML in which the activation state of UPR is well established.
ER stress induced activation of UPR pathway is an under investigated area in cancer.[138] So we thought of modelling of UPR signaling networks in the leukemic clones associated with CML to provide theoretical inputs for experimental investigation to explore the possible UPR signaling networks. Our aim was to provide theoretical inputs for the discovery of UPR associated therapeutic targets for further characterization and introduction of UPR inhibitors which are already being

developed by several pharmaceutical companies as a new line of therapy to treat CML patients since TKIs may cause cytotoxicity and rarely patients develop resistance. We carried out this modelling of UPR signaling network in CML.

It is known that ER stress induces cytoprotection in neoplastic cells, but the mechanisms remain to be investigated.[138] With a bioinformatics approach, we modelled ER stress induced cytoprotection in CML clones wherein IRE-1alpha causes abnormal mRNA degradation of p53 thus conferring inhibition of accurate execution of apoptosis or cell cycle arrest.

The known crucial criteria for assigning an mRNA as a target for degradation by IRE-1alpha are as follows[190]:

1. Proteins encoded by the mRNAs must be able to translocate to the ER

2. mRNA must contain CUGCAG consensus element in a hairpin loop structure

3. mRNA translation of the target mRNA must be stalled by activated PERK

Our analysis inluding from the previous studies has shown that *p53* mRNA possesses the so far known criteria for being an mRNA target of IRE-1 alpha for degradation. Thus we propose that a thorough investigation would be worthwhile to experimentally elucidate whether loss of p53 function during disease progression in MPNs is due to its mRNA degradation by IRE-1alpha. This area of investigation wherein ER stress induces a defective post-transcriptional regulation of p53 mRNA may hold promise to introduce small molecule inhibitors targeting endoribonuclease activity of IRE-1alpha to treat MPNs including tyrosine kinase resistant CML patients. Such an approach may be also extended to Ph^- MPN if an ER stress is induced by the different driver mutants. This could also be relevant to CALR mutant MPNs.

Perspectives

Although till now extensive investigation has been carried out to study the biological aspects of *CALR* del52 and ins5, it remains to be understood why *CALR* del52 and ins5 induce different disease phenotypes.
To answer this question, we ask the following questions:

1. In our experimental set up, we have used overexpression approach for both CALR mutants and MPL. So it is difficult to assess whether there is a difference in intensity of activation of MPL/JAK-STAT pathway induced by type-1 and type-2 mutants. So it is possible that del52 and ins5 CALR mutants cause different level of intensity of activation of MPL/JAK-STAT pathway, which may be responsible for different disease phenotypes in CALR mutated MPNs? It will be possible to understand to this question by working on the signaling in megakaryocytes derived from MPNs and from *CALR*del52 and ins5 knock-in mouse models.

2. It remains that the difference in disease phenotypes induced by type-1 and type-2 mutants may be due to activation of additional signaling pathways. One of the aspects concerns with high cytosolic calcium concentration in case of CALR del52 expressing cells but not at a similar level with CALR ins5. So we ask does the activation of calcium signalling contribute to progressive disease in case of del52 but not ins5? It will be important to study in details the calcium homeostasis because calreticulin by regulating the calcium in the ER can regulate also the calcium flux in the cell and also in the mitochondria regulating apoptosis. It will be important to study the calcium flux in different conditions as well in these consequences on mitochondria membrane potential. It will be also interesting to study by proteomics if the calreticulin wild type and mutants have different interactions with molecules invovled in calcium homeostasis such as Pmr1.

3. Accumulating evidences from the studies of glycoproteins point out that there is improper protein folding of glycoproteins in cells expressing type-1 and type-2 mutants. So we hypothesize that there is an ER stress induced activation of UPR in CALR mutant expressing cells. So it remains to be identified whether specific UPR signaling mechanisms are involved in progressive disease phenotype in case of *CALR*del52 but not ins5. So we ask does the differential activation of UPR cause the different disease phenotypes in CALR mutant MPNs?

4. Another important aspect of the biology of *CALR* mutated MPNs is the determination of cell compartment where the CALR mutants induce activation of MPL. This aspect is crucial since for example, in case of FLT3-ITD AML a severe and aggressive disease phenotype is observed also because the ligand independent of FLT3 receptor is induced in ER. There is increasing evidence that CALR mutants activate MPL on the cell surface but it is not excluded that a part of the signaling takes place in the cytoplasm including in the ER. This could be different between type 1 and type 2 mutants. Such studies can be performed by imaging using antibodies against the phosphorylated forms of JAK2 and MPL. However the main challenge will be to extend these studies to patient cells or to *CALR*del52 or ins5 knock-in mice.

5. It has been demonstrated that MPL defective endocytosis causes constitutive activation of MPL/JAK-STAT pathway and leads to myelofibrosis in mouse models. So we inferred that apart from ligand independent activation of MPL by CALR mutants there may be other contributing factors playing an important role in progression of CALR mutated ET to MF. It wil be important to study the endocytosis of MPL, its recycling or degradation in CALR mutated cells. Phosphatidylinositol 5-phosphate 4-kinase (PIP4K) is a negative regulator of endocytosis and has been implicated in several cancers due to its overexpression. It will be interesting to test the hypothesis that an increased expression of PIP4K may be present in CALR mutated ET and may cause disease progression to MF. So we seek to characterize PIP4K in the context of CALR mutated MPNs.

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Annexe 1: **Review (Introduction)**

Mosca M, Vertenoeil G, **Toppaldoddi KR**, Plo I, Vainchenker W:
Aspects biologiques de la voie JAK/STAT dans les néoplasmes myéloprolifératifs classiques négatifs pour BCR-ABL. Bull Cancer. 2016 Jun;103(6 Suppl 1):S16-28.

Annexe 2: Original paper (Part 1)

Katte Rao Toppaldoddi[*], Maira da Costa Cacemiro[*], Olivier Bluteau, Barbara Panneau-Schmaltz, Delphine Muller, Hana Raslova, Isabelle Plo, William Vainchenker[*] and Caroline Marty[*]: Functional characterization of rare calreticulin mutants. To be submitted

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Aspects biologiques de la voie JAK/STAT dans les néoplasmes myéloprolifératifs classiques négatifs pour BCR-ABL

Matthieu Mosca[1,2,3*], Gaëlle Vertenoeil [4,5*], Katte Rao Toppaldoddi[1,2,3], Isabelle Plo[1,2,3], William Vainchenker[1,2,3]

1. Inserm UMR 1170, Gustave Roussy, Villejuif, 94805, France
2. Université Paris-Saclay, UMR 1170, Gustave Roussy, Villejuif, 94805, France
3. Gustave Roussy, UMR 1170, Villejuif, 94805, France
4. Signal Transduction & Molecular Hematology Unit, Ludwig Institute for Cancer Research, Brussels B-1200, Belgium
5. De Duve Institute, Université catholique de Louvain, Brussels B-1200, Belgium

[*] ont également participé

Correspondance
William Vainchenker, Inserm UMR U1170, Gustave Roussy, 114, rue Edouard Vaillant, 94805 Villejuif, France
william.vainchenker@gustaveroussy.fr

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MPL
Mutations d'épigénétique

Résumé

Les syndromes myéloprolifératifs appelés maintenant les néoplasmes myéloprolifératifs (NMP) comportent plusieurs entités cliniques : la leucémie myéloïde chronique (LMC), les NMP classiques incluant la polyglobulie de Vaquez (PV), la thrombocytémie essentielle (TE), la myélofibrose primaire (MFP) et les NMP atypiques et inclassables. Le terme de NMP est le plus souvent utilisé pour les syndromes myéloprolifératifs classiques négatifs pour *BCR-ABL* (TE, PV, MFP). Ce sont des maladies clonales résultant de la transformation d'une cellule souche hématopoïétique et aboutissant à la production anormale de cellules myéloïdes. Les anomalies génétiques responsables de cette myéloprolifération sont appelées des anomalies « drivers ou motrices » et ont toutes pour conséquence des dérégulations de la voie des récepteurs aux cytokines/JAK2/STAT. Parmi elles, les mutations de *JAK2*, du récepteur à la thrombopoïétine (*MPL*) et de la calréticuline (*CALR*) sont retrouvées dans environ 90% des cas. Ces mutations motrices des NMP peuvent être associées à d'autres mutations également trouvées dans d'autres hémopathies malignes, surtout dans les MFP. Ce sont des maladies chroniques dont les risques majeurs sont les thromboses, les hémorragies et les cytopénies pour les MFP et à plus long-terme la progression vers une myélofibrose et la transformation vers des leucémies. Les thérapeutiques récentes se sont focalisées sur le ciblage de la voie de signalisation JAK2 directement par des inhibiteurs de JAK2 ou indirectement. L'interféron α est également utilisé et permet dans certains cas une rémission hématologique et moléculaire des patients.

Keywords
Myeloproliferative neoplasms
JAK2V617F
Calreticulin
MPL
Epigenetic mutations

Summary

Biological aspects of JAK/STAT signaling in BCR-ABL-negative myeloproliferative neoplasms

Myeloproliferative disorders more recently named Myeloproliferative neoplasms (MPN) display several clinical entities: chronic myeloid leukemia (CML), the classical MPN including polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF) and atypical and unclassifiable NMP. The term MPN is mostly used for classical *BCR-ABL*-negative (*myeloproliferative disorder*) (ET, PV, PMF). These are clonal diseases resulting from the transformation of an hematopoietic stem cell and leading to an abnormal production of myeloid cells. The genetic defects responsible for the myeloproliferative abnormalities are called « driver » mutations and all result in deregulation of the cytokine receptor / JAK2 / STAT axis. Among them, *JAK2*, the thrombopoietin receptor (*MPL*) and calreticulin (*CALR*) mutations are found in around 90% of the cases. These driver MPN mutations can be associated with other driver mutations also found in other hematological malignancies, especially in PMFs. These are chronic diseases with major risks being thrombosis, hemorrhage and cytopenias for PMF and the long-term progression to myelofibrosis and the transformation to leukemia. Most recent therapeutic have focused on targeting the JAK2 signaling pathway directly by inhibitors of JAK2 or indirectly. Interferon α allows in some cases hematologic and molecular remission patients.

Introduction

Les syndromes myéloprolifératifs appelés maintenant les néoplasmes myéloprolifératifs ou plus rarement néoplasies myéloprolifératives (NMP) comportent quatre entités cliniques : la leucémie myéloïde chronique (LMC), les NMP classiques incluant la polyglobulie de Vaquez (PV), la thrombocytémie essentielle (TE), et la myélofibrose primaire (MFP). En fonction de la classification, d'autres maladies étroitement liées peuvent être incluses comme la mastocytose systémique ou l'hyperéosinophilie idiopathique et la leucémie à polynucléaire neutrophile. Le terme de NMP est le plus souvent utilisé pour les syndromes myéloprolifératifs classiques négatifs pour *BCR-ABL*. Ce sont des maladies clonales résultant de la transformation d'une cellule souche hématopoïétique (CSH), cette transformation est associée à une hypersensibilité ou à une indépendance aux cytokines, qui est la caractéristique biologique des NMP. Il en résulte une surproduction de cellules myéloïdes. Les anomalies génétiques responsables de cette myéloprolifération sont appelées des anomalies *drivers* ou motrices. Les mutations motrices réellement responsables de l'aspect « myéloprolifération » ont toutes pour conséquence des dérégulations de la voie des récepteurs aux cytokines/JAK2/STAT. Ces mutations sont caractéristiques de ces NMP, mais peuvent être retrouvées dans des maladies apparentées comme les anémies sidéroblastiques réfractaires avec thrombocytose. Parmi elles, les mutations de *JAK2*, du récepteur à la thrombopoïétine (*MPL*) et de la calréticuline (*CALR*) sont responsables de 90% des cas. Ces mutations motrices des NMP peuvent être associées à d'autres mutations également trouvées dans d'autres hémopathies malignes et qui touchent les composants de l'épigénétique ou de l'épissage ou certains facteurs transcriptionnels. Elles sont surtout retrouvées dans les MFP. Ce sont des maladies chroniques dont les risques majeurs sont les thromboses, les hémorragies et à plus long-terme la progression vers une myélofibrose pour les PV et les TE ou les cytopénies pour la MFP et la transformation vers des leucémies secondaires. Les traitements classiques se sont avérés efficaces pour la prévention des thromboses, mais peu efficaces sur l'évolution à long terme de ces pathologies. Les thérapeutiques plus récentes se sont focalisées sur le ciblage de la voie de signalisation récepteurs de cytokine / JAK2 directement par des inhibiteurs de JAK2 ou indirectement en bloquant d'autres voies. Ces thérapies améliorent les symptômes de la MFP, mais jusqu'à présent ont peu d'effet sur la progression. L'interféron α (IFNα) est aussi utilisé et permet dans certains cas une rémission hématologique et moléculaire des patients essentiellement dans les PV et les TE.

Les néoplasmes myéloprolifératifs classiques

Les NMP classiques sont des hémopathies malignes clonales dues à la survenue d'anomalies génétiques au niveau des CSH, responsables de la production exagérée d'une ou plusieurs lignée(s) myéloïde(s). Elles incluent la PV, la TE et la MFP [1]. Tous les NMP excepté la MFP sont associés à une myéloprolifération pure, sans dysplasie, conduisant à une augmentation de la production de cellules sanguines matures, qui prédomine généralement sur une lignée. Trois lignées hématopoïétiques sont principalement touchées : i) la lignée mégacaryocytaire / plaquettaire dans la TE, ii) la lignée érythrocytaire dans la PV, iii) les lignées mégacaryocytaires et granuleuses dans la MFP. Ces trois maladies peuvent évoluer de l'une vers l'autre à plus ou moins long terme, mais généralement de façon très lente sur des dizaines d'années. Notamment, la TE peut progresser soit directement vers la MF soit évoluer en PV puis en MF. La PV

peut également évoluer vers la MF. Ainsi, les données actuelles montrent que ces maladies sont en continuum [2]. La MFP est la forme la plus avancée et sévère de NMP par définition associée à une fibrose médullaire et le plus souvent à une hématopoïèse extramédullaire et une splénomégalie. Elle est considérée par certains comme l'équivalent d'une forme subaiguë de NMPs. Les complications des NMP classiques comportent des risques accrus de thrombose artérielle ou veineuse et la progression possible vers la leucémie. La fréquence de développement de celle-ci est très variable et dépend du type de NMP allant de 1 % dans la TE, 4 % dans la PV et 20 % dans la MFP sur une période de 10 ans. Ces chiffres sont actuellement discutés, et dépendent des traitements mais le risque augmente encore beaucoup après 10 ans [3]. Ainsi, les NMP peuvent être considérés comme la phase initiale ou pré-leucémique d'une leucémie. Leur étude détaillée est importante pour comprendre les mécanismes de la leucémogenèse allant de l'initiation à la progression, ce d'autant que les données récentes suggèrent que la plupart des leucémies sont précédées d'une longue phase d'hématopoïèse clonales.

Le panorama des anomalies génétiques responsables de ces NMP est connu en grande partie grâce notamment aux technologies de séquençage de nouvelle génération (NGS). Les anomalies responsables de la myéloprolifération des NMP classiques affectent la voie de signalisation récepteur de cytokine/JAK2/STAT et comprennent trois grands groupes : ceux mutés pour *JAK2*, ceux mutés pour le récepteur à la thrombopoïétine (*MPL*) et ceux mutés pour la calréticuline (CALR) [4, 5].

Rôle physiologique de la voie JAK/STAT

Les Janus kinase (JAK)

La famille des JAK regroupe 4 membres : JAK1, JAK2, JAK3 et TYK2. Ce sont des protéines cytoplasmiques à activité tyrosine kinase non-récepteur [6]. Elles sont fixées aux récepteurs de cytokines de type I et II qui sont dépourvus d'activité enzymatique, afin de permettre la transduction du signal. Ces protéines sont caractérisées par 7 domaines présentant des similarités de séquence, appelés JH1-JH7 (*JAK homology*) et organisés en 4 grands domaines. En N-terminal (JH7 à JH5) se trouve un premier domaine appelé FERM (Band-4.1, ezrine, radixine, et moésine) qui permet la liaison à la « box 1 » située dans la région juxta-membranaire du récepteur de la kinase associée. Au niveau de JH3 et JH4 se situe un deuxième domaine d'homologie à SH2 (*Src Homology 2*) qui est structurellement important pour la spécificité d'interaction avec le récepteur et pour la liaison à des résidus tyrosines phosphorylés d'autres protéines. La région C-terminale contient deux domaines conservés : le domaine catalytique JH1 et le domaine JH2 ou domaine pseudokinase, qui régule négativement JH1. L'interaction entre les domaines JH1 et JH2 est nécessaire à une activation optimale de JAK2. Bien que le JH1 contienne le domaine de liaison à l'ATP, le JH2 est également capable de le fixer [7]. De plus, le JH1 possède un site catalytique (boucle d'activation).

Axe de signalisation récepteurs / JAK2 / STAT

Il existe différentes associations entre les JAK et les récepteurs de cytokines selon le type cellulaire. En ce qui concerne les NMP, trois lignées myéloïdes sont principalement touchées : les lignées érythrocytaire, mégacaryocytaire et granuleuse. Les principaux récepteurs qui gouvernent la différenciation de ces lignages hématopoïétiques sont les récepteurs à la thrombopoïétine (TPO) (MPL) pour la lignée mégacaryocytaire, à l'érythropoïétine (EPOR) pour la lignée érythrocytaire et au G-CSF (*Granulocyte-colony stimulating factor*) (G-CSFR) pour la lignée granuleuse. Ces trois récepteurs font partie de la famille des récepteurs homodimériques de type I et sont caractérisés par la présence dans leur partie extracellulaire de 4 résidus cystéine conservés, d'un motif WSXWS et de domaines fibronectine de type III, ainsi que des box1 et 2 dans la partie intracellulaire juxta-membranaire. JAK2 est la protéine kinase qui s'associe principalement à ces récepteurs, même si TYK2 se fixe aussi à MPL. L'interaction se fait entre le domaine FERM de JAK2 et la « box » 1 de la partie juxta-membranaire du récepteur.

Tout d'abord, JAK2 joue un rôle de chaperon au niveau du réticulum endoplasmique en se fixant à ces récepteurs favorisant ainsi leur stabilité et leur montée à la surface cellulaire par la voie canonique (réticulum endoplasmique, appareil de Golgi, vésicules, membrane plasmique). Une fois ancrée à la surface cellulaire, JAK2 reste associé au récepteur. La fixation de la cytokine (EPO, TPO, G-CSF) sur le récepteur entraîne soit une homodimérisation voir une structure plus complexe (G-CSFR), soit des changements conformationnels dans le cas d'homodimère préformé (EPOR ou MPL). Ceci provoque l'activation de JAK2 qui s'autophosphoryle au niveau des résidus Y1007 et 1008, situés dans la boucle d'activation du domaine kinase, puis la transphosphorylation des tyrosines conservées dans la partie intra-cytoplasmique des récepteurs, permettant ainsi le recrutement de protéines possédant un domaine SH2, comme des molécules de signalisation impliquées dans les voies STAT, MAPK et PI3K [8]. En ce qui concerne les STAT, ces récepteurs activent principalement STAT1, 3 et 5 mais à différents niveaux. MPL active STAT1, 3, et 5 quand EPOR active majoritairement STAT5 et G-CSFR, STAT1 et 3. Une fois les STAT phosphorylées, elles s'homo ou hétéro-dimérisent et migrent vers le noyau où elles agissent en tant que facteur de transcription sur des gènes cibles comme p21, PIM1, SOCS1, 3, et CIS. La voie des MAPK est impliquée dans la prolifération, la différenciation et la survie cellulaire. Cette cascade de signalisation est activée via la protéine adaptatrice GRB2, recrutée au niveau du récepteur qui à son tour se lie à SOS et entraîne l'activation de RAS/RAF/MEK/ERK. La voie de la PI3K joue plutôt ici un rôle dans la survie ainsi que dans le contrôle des stress cellulaires. L'activation de la cascade se fait par fixation de la sous-unité p85 de la PI3K au récepteur qui permet l'activation de l'activité catalytique de la p110 et la phosphorylation des protéines d'aval comme AKT, MTOR, S6K ou RSK.

L'activation du signal est ensuite contrôlée très finement par régulation négative pour éviter une sur-signalisation [8]. En particulier, la protéine tyrosine phosphatase SHP1 se lie à la fois

à JAK2 et au récepteur activé provoquant sa déphosphorylation et la terminaison du signal. Les protéines SOCS, en particulier SOCS1 et SOCS3, régulent négativement la signalisation due à JAK2. SOCS1 et SOCS3 peuvent inhiber directement l'activité de JAK2, mais elles peuvent aussi entrer en compétition allostérique avec les STAT pour la liaison au récepteur. Enfin, elles peuvent recruter le complexe ubiquitine ligase et induire la dégradation par le protéasome de JAK2. LNK (codée par *SH2B3*) est aussi une protéine adaptatrice qui permet la régulation négative du signal en inhibant l'activation de JAK2 via EPOR ou MPL. CBL est une E3-ligase qui induit l'ubiquitination de JAK2 et du récepteur associé et entraîne leur dégradation par le protéasome et la voie des lysosomes. Enfin, les PIAS régulent négativement la voie JAK2/STAT par inhibition directe de la liaison des STAT à l'ADN ou en entrainant la sumoylation des STAT.

Ainsi, toutes ces molécules d'activation ou de contrôle de l'axe de signalisation récepteur de cytokine/JAK2/STAT sont susceptibles d'être impliquées dans la pathogenèse des NMP classiques. De fait, il a été identifié dans les NMP classiques, des mutations gain-de-fonction de *JAK2*, de *MPL* et des mutations perte-de-fonction de *LNK* et *CBL*. De façon surprenante des mutations inattendues dans le gène de la *CALR,* une protéine chaperon du réticulum, et sans lien direct avec cette voie, ont aussi été mises à jour ces dernières années.

Mutations de signalisation trouvées dans les NMP (Figure 1)

Mutations JAK2 V617F

La proximité physiopathologique des NMP BCR-ABL négatifs a été mise en exergue par la découverte d'une mutation récurrente de JAK2 au niveau de l'exon 14, la mutation JAK2V617F. Cette découverte est l'œuvre simultanée de différentes équipes, via des approches expérimentales distinctes [9]. La mutation *JAK2*V617F est retrouvée dans plus de 95 % des cas de PV et de 50 à 60 % des cas de TE et PMF, soit dans plus de 60 % de la totalité des NMPs. Cette mutation résulte du remplacement d'une guanine en thymidine, entraînant la substitution d'une valine par une phénylalanine en position 617, dans le domaine pseudokinase de JAK2, le domaine JH2. La mutation *JAK2*V617F entraîne une rigidification de l'hélice alpha C de ce domaine JH2, favorisant la transphosphorylation de JH1 et donc l'activation constitutive de JAK2 [10]. Cette modification structurale est liée dans la cas de JAK2V617F à de nouvelles interaction entre les phénylalanines 617 et 595. De manière intéressante, la plupart des mutations oncogéniques de JAK2 (voir mutations de l'exon 12) aboutissent à des modifications structurales proches suggérant qu'il serait possible de développer des inhibiteurs des formes oncogéniques de JAK2. L'activité kinase de JAK2V617F nécessite cependant la présence d'un récepteur homodimérique pour être constitutivement active. *In fine*, JAK2V617F induit en l'absence de cytokine une activation des voies de signalisation d'aval, telles les voies STAT5/3/1, MAPK, PI3K [9]. Parmi ces voies, STAT5 semble prépondérante dans la survenue des NMP car sa présence est nécessaire à la transformation par JAK2V617F dans les lignées cellulaires comme dans les modèles murins [11]. STAT1 est important pour la mégacaryopoïèse puisque le KO développe un phénotype érythrocytaire [12]. Le KO de STAT3 entraîne une plus grande thrombocytose. Ce résultat inattendu est indirectement liée à une surexpression de STAT1 et une augmentation de son activation [13].

Dans les modèles murins rétroviraux, l'expression de JAK2V617F induit un syndrome myéloprolifératif, essentiellement de type polyglobulie vraie, avec évolution vers une myélofibrose [14]. La sévérité de la maladie varie cependant avec le fond génétique utilisé. Par la suite, dans des modèles transgéniques où le niveau de JAK2V617F est régulé de manière précise, on note le développement d'une maladie de type PV lorsque JAK2V617F est exprimé à des niveaux similaires à ceux de JAK2 endogènes, tandis qu'un phénotype de type TE est observé quand les niveaux de JAK2V617F sont inférieurs à ceux de JAK2 endogène [15]. Ces modèles murins permettent de prouver que la mutation JAK2V617F est suffisante pour induire un syndrome myéloprolifératif chez la souris. Par la suite, 4 modèles plus physiologiques de knock-in de JAK2V617F ont été mis au point [16]. Dans ceux-ci l'expression de JAK2V617F murin à l'état hétérozygote entraîne l'apparition d'un phénotype de type PV, tandis que celle de JAK2V617F humain est associée à une maladie mimant une TE très modérée à l'état hétérozygote et plus une PV à l'état homozygote [17].

Chez les patients, la plupart de ceux atteints de PV ou de MF surtout post-PV ou post-TE portent la mutation à l'état homozygote, suite à une recombinaison mitotique, alors que la plupart des patients atteints de TE sont hétérozygotes pour la mutation JAK2V617F bien que de rares clones homozygotes puissent être détectés [18].

Mutations JAK2 exon 12

Suite à la découverte de la mutation *JAK2*V617F, des efforts ont été entrepris afin d'identifier les mutations en cause dans les PV, TE et MFP *JAK2*V617F négatifs. Le séquençage de tous les exons de *JAK2* a permis de révéler des mutations gain-de-fonction de *JAK2* au niveau de l'exon 12 dans environ 3-4 % des patients *JAK2*V617F négatifs atteints de PV uniquement [19]. Pratiquement tous les patients atteints de PV présentent donc une mutation au niveau de *JAK2*, que ce soit au niveau de l'exon 14 (*JAK2*V617F) ou de l'exon 12. Ces dernières mutations ne surviennent pas dans le domaine pseudokinase de JAK2, mais dans une région hautement conservée, immédiatement adjacente à celui-ci et s'étendant des résidus 536 à 547. Il y a plus d'une trentaine de mutations actuellement décrites, consistant en des substitutions, délétions et duplications. La plus fréquente d'entre elles est la délétion N542-E543.

Sur le plan structural, bien que ne survenant pas dans le domaine pseudokinase de JAK2 mais, dans une région de liaison entre celui-ci et le domaine SH2, les mutations au niveau de l'exon 12 ont un effet similaire à la mutation *JAK2*V617F. En effet, dans une représentation en 3 dimensions, les résidus concernés se trouvent dans une boucle localisée, comme celle contenant V617, à l'interface entre les domaines kinase et pseudokinase

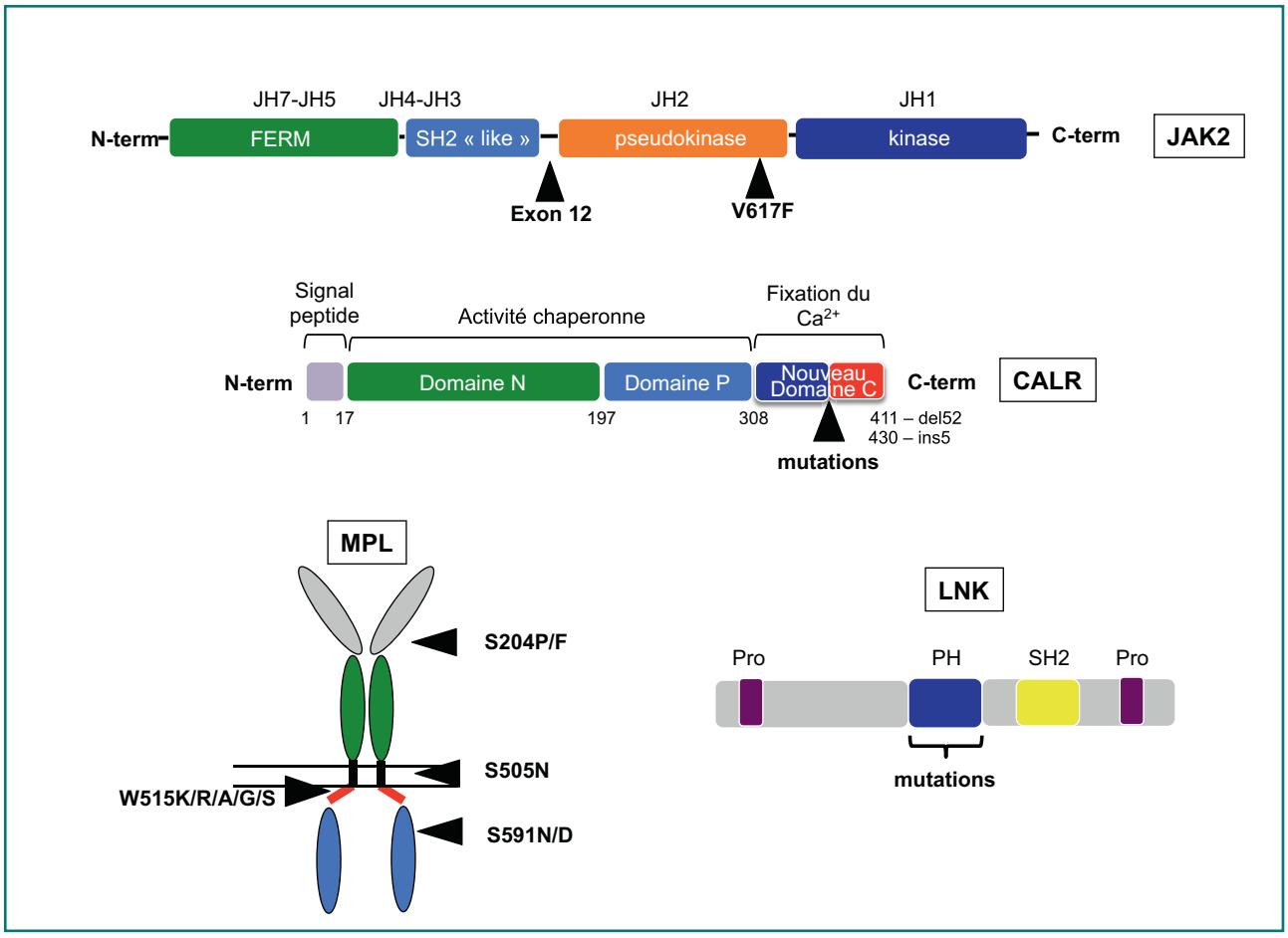

FIGURE 1

Mutations motrices de signalisation identifiées dans les néoplasmes myéloprolifératifs (NMP).

Les principales mutations motrices dans les NMP affectent l'axe de signalisation récepteur de cytokine/JAK2/STAT. Les mutations touchent 4 molécules principales : JAK2, MPL, CALR, LNK.

Les mutations de *JAK2* (*JAK2*V617F ou exon 12) expliquent la majorité des PV. JAK2V617F est également présente dans les TE et les MFP (55%). Les mutations de *MPL* (3-6%), de *CALR* (25-30%) et de *LNK* (2%) sont exclusivement trouvées dans les TE et MFP. Les mutations de *MPL* touchent principalement le codon W515 mais aussi S505 et plus rarement d'autres codons (S204, Y591). Les mutations de la *CALR* sont nombreuses (plus d'une cinquantaine) mais toutes ont pour conséquence la génération d'une nouvelle séquence en C-terminal quasi identique pour tous les mutants et la perte du signal KDEL de rétention dans le réticulum endoplasmique. Les mutations de *LNK* entrainent des modifications principalement au niveau du domaine PH.

de JAK2. Les différentes mutations pourraient perturber de la même manière l'action inhibitrice du domaine pseudokinase sur le domaine kinase.

Sur le plan clinique, les patients porteurs des mutations de l'exon 12 sont diagnostiqués à un âge plus précoce, et ils présentent un phénotype surtout érythrocytaire, avec un taux d'Hb accru par rapport aux patients *JAK2*V617F tandis que leurs taux de plaquettes et de leucocytes sont généralement normaux ou inférieurs. Cependant, il ne semble pas avoir de différences dans l'évolution clinique de ces patients comparés aux patients *JAK2*V617F en terme de survenue de thrombose, de myélofibrose secondaire et de leucémies aiguës [20]. Les mutations de *JAK2* au niveau de l'exon 12 sont par ailleurs retrouvées dans la majorité des cas à l'état hétérozygote.

Récepteur à la thrombopoïétine – MPL

La mise en évidence des mutations dans le gène de *JAK2* et les éléments de plus en plus concordants concernant l'implication de la voie JAK2/STAT dans les NMP a conduit d'autres équipes à rechercher des mutations dans les récepteurs associés à JAK2, comme MPL, EPOR et G-CSFR. Ceci a permis la mise en évidence d'une mutation gain-de-fonction de *MPL* en position 515, appelée *MPL*W515L [21], induisant chez la souris un NMP caractérisé par une hyperleucocytose, une thrombocytose, une splénomégalie et une fibrose médullaire. Par la suite, d'autres mutations ont été décrites, les mutations W515K/R/A/G/S [22]. Le tryptophane en position 515 fait partie d'un domaine amphiphatique situé à la jonction entre les domaines transmembranaire et cytoplasmique de MPL (RWQFP). Il a été démontré qu'il prévient la dimérisation de MPL

en l'absence de cytokine alors que sa substitution par une leucine, lysine, arginine ou alanine, entraîne l'activation constitutive du récepteur [23]. La mutation S505N a également été décrite dans de rares cas sporadiques de TE et de MF bien qu'elle soit surtout décrite dans des cas familiaux [24]. Celle-ci est localisée dans le domaine transmembranaire du récepteur et induit également sa dimérisation et son activation en l'absence de ligand. Enfin de très rares mutations de *MPL* en dehors de l'exon 10 (T119I, S204F, E230G, Y591D/N, S204P) ont récemment été mises en évidence dans les TE et MF dites « triple négatives » [25, 26]. Ces mutations ont une activité gain-de-fonction, mais plus faible que celles des mutations de *MPL*W515 et pourraient être associées à d'autres événements moléculaires pour induire le phénotype.

Les mutations de *MPL* sont retrouvées dans environ 5 % des MF et 3 % des TE, la mutation W515L étant la plus fréquente.

Sur le plan clinique, les patients présentant une TE *MPL* mutée ont généralement un âge supérieur au moment du diagnostic, un taux d'hémoglobine plus bas et une thrombocytose supérieure par rapport aux patients *JAK2*V617F [27]. Bien qu'ils semblent présenter des anomalies plus fréquentes de la microcirculation et un risque plus élevé de survenue de thrombose artérielle, il ne semble pas y avoir de différence en termes de survie, d'évolution leucémique ou fibrotique. Les patients atteints de myélofibrose et présentant une mutation de *MPL* sont quant à eux plus souvent des femmes âgées, avec une anémie plus sévère et une plus grande probabilité de devoir bénéficier d'un support transfusionnel régulier [28].

Mutations de *CALR*

En décembre 2013, deux groupes ont rapporté de manière indépendante la découverte de mutations dans l'exon 9 du gène *CALR* et qui consistent en des insertions ou des délétions entrainant un décalage du cadre de lecture [4,5]. Ces mutations de *CALR* ont été trouvées dans plus de 50% des TE et des MFP sans mutation de *JAK2* ou de *MPL* et au total dans environ 25 à 30 % des patients présentant une TE ou une MFP. Ces mutations sont également retrouvées dans les anémies sidéroblastiques réfractaires avec thrombocytose (RARS-T), mais avec une fréquence très faible par rapport à *JAK2*V617F [4]. De rares cas de PV ont été décrits, mais souvent associés à une thrombocytose, et dans certains de ces cas il pourrait exister des clones indépendants *JAK2* et *CALR* [4,29,30].

Plus de 50 types de mutations différentes ont été détectées à l'heure actuelle, mais les mutations les plus fréquentes et comptant pour plus de 80-90 % des cas sont soit une délétion de 52 bp (appelée mutation de type 1 ou CALRdel52) ou une insertion de 5 bp (ou mutation de type 2 ou CALRins5). Ces mutants n'ont pas la même prévalence parmi les TE et les MF : le mutant CALRdel52 est plus fréquemment associé aux MFP que la CALR ins5 tandis que la fréquence de ces deux mutations est proche en cas de TE [31]. Ces mutations sont bien responsables du phénotype de la maladie puisque les modèles de souris par transplantation de moelle transduite avec les mutants CALRdel52 et à un moindre degré CALRins5, développent une thrombocytose pure. De plus, les souris CALRdel52 récapitulent l'évolution vers la myélofibrose. Ceci est vrai aussi pour la CALRins5 avec une pénétrance beaucoup plus faible [32]. Ainsi, la myélofibrose pourrait être une évolution naturelle de la TE en cas de mutation de *CALR*.

Toutes les mutations de *CALR* génèrent une protéine mutante avec une nouvelle séquence peptidique C-terminale quasi-identique pour tous les mutants. Ceci aboutit à une région C-terminale positivement chargée dépourvue du motif KDEL, signal de rétention dans le réticulum endoplasmique alors que celle de la CALR sauvage est fortement chargée négativement. *A priori*, la CALR avait peu de lien avec l'hématopoïèse en général et les NMP en particulier. En effet, il s'agit d'une protéine chaperon du réticulum endoplasmique, qui est impliquée dans le repliement des protéines N-glycosylées, et l'homéostasie du calcium ainsi que dans les réponses immunitaires (via sa capacité à être transloquée à la surface des cellules apoptotiques) [33]. Cependant, il a été montré que la CALRdel52 active STAT5 en absence de cytokine dans des lignées cellulaires [4]. Récemment, nos laboratoires et d'autres groupes ont confirmé ces données en montrant que CALRdel52 et ins5 activent la voie JAK2/STAT en activant spécifiquement MPL et à un niveau moindre le G-CSFR, essentiellement pour la CALRdel52. Ceci se fait par interaction du domaine d'interaction aux lectines (domaine N) de la CALR et les sites de glycosylation dans la partie N-terminale de MPL [32,34-36]. L'activation des voies de signalisation serait plus liée à l'interaction entre une région particulière de MPL et les charges positives de la nouvelle partie C-terminale. De plus, la CALR mutante est sécrétée, pouvant potentiellement activer des cellules de l'immunité, voir des cellules hématopoïétiques. MPL est également détectée à la surface cellulaire, mais de façon partiellement glycosylée associée aux CALR mutantes suggérant une anomalie de maturation et éventuellement d'adressage à la surface.

Sur le plan clinique, les patients atteints de TE et présentant une mutation de *CALR* ont généralement un taux de plaquettes accru, mais auraient un risque thrombotique plus faible que les patients JAK2V617F [4]. Par ailleurs, ils présentent une charge allélique très supérieure aux patients présentant une TE *JAK2*V617F, ce qui pourrait suggérer un avantage sélectif des cellules CALR mutées au niveau du compartiment des CSH. Les patients *CALR* mutés atteints de MF présentent quant à eux une survie accrue. Ce dernier point est cependant limité aux cas de mutation de type 1 [37].

LNK

Comme mentionné plus haut, LNK ou SH2B3 est une protéine adaptatrice, inhibitrice de la voie JAK-STAT. Elle se lie à la tyrosine 813 phosphorylée de JAK2 via son domaine SH2 et entraîne l'inhibition de la voie, que celle-ci soit activée par la liaison du ligand à son récepteur (MPL ou EPOR) ou par les mutations *JAK2*V617F ou *MPL*W515. Les mutations de *LNK* ont été décrites pour la première fois en 2010, chez 2 patients, l'un atteint de MF et l'autre de TE [38]. Par la suite d'autres mutations ont été découvertes, chez des patients atteints de TE, de PV, ainsi que chez des patients atteints d'érythrocytose idiopathique [39]. Ces mutations sont rares en phase chronique des NMP (moins de 2 % des cas), mais semblent

être plus fréquentes lors de la transformation leucémique (13 %). La plupart des mutations décrites surviennent dans le domaine PH, dans un « hot spot » s'étendant des résidus E208 à G234, bien que des mutations en dehors de ce domaine aient également été décrites. Etant donné que ce domaine PH pourrait être impliqué dans la localisation membranaire de la protéine, ceci devrait aboutir à une localisation anormale de la protéine mutée dans le cytoplasme. La plupart des mutations sont trouvées à l'état hétérozygote, la question étant de savoir si cela correspond à une haplo-insuffisance ou à un effet dominant négatif. Les souris *Lnk*$^{-/-}$ présentent un syndrome myéloprolifératif caractérisé par une thrombocytose, une splénomégalie et une fibrose médullaire tandis que les souris *Lnk*$^{+/-}$ présentent un phénotype intermédiaire, ce qui est en faveur de l'haplo-insuffisance [40]. Cependant, étant donné que le domaine de dimérisation N-terminal demeure intact dans les mutants LNK, ceux-ci pourraient lier et séquestrer la protéine WT dans le cytoplasme, entraînant donc un effet dominant-négatif [38]. Cette dernière étude a également montré que les mutants gardaient la capacité de se lier à JAK2 tandis que leur localisation ne semblait pas affectée, ce qui va à l'encontre de l'hypothèse initialement formulée. En fait, dans un certain nombre de cas, les mutations de *LNK* sont associées à d'autres mutations motrices comme *JAK2*V617F.

Patients triple-négatifs

La majorité des patients présentant un NMP présentent donc des mutations activatrices de la voie JAK-STAT, que ce soit au niveau du récepteur (mutation de MPL au niveau de l'exon 10), de JAK2 (JAK2V617F et au niveau de l'exon 12), de CALR (exon 9) ou des régulateurs négatifs de la voie (LNK). Les NMP ne présentant pas de mutation de *JAK2*, de *MPL* ou de *CALR* sont appelés triple-négatifs et représentent de 7 à 10 % des cas. Une étude récente a cependant montré que même ces patients triple-négatifs montrent des signes d'activation de la voie JAK-STAT [41].
Au total, toutes les mutations de signalisation identifiées dans les NMP affectent l'axe MPL/JAK2/STAT et sont des mutations dites motrices de la myéloprolifération et de la maladie comme l'ont démontré les nombreux modèles de souris. Toutefois, il existe une grande hétérogénéité de ces maladies et de leurs évolutions qui peuvent être en partie expliquées par d'autres événements génétiques acquis ou germinaux.

Les mutations acquises associées aux NMP (Figure 2)

Un certain nombre de mutations impliquées dans l'épigénétique ou les composants de l'épissage ont été identifiées dans les NMP. Ces mutations ne sont pas spécifiques de ces maladies puisqu'elles sont aussi présentes dans d'autres hémopathies malignes (NMP/SMD, SMD, LAM) et ne sont pas responsables de la myéloprolifération, mais modifient plutôt le phénotype de ces maladies.

Les facteurs de régulation épigénétique

Des mutations du gène ***TET2*** ont été identifiées dans les NMP de façon assez fréquente allant de 4 à 11 % dans les TE, 15 %

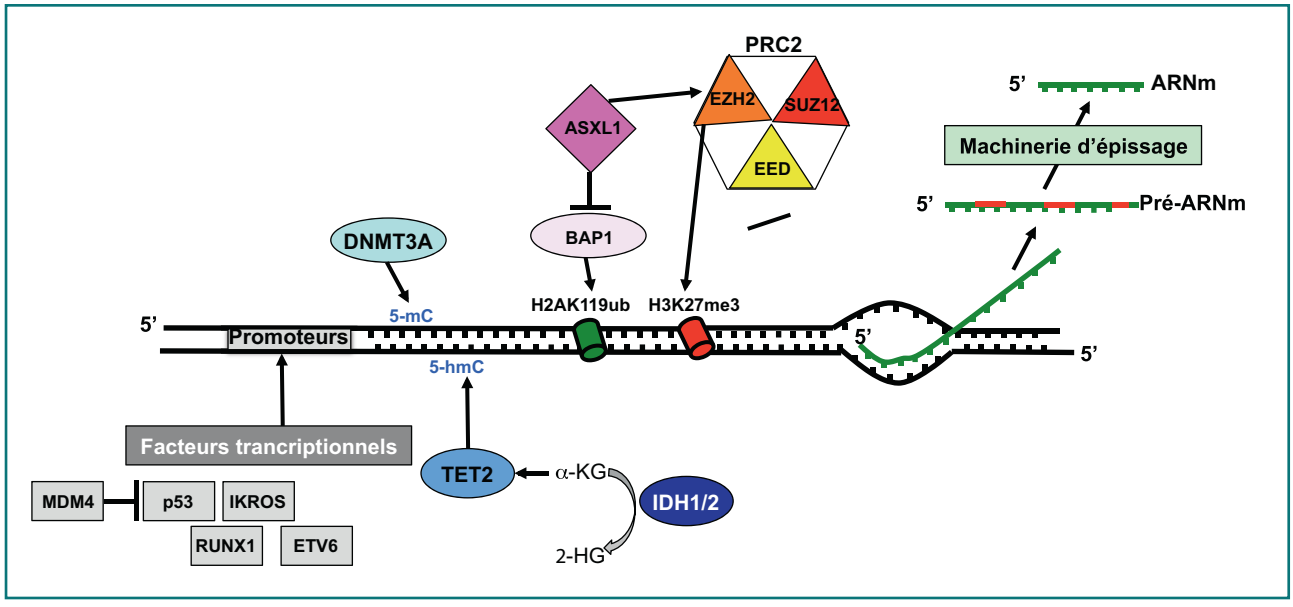

FIGURE 2

Mutations associées aux mutations de signalisation dans les néoplasmes myéloprolifératifs (NMP).

Les mutations associées aux NMP affectent des composants de la machinerie d'épigénétique ou d'épissage. Lors de la transformation des NMP, des anomalies dans les facteurs de transcription apparaissent également. Parmi les molécules d'épigénétique mutées dans les NMP, on trouve *TET2*, *IDH1/2*, *DNMT3A*, *EZH2* et *ASXL1*. Un certain nombre d'anomalies dans les molécules de la machinerie d'épissage sont également trouvées incluant *SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*, *PRPF40B* et *SF1*. Enfin, parmi les facteurs transcriptionnels, on trouve fréquemment des mutations de *P53* ou des amplifications de son régulateur négatif *MDM4*, des mutations de *RUNX1*, et plus rarement des délétions d'*IKZF1* ou *ETV6*.

dans les PV, 20 % dans les MFP et 26 % des leucémies post-NMP [14,42,43]. Les mutations de *TET2* sont perte-de-fonction et incluent des mutations faux-sens au niveau de région conservée ou de sites d'épissage, des mutations non-sens, des délétions ou des insertions résultant d'un décalage du cadre de lecture. Il a été décrit comme suppresseur de tumeur puisqu'il existe des mutations bi-alléliques. TET2 appartient à une famille de trois membres incluant TET1, TET2 et TET3. Les protéines correspondantes possèdent une fonction de 5-méthylcytosine dioxygénase dépendante de l'α-kétoglutarate (α-KG) et du fer, permettant la transformation de la 5-méthylcytosine (5mC) en 5-hydroxylméthylcytosine (5-hmC). Elles participent donc à la régulation de l'expression génique. Les mutations de *TET2* sont associées à un défaut d'activité catalytique et globalement, les patients *TET2* mutés présentent un niveau de 5-hmC plus faible que des donneurs sains [44]. Les études sur la fonction de TET2 dans les systèmes hématopoïétiques humain et murin montrent que le défaut de TET2 entraîne une amplification des progéniteurs et induit la différenciation monocytaire aux dépens des différenciations granuleuse, lymphoïde et érythroïde [45]. En effet, les modèles *in vivo* murins déficients en *TET2* récapitulent la plupart des hémopathies incluant la LMMC dans de nombreux cas et sont caractérisés par une amplification du nombre de progéniteurs et des CSH avec de hauts potentiels d'auto-renouvellement et de capacité de repopulation à long-terme [45]. Ainsi, ces données montrent que le défaut de TET2 pourrait favoriser le développement des NMP et d'autres hémopathies malignes en favorisant la dominance clonale au niveau d'une CSH mutée. Il faut aussi souligner que les mutations de *TET2* sont trouvées dans la population générale de sujets âgés et associées à une hématopoïèse clonale [46].

Ces mutations peuvent apparaître le plus souvent avant les mutations de signalisation comme *JAK2*V617F, mais également après. Elles apparaissent plutôt après les mutations de *CALR* [30]. L'ordre des mutations est important dans les NMP et pourrait avoir un impact sur le phénotype de la maladie. En effet, une étude a montré que l'acquisition d'une mutation de *TET2* avant *JAK2*V617F résultait en un phénotype de TE alors que lorsque *JAK2*V617F précédait la mutation de *TET2*, les patients développaient plus fréquemment une PV [47]. De plus, au cours de leur progression, les patients peuvent acquérir des mutations de *TET2* qui sont plutôt associées à un mauvais pronostic [30].

Les mutations de ***DNMT3A*** sont parmi les plus récurrentes avec celles de *TET2* dans les NMP. Les mutations de *DNMT3A* se retrouvent à 3 % dans les TE, 7 % dans les PV, 7-15 % dans les MFP et 14 % dans les leucémies post-NMP [5,30]. La mutation la plus récurrente permet le changement du résidu R882 de la protéine. Dans tous les cas, la mutation n'est retrouvée qu'à l'état hétérozygote, indiquant un effet d'haploinsuffisance. DNMT3A appartient à la famille des protéines méthyltransférases, responsable de l'ajout *de novo* d'un groupement méthyl aux cytosines des îlots CpG. Son rôle sur l'hématopoïèse semble très similaire à TET2 puisque le modèle murin déficient en Dnmt3a présente une augmentation des CSH dans la moelle osseuse tout en diminuant leur potentiel de différenciation. Ceci était corrélé avec une sur-régulation des gènes de multipotence des CSH et une sous-régulation des facteurs de différenciation, mettant en exergue le rôle de DNMT3A en tant que répresseur épigénétique de gènes spécifiques des CSH [48]. Comme pour les mutations de *TET2*, l'ordre d'apparition des mutations semble important.

Les mutations d'***EZH2*** *(Enhancer of Zeste Homolog*) sont aussi décrites dans de nombreuses pathologies myéloïdes et lymphoïdes malignes. Dans les NMP, elles sont surtout présentes dans les MFP (13 %) [5,30]. Les mutations d'*EZH2* sont de type perte-de-fonction incluant souvent des délétions. Ce sont des anomalies qui apparaissent généralement après les mutations de signalisation et qui sont de mauvais pronostic dans les MFP. Elles favoriseraient un phénotype mégacaryocytaire plutôt qu'érythroïde en association avec *JAK2*V617F entraînant des TE ou MFP. EZH2 fait partie du complexe PRC2 (Polycomb Repressive Complex) qui catalyse la triméthylation de la Lysine 27 de l'histone H3 (H3K27me3) avec EZH1, SUZ12, EED et RBAP46/48. Il s'agit d'un complexe protéique qui joue un rôle essentiel dans le développement, le destin cellulaire et la prolifération, et qui est connu comme un répresseur épigénétique global de la transcription. D'autre part, certaines délétions ou mutations somatiques recensées chez d'autres membres du complexe PRC2 (SUZ12, JARID2...) sont observées dans certaines PV ou d'autres NMP/SMD, démontrant ainsi que la fonction du complexe PRC2 peut être compromise par la mutation de différents gènes [49].

Les mutations d'***ASXL1*** (*Additional Sex Comb Like 1*) ont été trouvées dans les hémopathies myéloïdes malignes notamment, dans de nombreux SMD et SMD/NMP (LMMC). Dans les NMP, elles sont très rares dans les TE et PV (< 8 %) et surtout présentes dans les MFP (13-26 %) où elles sont les plus fréquentes parmi les mutations des gènes impliqués dans l'épigénétique [50]. De plus, 22-38 % des patients atteints d'une leucémie post-MFP possèdent cette mutation, établissant un rôle de ce gène dans la transformation leucémique. Les mutations d'*ASXL1* sont donc corrélées à un très mauvais pronostic de survie et une évolution vers les leucémies [51]. Les souris *aslx1*$^{-/-}$ présentent notamment un phénotype de SMD, ce qui peut laisser supposer que la perte d'ASXL1 pourrait induire des phénotypes dysplasiques [52]. ASXL1 est une protéine nucléaire présente dans les complexes Polycomb, qui est impliquée dans au moins deux processus de contrôle épigénétique de répression, en agissant sur PRC1 et sur PRC2. En effet, d'une part, la perte d'ASXL1 induit l'activation de la protéine BAP1, une E3 ubiquitine ligase de l'histone H2A, ayant pour conséquence une inhibition de H2AK119Ub. D'autre part, ASXL1 se fixe à EZH2 et la délétion d'ASXL1 induit une perte de la marque H3K27me3 générée par PRC2.

Le dépistage des mutations de ***IDH1/2*** dans deux cohortes indépendantes a révélé peu de mutations en phase chronique des NMP (0,8 % des TE, 1,9 % des PV et 4 % des MFP) [5,30]. En revanche ces mutations sont très enrichies en phase blastique des NMP (21 %) indiquant un très mauvais pronostic de survie et de progression vers les leucémies. Les mutations sont généralement faux-sens hétérozygotes et ont pour conséquence une substitution sur les résidus R132 pour IDH1 et R140 et R172 pour IDH2. IDH1 et 2 codent les enzymes isocitrate déhydrogénase 1 et 2 qui

participent à la catalyse de la décarboxylation oxydative de l'isocitrate en *a*-KG et leur activité est nécessaire à la protection cellulaire contre le stress oxydatif. Les enzymes mutées *IDH1/2* ont une affinité réduite à l'isocitrate, mais possèdent une nouvelle activité catalytique consistant en la synthèse de 2-hydroxyglutarate (2-HG) à partir du α-KG. Ainsi la forte diminution de la quantité d'α-KG diminue l'activité enzymatique de TET2 et d'autres enzymes dépendant de ce cofacteur. En effet, il a été montré que les mutants *IDH2* altèrent l'activité catalytique de TET2 et sont mutuellement exclusifs dans les LAM. Comme la déplétion de TET2, celle d'IDH1/2 altère la différenciation hématopoïétique et augmente le nombre de cellules progénitrices ou souches [53]. Enfin, il a été montré que 2-HG est un antagoniste de l'α-KG et qu'il intervient dans des altérations des histones et de la méthylation de l'ADN. Tous ces résultats suggèrent un mécanisme commun de leucémogenèse des mutations *IDH1/2* et *TET2*.

Les gènes de la machinerie de l'épissage

L'épissage de l'ARN est un processus fortement régulé faisant intervenir de petites protéines ribonucléiques (snRNP) et d'autres composants protéiques dans le but de transformer les pré-ARNm en des ARNm matures (élimination des introns). Chez les patients atteints de NMP des anomalies correspondant à des mutations de cette machinerie sont retrouvées dans 9,4% des cas, presque exclusivement des MFP [54].

SF3B1, un gène clé de l'épissage, est muté tout particulièrement dans les SMD, mais aussi dans 3 % des TE et dans 4 à 6,5 % des MFP. Néanmoins, les mutations de *SF3B1* ne semblent pas influencer la survie des patients et ne sont pas associées aux thromboses, mais à la présence de sidéroblastes en couronne posant le problème de la continuité entre TE et RARS-T.

Les mutations de ***SRSF2*** (*Serine/Arginine-Rich Splicing Factor 2*) sont retrouvées dans 17 % des MFP et dans 19 % des leucémies post-NMP. Elles sont significativement associées aux mutations d'*IDH* mais aussi de *TET2* (dans les LMMC), suggérant que *SRSF2* muté est aussi impliqué dans le phénomène de transformation leucémique des NMP. SRSF2 possède un domaine RRM (*RNA Recognition Motif*) qui se fixe à l'ARN, et un domaine RS (riche en sérines) qui permet le recrutement des autres composants protéiques du complexe d'épissage.

D'autres gènes de cette machinerie tels que *U2AF1*, *ZRSR2, PRPF40B* et *SF1* sont retrouvés mutés dans moins de 2 % des NMP, mais les conséquences fonctionnelles de ces mutations dans ces pathologies restent encore obscures bien qu'il existe une corrélation entre la présence de mutations *de U2AF1* et l'importance des anémies dans les MFP.

Les autres molécules impliquées dans la transformation leucémique

TP53 code le suppresseur de tumeur P53, impliqué dans les mécanismes de réparation de l'ADN, l'arrêt du cycle cellulaire, l'apoptose et la sénescence après dommage génomique et est fréquemment dérégulé dans de nombreux cancers. Les mutations de *P53* sont identifiées dans 5 à 10% des LAM *de novo*. Bien que présentes dans 3,1 % des phases chroniques des NMP, elles sont fortement associées à la transformation des NMP en LAM (20 à 27 %) [55]. Ces mutations sont généralement homozygotes. De plus des amplifications du chromosome 1q ont été retrouvées dans 18 % des leucémies post-NMP alors qu'elles sont présentes dans 0,2 % des cas de NMP chroniques. La région minimale amplifiée sur ce chromosome comporte *MDM4*, un inhibiteur de l'activité transcriptionelle de P53, souvent amplifié dans différents types de cancers. L'amplification de *MDM4* et les mutations de *P53* sont donc des évènements mutuellement exclusifs, mettant en exergue un rôle important de cet axe dans la transformation des NMP en LAM.

Le facteur de transcription **RUNX1** (AML1 ou encore CBFA2) ainsi que CBFβ (*Core-Binding Factor Beta*), son partenaire hétérodimérique, compose le CBF (*Core Binding Factor*), est essentiel à l'hématopoïèse définitive. La dérégulation fonctionnelle de RUNX1 est une des principales causes des leucémies. Certaines altérations génétiques acquises de *RUNX1* sont fréquemment retrouvées dans les LAM et les SMD. Les mutations de *RUNX1* sont rares dans les NMP, mais sont significativement associées à une progression des NMP en leucémies dans 27 à 37 % des cas [30].

IKZF1 code pour IKAROS, un facteur de transcription, impliqué dans le remodelage de la chromatine lors de la lymphopoïèse normale, ainsi que dans la myélopoïèse et l'activité des CSH. Les modèles murins déficients en *Ikaros* présentent de sévères défauts lymphoïdes entraînant des lymphomes ou des leucémies lymphoïdes. Dans une cohorte de patients atteints de NMP, les délétions d'*IKZF1* (délétion commune de del 7p) sont présentes dans moins de 1 % des NMP en phase chronique, et dans 20 % des patients atteints de leucémies post-NMP [56].

On trouve également de rares mutations d'***ETV6***, un gène membre de la famille des facteurs de transcription ETS. La partie N-terminale de cette protéine est impliquée dans les interactions protéine-protéine et la partie C-terminale permet la fixation à l'ADN. Les modèles murins KO suggèrent qu'Etv6 est nécessaire à l'hématopoïèse normale et la maintenance du développement du réseau vasculaire. Ce gène est aussi connu pour être impliqué dans de nombreux réarrangements chromosomiques associés à des leucémies. ETV6 interagit avec Grb2, FLI1 et HTATIP et peut former un gène de fusion avec ABL1 (gène ETV6/ABL1).

Autres facteurs participant à l'hétérogénéité des NMP

Des facteurs héréditaires pourraient également participer à l'initiation, au développement et à l'hétérogénéité des NMP. Des allèles de susceptibilité correspondant à des polymorphismes ou à des haplotypes contribuent au développement des NMP [57]. L'haplotype 46/1 ou GGCC favorise l'acquisition en *cis* de *JAK2*V617F et des mutations *MPL*W515 avec une pénétrance faible. Cet haplotype correspondrait à un rtQTL (*replication timing quantitative trait loci*) qui favoriserait l'utilisation d'une origine de réplication particulière et créerait des interférences entre les machineries de réplication et de transcription, entraînant l'apparition de mutations de *JAK2* [58]. L'haplotype

46/1 n'a pas d'impact sur les mutations de *CALR* [59]. Une association entre un variant intronique de *TERT (telomerase reverse transcriptase)*, une transcriptase inverse appartenant au complexe de la télomérase, et les trois types de NMP (TE, PV et MFP) a été plus récemment mise en évidence [60]. Ce variant est également associé à des taux d'hémoglobine, de plaquettes et de leucocytes plus élevés chez les sujets sains, suggérant que son rôle s'exercerait au niveau d'un progéniteur myéloïde commun. TERT rs2736100 est retrouvé indifféremment en présence de *JAK2*V617F ou de mutations de *CALR*. Plus récemment, l'association entre plusieurs SNP et l'apparition de NMP a été rapportée : rs12339666 situé dans *JAK2* et rs2201862 en aval de *MECOM* qui s'associent plus fréquemment avec les NMP *JAK2*V617F négatifs, ainsi que rs9376092, localisé entre les gènes *HBS1L* et *MYB*, qui semble favoriser les TE *JAK2*V617F [61]. Ces variants sont fréquents dans la population générale, mais leur pénétrance faible et ils n'influencent que modérément le développement des NMP. Dans des familles de NMP, il a été récemment caractérisé des facteurs de prédisposition qui contribuent de façon importante au développement des NMP. Une découverte récente du groupe de R. Kralovics dans les familles développant des myélofibroses a récemment identifié *RBBP6* comme étant un gène de prédisposition [62]. Notre laboratoire a également identifié quatre familles de même origine géographique qui se distinguent des autres cas familiaux de NMP par leurs caractéristiques cliniques. Il s'agit de grandes familles de TE présentant une évolution très active de leur pathologie vers la MFP et la leucémie. Les analyses pan-génomiques ont mis en évidence une anomalie germinale qui consiste en une région de 700 kb dupliquée en tandem, présente chez tous les sujets atteints. Cette région contient 6 gènes (*TCL1A*, *BDKRB1*, *BDKRB2*, *ATG2B*, *GSKIP*, *AK7*) dont 2 (*ATG2B*, *GSKIP*) sont exprimés dans l'hématopoïèse myéloïde et sont impliqués dans le développement de ces maladies très évolutives [63].

Traitement des NMP

Plusieurs traitements sont utilisés dans les TE, PV, MFP afin de contrôler les complications thrombo-emboliques. Les antiagrégants plaquettaires sont préconisés ou bien des saignées itératives dans les cas de PV pour diminuer l'hématocrite. L'hydroxyurée (Hydrea®) est utilisée dans tous les cas de NMP à risque élevé de complications pour diminuer la prolifération cellulaire et donc la leucocytose, la thrombocytose, et la polyglobulie [64]. Ces traitements agissent donc sur les paramètres sanguins, mais n'ont pas apparemment d'effet sur la maladie elle-même ou sur les anomalies moléculaires. Le risque du traitement par l'Hydréa® d'augmenter la fréquence des leucémies à long terme est discuté alors que l'augmentation des leucémies par le pipobroman, le chloraminophène ou le Phosphore radioactif a été démontrée si bien que ces médicaments sont sortis de l'arsenal thérapeutique habituel des NMPs. L'anagrélide conserve des indications dans les TE avec des thrombocytoses importantes.

Etant donné que toutes ces maladies sont dues à l'activation de la voie JAK2/STAT, des inhibiteurs de JAK2 ont été plus récemment développés par de nombreuses compagnies pharmaceutiques. Ces inhibiteurs pharmacologiques de spécificité variable pour JAK2 et non sélectif de JAK2V617F ont été utilisés dans les MFP, puis dans les PV et les TE graves. Le ruxolitinib est le seul médicament à avoir obtenu l'autorisation de mise sur le marché en Europe et aux USA [65]. Un certain nombre d'autres inhibiteurs ont été arrêtés pour toxicité neurologique et cardiaque comme le fédratinib ou le pacritinib. Le ruxolitinib réduit beaucoup les symptômes de la maladie dans les MFP, en particulier la taille de la rate et la cachexie et augmente la qualité de vie. Ces effets semblent être liés aux effets sur le syndrome inflammatoire associés aux NMP. Ce syndrome est la conséquence de la sécrétion de cytokines inflammatoires par les cellules du clone et par des cellules n'appartenant pas aux clones à la fois hématopoïétiques comme les cellules du système immunitaire ou non hématopoïétiques. Ces cytokines comme par exemple l'IL-6 induisent un syndrome inflammatoire via les voies JAK1/JAK2/STAT3 si bien que l'inhibition simultanée de JAK1 et JAK2 par le ruxolitinib explique ces effets anti-inflammatoires [66]. Même s'ils affectent surtout les symptômes, ces inhibiteurs agissent aussi sur le clone muté, mais après de nombreuses années de traitement. Ce traitement a été aussi utilisé chez des patients atteints de PV résistants ou intolérants à l'hydroxyurée et montre une diminution de la splénomégalie dans 40 % des cas et une normalisation du taux d'hématocrite dans 60 % des cas. Il est possible que dans les PV, le ruxolitinib ait plus d'effets sur le clone JAK2V617F que dans la myélofibrose, ce qui pourrait être expliquée par le fait que la myélofibrose est un NMP plus complexe que les PV et TE et se rapproche pour certaines d'entre elles des SMD.

D'autres molécules, notamment des inhibiteurs de télomérases tel que l'imetelstat, dont les résultats préliminaires semblent prometteurs, des inhibiteurs d'histones déacétylases (HDAC) ou d'HSP90 (*Heat Shock protein 90*), sont actuellement en cours de développement, seuls ou en association, dans la MFP [64].

L'IFNα est utilisé dans les patients TE ou PV pour réduire la thrombocytose et l'hématocrite. Il est capable d'induire non seulement une réponse hématologique, mais également une réponse moléculaire et partielle ou complète sur le clone *JAK2*V617F et sur le clone *CALR*m dans à peu près 20-30% des cas [67]. Cependant, c'est un traitement à long terme, lourd et avec des effets secondaires parfois non négligeables, même si le développement de l'IFNα pégylé les a diminués. De plus, il existe un certain nombre de patients (20 %) qui restent résistants à ce traitement. Chez la souris knock-in *Jak2V617F*, l'IFNα récapitule la réponse hématologique et moléculaire en ciblant les CSH *Jak2*V617F ce qui signifie que potentiellement un traitement par l'IFNα pourrait guérir la maladie [16]. Le mécanisme de ce ciblage reste toutefois inconnu et doit à l'avenir être compris pour permettre de trouver des alternatives à ce traitement ou diminuer la durée du traitement ou encore trouver des alternatives aux résistances.

Conclusion

La grande majorité des NMP classiques ont été identifiés comme des maladies de la voie récepteur de cytokine/JAK2/STAT même si dans 10 % des cas l'anomalie motrice n'a pas encore été découverte. La TE et la PV sont les formes les moins évoluées et les plus simples sur le plan génétique contrairement à la MFP qui présente souvent un certain nombre d'anomalies associées comme les mutations de l'épigénétique ou d'épissage. Ces maladies évoluent à plus long terme vers des leucémies secondaires ce qui font des NMP un excellent modèle pour étudier le mécanisme de leucémogenèse à des temps précoces. Un bon nombre de questions se pose encore. En particulier, comment apparaissent ces maladies ? Comment se fait l'apparition des mutations motrices et comment s'amplifient-elles au niveau des CSH pour induire une maladie ? Pourquoi les différentes mutations motrices induisent des maladies différentes ? Comment se font les coopérations entre les différentes anomalies génétiques pour induire les leucémies ? Enfin, comment des traitements comme l'IFN*a* agissent pour éventuellement guérir les malades ?

Abréviations : NMP: néoplasme myéloprolifératif; TE: thrombocytémie essentielle; PV: polyglobulie de Vaquez, MFP: myélofibrose primaire; MF: myélofibrose; CSH: cellule souche hématopoïétique, LMMC: leucémies myélomonocytaire chronique, SMD: syndrome myelodysplasique; LAM: leucémie aiguë myéloïde; IL: interleukine; CALR: calreticulin; OMS: organisation mondiale de la santé; NGS: next-generation sequencing; IFN: interferon, MPL : récepteur à la thrombopoïétine, EPOR : récepteur à l'érythropoïétine, G-CSFR : récepteur au granulocyte colony stimulating factor, JAK : Janus activated kinase, HDAC histones déacétylases, ou d'HSP90 : Heat Shock protein 90

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Rare type 1-like and type 2-like calrecticulin mutants induce similar myeloproliferative neoplasms than type 1 and type 2 mutants in mice

Running title: Functional characterization of rare calreticulin mutants

Katte Rao Toppaldoddi[*1,2,3], Maira da Costa Cacemiro[*1,2,3], Olivier Bluteau[1,2,3], Barbara Panneau-Schmaltz[1,2,3], Delphine Muller[1,2,3], Hana Raslova[1,2,3], Isabelle Plo[1,2,3], William Vainchenker[*1,2,3] and Caroline Marty[*1,2,3,4]

1 INSERM, UMR1170, Gustave Roussy, Villejuif, France
2 Université Paris-Saclay, UMR 1170, Gustave Roussy, Villejuif, France
3 Gustave Roussy, UMR 1170, Villejuif, France
4 Corresponding author
* These authors equally contribute

Corresponding author:
Caroline Marty, 114 rue Edouard Vaillant, 94805 Villejuif, France; Phone: (+33) 1 42 11 53 63; Fax: (+33) 1 42 11 52 40; Email: cmarty1@gmail.com

Key points

- CALR type 1 and type 2 mutants drive an ET-like disorder in a retroviral mouse model resembling del52 and ins5 phenotypes, respectively.
-Type 1 and type 1-like CALR mutants induce an ET evolving into MF in transplanted mice.

Abstract

Frameshift mutations in the calreticulin (*CALR*) gene have been identified in 30% of essential thrombocythemia and myelofibrosis patients. The two most frequent are *CALRdel52* (type 1) and *CALRins5* (type 2) but many other rarer mutations exist and each represents less than 2% of *CALR* mutations in patients. They are structurally classified as type 1- and type 2-like *CALR* mutations but it is not known whether they are equally functionally related to *CALRdel52* and *CALR ins5*, respectively. We modeled two type 1-like mutations, *del34* (E369fs*50) and *del46* (L367fs*48) and of one type 2-like mutation *del19* (K374fs*50) in cell lines and in mice. All CALR mutants specifically activated cytokine-independent cell growth in presence of the TPO receptor (MPL) only, and induced a constitutive activation of JAK2 and STAT5/3/1. Lethally irradiated mice that were engrafted with bone marrow transduced with the wild-type (*CALR*wt) or the different *CALR* mutations developed thrombocytosis but to a much lesser extent in presence of type 2 and type 2-like *CALR* mutations. In contrast to type 2-like mice, type 1-like mice developed a myelofibrosis after 10 months of engraftment associated with splenomegaly, a marked osteogenesis in bone marrow and a thickening of reticulin fiber network. Like observed for type 1 mutant, type 1-like *CALR* mutations induced an expansion at early stage of hematopoiesis compare to type 2 and type 2-like mutations. Thus, type 1-like and type 2-like CALR mutants structurally and functionally resemble type 1 and type 2, respectively.

Introduction

The acquisition of somatic mutations in the hematopoietic stem cell (HSC) compartment leading to the constitutive activation of the JAK-STAT signaling pathway drives the development of myeloproliferative neoplasms (MPNs).[1, 2]

Classical MPNs are characterized by the hyperplasia of one main or the three myeloid lineages. They includes three disorders, polycythemia vera (PV) that displays an increased red blood cell count, essential thrombocythemia (ET) and myelofibrosis (MF) both characterized by a megakaryocytic hyperplasia and an increased platelet count in ET and bone marrow fibrosis and granulocytic proliferation in MF. The gain-of-function mutation in *JAK2* (*JAK2*V617F) is present in the majority of PVs, ETs and MFs. The second most frequent driver mutations inducing approximately 30% of ET and MF are located in the calreticulin (*CALR*) gene.[3, 4]

More than 50 different *CALR* mutations have been reported, the two most frequent ones are a deletion of 52 base pairs (del52) L367fs*46 also called type 1 and an insertion of 5 base pairs (ins5) K385fs*47 or type 2. They represent together more than 80% of all *CALR* mutations.

These mutants specifically activate the thrombopoietin (TPO) receptor (MPL) in a TPO-independent manner through binding to the N-glycosylated residues of its extracellular domain.[5] The activation by itself requires the new C-terminus domain of CALR mutants.[6, 7]

In retroviral mouse models, we previously showed that CALR mutants induce a thrombocytosis, but to a lesser extent in presence of ins5 compared to del52.[8] Moreover, del52 mice evolved more frequently into MF than ins5 mice, mimicking what is observed in patients.[9,10] Indeed, *del52* and *ins5* are both frequent in ET whereas *del52* is largely predominant in MF.[11, 10, 12]

Other mutants of CALR each represent 0.3 to 1.7% of CALR mutations in MPNs. They have been classified as type 1- or type 2-like mutations according to the absence or presence of a residual wild-type calcium-binding motif and the modification of the a helix structure.[10, 13]

This classification based on the structure of the CALR mutants appears to correlate with the phenotype of the patients, type 1 and type 1-like mutants being more predominant in MF and associated with a better prognosis in MF.[14] However there is no direct demonstration that type 1-like and type-like 2 mutants have different pathogenic capacities as shown for type 1 (del52) and type 2 (ins5) mutants.[8]

In this work, we investigated whether the oncogenic activity of two type 1-like mutations, *del34* (E369fs*50) and *del46* (L367fs*48) and of one type 2-like mutation *del19* (K374fs*50) was mediated by MPL, like *de52* and *ins5*. In Ba/F3 cell lines, all mutants activated MPL-

dependent JAK/STAT signaling pathway to a similar extent and in a TPO-independent fashion. We verified whether the structural-related classification of rarer CALR mutants could be confirmed *in vivo* in mice. We developed retroviral mouse models of *del19*, *del34* and *del46*. Mice were followed for up to 10 months and hematopoiesis was analyzed in bone marrow and spleen and compared to *del52*, *ins5* and wild-type *CALR* (*CALR*wt) models. We demonstrate that *del34* and *del46* developed thrombocytosis of similar severity with a progression to MF than *del52* whereas *del19* phenotype was closed to *ins5*.

Methods

Retroviruses

Human *CALRdel52*, *CALRins5*, *CALRdel19*, *CALRdel34*, *CALRdel46*, *CALRdel9* and *CALR*wt were inserted into a pMSCV-IRES-GFP retroviral vector. *CALRdel9* has ben described in some patients but appear more as a polymorphism. Vesicular stomatitis virus glycoprotein (VSVG) pseudotyped viral particles were produced into 293EBNA cells as described previously (marty et al Blood 2014).

Modeling in Ba/F3 cells

As described previously, Ba/3 cells were transduced to express human receptors to TPO (FLA-tagged MPL), to erythropoietin (HA-tagged EPOR) and to the granulocyte colony-stimulating factor (G-CSFR) as well as the various human CALR constructs.

Clonogenicity assay was conducted by sorting one cell/well in a 96-well plate in the presence of the absence of IL-3. Colony-forming cells were numbered after a week of culture.

Viability assay using the Premix WST-1 Cell Proliferation Assay System (Clontech) was conducted to measure dose-dependent cell proliferation to TPO.

Western blot served to assess MPL signaling using Abs against the phosphorylated forms of JAK2 (Tyr 1054/1055), STAT1 (Tyr701), STAT3 (Tyr705) and STAT5 (Tyr694), extracellular signal-regulated kinase 1/2 (ERK1/2 Thr202/Tyr204) and AKT (Thr308) and against the pan forms (Cell Signaling Technology, Ozyme). MPL was revealed using an Ab from Upstate (Millipore), anti-total CALR Ab was from abcam and anti-β-Actin Ab was from Sigma and serves as a loading control.

Bone marrow transduction and engraftment

All procedures were approved by the Gustave Roussy Ethics Committee (protocol 2016-066-7171). Bone marrow (BM) was collected from donor C57bL/6 mice (Janvier). Lineage-

negative (Lin^-) cells were purified and cultured for 2 days in presence of a cocktail of cytokines (SCF, IL-3, TPO, FLT3L, IL-6) before infection with the different viruses. Approximately 4 X 10^5 transduced cells were injected intravenously into lethally irradiated (9.5Gy) recipient mice.

Analysis of mice

Blood was collected in citrated tubes and red blood cell (RBC), white blood cell (WBC) and platelet counts were measured using an automated counter (MS9, Schloessing Melet). BM cells were collected by flushing both femurs and tibias. Spleen single-cell suspensions were prepared.

For *histology analysis*, tibia and spleen sections were fixed in 4% formaldhehyde, decalcified and paraffin embedded. Sections were stained with hematoxylin/eosin/safran (HES) for cytology, megakaryocytes (MK) and reticulin fibers were revealed with Von Willebrand factor immunostaining and Gordon and Sweet silver staining, respectively.

MK progenitor cell (CFU-MK) assays were carried out in duplicate by plating BM and spleen nucleated cells in serum-free fibrin clot assays with SCF, IL-6 and the absence or the presence of TPO. CFU-MK-derived colonies were revealed at day 7 by acetylcholinesterase staining.

Flow cytometry (LSRFortessa, FACSCanto I, BD Biosciences) was used to determine the percentages of green fluorescent protein (GFP)-positive infected cells and cell content of BM and spleen with appropriate antibodies (Abs). Lin- cells were negatively selected with allophycocyanin-conjugated anti-CD3, B220, Gr-1, Mac-1 and Ter-119 Abs, $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ (LSK) population was stained with Abs against Sca-1 (phycoerythrin-Cy7) and c-Kit (PerCP-Cy5.5) and the $LSKCD48^-CD150^+$ (SLAM) population enriched in HSCs was isolated with anti-CD48 (Pacific Blue) and CD150 (Brilliant Violet 510) Abs. Anti-CD41 (phycoerythrin) was used to label MK progenitor (MkP) cells with the $LKSca\text{-}1^-CD150^+CD41^+$ combination. BM and spleen precursors were labeled with fluorochrome-conjugated Abs against CD41 and CD42 (megakaryocytic lineage), CD71 and Ter-119 (erythroid lineage), Mac-1 and Gr-1 (granulocytic lineage), B220 and CD3 (lymphoid lineages).

Results

Rare mutants of CALR specifically activate the thrombopoietin receptor MPL

Rare mutants of CALR have been structurally classified as type 1- and type 2-like based on structural characteristics with the 2 most frequent CALRdel52 (type 1) and CALRins5 (type 2) prototypes (Figure 1A).[10] In order to test whether this classification could also be verified

functionally, we modeled three *CALR* mutations, two type 1-like, *del34* and *del46*, and one type 2-like, *del19*, in Ba/F3 cell lines that expressed or not either the receptor to erythropoietin (EPOR), the granulocyte-colony stimulating factor (G-CSFR) or MPL. *CALRdel9*, an in frameshift deletion, was also tested. CALR mutants resulted in cytokine-independent growth in around 100% of the Ba/F3 cells expressing MPL assayed by limited dilution experiments with no significant differences between mutants (Figure 1B). CALR mutants did not induce autonomous growth of parental or EPOR- or G-CSFR-expressing Ba/F3 cells. As expected, the CALRdel9 mutant devoid of a +1 frameshift did not induce any cytokine-independent growth whatever the receptors (MPL, EPOR and G-CSFR).

We assessed the activation of MPL signaling induced by CALR mutants in Ba/F3-MPL cells by Western blot (Figure 2A). All CALR mutants tested induced constitutive activation of JAK2 and STAT1, 3 and 5 compared to CALRwt. There was a slight induction of ERK phosphorylation with CALR mutants, but no significant activation of the PI3K/AKT pathway. Addition of TPO provided only a minor increase in the phosphorylation of these proteins compared to CALRwt except in cells expressing del19 that were more sensitive to TPO stimulation. Moreover, a MTT-like assay showed that independently of the CALR mutant, Ba/F3 cells presented a TPO-independent proliferation (Figure 2B).

Type 1- and type 2-like CALR mutants induce thrombocytosis in mice resembling del52 and ins5 phenotypes, respectively

We have previously shown that del52 and ins5 CALR mutants recapitulate an ET-like phenotype *in vivo* in a retroviral mouse model, but with different intensity and progression into MF.[8] To examine whether del19 behaves like ins5 and both del34 and del46 like del52 in mice, we performed bone marrow transplantation (BMT) after retroviral transduction of either *CALR*wt or the respective *CALR* mutations into Lin^- BM cells. We engrafted a 40-60% of GFP^+ cell population representing the percentage of initial $CALR^+$ mutated cells. Type 1-like mutants induce a strong thrombocytosis in mice compared to CALRwt whereas platelet counts only slightly raised above the control in *ins5* and *del19* mice (Figure 3).

Type 1-like CALR mutants develop a post-ET myelofibrosis

Mice expressing type 1-like CALR mutants of ages above 9 months post-BMT presented an significant increase in spleen weight (Figure 4A) associated with a decrease in BM cellularity (Figure 4B) compared to CALRwt and type 2-like mutant mice. Histopathology of BM (Figure 4C) and spleen (data not shown) revealed expanding osteogenesis in BM associated

with clusters of megakaryocytes (MK) in BM and spleen in type 1-like *CALR* mutated mice. Silver staining revealed thickening of the reticulin fiber network in both BM and spleen, while no fibrosis was observed in *CALR*wt, *ins5* or *del19* mice.

Type 1-like *CALR* mutations lead to an early clonal advantage

We engrafted approximately 40-60% of retrovirally transduced GFP^+ cells in recipient mice. After 9 months, 100% of the HSC-enriched cell population (SLAM) were GFP^+ in BM of type 1-like *CALR* mutated mice while *CALR*wt and type 2-like *CALR* mutated mice remained to levels similar to the initial graft (Figure 5A). Accordingly, there was an increase in frequency of SLAMs (Figure 5B) confirmed in early hematopoietic progenitor LSKs (Figure 5C) in BM of type 1-like *CALR* mutated mice. These data suggest that *CALR* type1-like mutations provide a strong competitive advantage to the mutated clone in wild-type hematopoiesis at the HSC level. This clonal advantage was visible in blood with WBCs of almost exclusively virus-transduced origin, especially for del52 (Figure 5D).

Type 1-like *CALR* mutations induce an early amplification of the MK lineage while type 2-like mutations induce a late platelet amplification

As expected, type 1-like *CALR* mutations provided a competitive advantage to the MK lineage with 100% of the megakaryocytic progenitors (MkPs) detected in BM being GFP^+ at 9 months post-BMT (Figure 6A). This was accompanied with an increased frequency of MkPs and MKs ($CD41^+CD42^+$) in BM of type 1-like *CALR* mutated mice (Figures 6B and C). Interestingly, after BMT there was a rapid and strong increase in the percentages of GFP^+ platelets detected in blood of type 1-like *CALR* mutated mice and a mild one in type 2-like *CALR* mutated mice (Figure 6D). This suggests that type 2-like mutants induce a very late amplification in megakaryopoiesis, only visible at the late stages of megakaryopoiesis. Moreover, MK progenitors (CFU-MKs) expressing the type 1- and type 2-like CALR mutants displayed independency and hypersensitivity to TPO that however was not significant compared to the control (Figure 6E).

Discussion

In this study we demonstrate that the structurally-based classification of rare mutants of CALR, related to the two most frequent mutations found in patients, *del52* and *ins5*, can be functionally validated in cell lines and *in vivo* in mice. The main structural difference between the two types of mutants is the retention of a wild-type residual calcium-binding motif in ins5

that is absent in del52. [3, 4, 10] Bioinformatic studies have also shown that the type 1 mutant has more altered structural properties than type 2 mutant.[13]

Using Ba/F3 cell line models we demonstrated that MPL is essential in autonomous cell growth and in activation of the JAK2/STAT signaling axis whatever the mutant used further suggesting that he electrostatic charge of the C-terminus is the common feature responsible for MPL activation.[6, 7, 15]

In addition, all CALR mutants activate the MPL/JAK2 pathway to the same extent in this cell model. This may suggest that the differences between type 1- and type 2-like mutants are not related to the level of MPL/JAK2 activation. Knowing that the main difference between type 1 and type 2 mutants concerns the size of the residual polypeptides arising from exon 9, a likely hypothesis is that the phenotypic difference is related to calcium binding and signaling although endogenous CALRwt is normally expressed in the cells as suggested in patient megakaryocytes.[10] Another hypothesis to explain phenotypic differences may be related to differences in the structure of the 2 types of mutants that may modify their direct interaction with MPL or also by differences in the secretion of the CALR mutants. Indeed, experiments in cell lines show that the great majority of CALR mutants is secreted explaining that a low level of the protein is detected in the cytoplasm by Western blot experiments. There are some evidence for both hypothesis. The type 1 mutation is capable to activate the G-CSFR *in vitro* at a much higher extent than type 2 mutation.[5] In Ba/F3 cells expressing N-terminus tagged CALR mutants, we have preliminary results showing that type 2 mutant is more secreted than type 1 mutant. In the present retroviral model in Ba/F3 cells, subtle differences may be minimized by the retroviral-induced overexpression. Thus, it will be important to precisely model and study the interaction of the different CALR mutants with MPL and to investigate in details if there are differences in their capacities to be secreted.

We showed that ectopic expression of these CALR mutants in mice is sufficient to drive an ET-like phenotype evolving into MF for type 1-like *CALR* mutated mouse models. The disease was much more severe in presence of *del34* and *del46* than with *del19*. Although both type 1 and type 2-like mutations are present in the HSC compartment, only type 1-like mutations present a clonal dominance at this early stage of hematopoiesis. In previous work, we had shown that *ins5* induced a competitive advantage at late stages of megakaryopoiesis probably due to hypersensitivity to TPO of the MK progenitors. In this study, amplification of the MK lineage was moderately seen at the platelet level for *ins5* and *del19*. We detected no significant increase in MK frequency in BM. One of the main differences between these two studies might rest on differences in the percentages of GFP^+ engrafted cells that were around

40% here and above 50% in previous experiment. Thus, a minimal level of *CALRins5* clones might be necessary to induce a more robust phenotype in mice. Indeed, when *CALRins5* patients develop disease, allele burden has often reached a 50% level (100% heterozygote mutated clones). It will be important to engraft 100% of mutated *ins5* clones in mice to test whether when clonal dominance is reached, mice develop a more severe thrombocytosis.

The present retroviral mouse models have some similarities but also some divergences with the human diseases. In both human and mouse, the type 1 and type1-like l *CALR* mutations lead to an ET with a frequent progression to MF whereas type 2 and type-2 like mutants are mainly responsible for a thrombocytosis. In contrast to mouse, the human type 2 or type-2 like human disease is associated with an important thrombocytosis and a similar clonal dominance at the level of HSC. It remains to determine whether these differences are related to the species or to the use of retrovirus. Results obtained with *CALRdel52* transgenic mice[16] as well as with *CALRdel52* knock-in mice (unpublished result) suggest that CALR mutants overall induce a milder myeloproliferative disorder than in human. However, the present retroviral mouse models strongly suggest that type 1 and type 2 mutants have stronger oncogenic activities than type 2 and type 2-like mutants. This seems to be interdiction with the human disease where only type 1 and type1-like MF have an improved prognosis. As it has been demonstrated that the prognosis of MF is in great part dependent on the number of driver mutations and also on their types, our results may suggest that the good prognosis of *CALR* type 1 and type 1-like mutations are related to the fact that it may be the natural history of the disease whereas type 2 or type-2 like mutants require several additional mutations to induce a MF. In a similar manner, *ins5* and *del19*, like *JAK2*V617F, might be associated to other mutations (for instance in epigenetic regulators) for emergence and dominance of the CALR-mutated clone in contrast to type 1-like mutation. This hypothesis can be easily tested in the future.

Future functional and biochemical studies will be required to precisely understand the mechanisms responsible of the phenotypic differences between type 1 and type 2 mutations.

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Authorship Contributions

I.P., C.M. and W.V. conceived and designed the study, interpreted the data and wrote the paper. K. R. T. and M.C.C. performed cell lines and mouse experiments, respectively and analyzed the data. O.B. cloned the *CALR* constructs. B.P-S. contributed to mouse analysis and D.M. did engraftment. H.R. gave essential inputs and wrote the paper.

Disclosure of Conflict of Interest

The authors declare that there are no conflicts of interest.

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Figure Legends

Figure 1. Requirement of MPL for type 1-like and type 2-like mutants of CALR to induce cytokine-independent growth of Ba/F3 cells. (A) Schematic representation of the C-terminal type 1- and type 2-like protein sequences of the CALR mutants. The underline 36-amino acid sequence is common to all mutants. +1 framshift results is the new sequence shown in bold, compared to wild-type sequence. In the square is the EEED calcium-binding

motif retained in type 2-like but not in type 1-like CALR mutants. **(B)** Ba/F3 parental cells were transduced to express EPOR, the G-CSFR or MPL and each of the CALR mutants del19, del34 and del46 or wild-type CALR (wt). Cells were sorted at one cell per well in a 96-well plate in the presence (plain) or not (shaded) of IL-3. Cells were grown for a week before counting the colony-forming cells. Clonogenicity is expressed in percentages of seeded cells. Histograms shown are representative of 2 independent experiments.

Figure 2. Activation of MPL signaling by CALR mutants. (A) Ba/F3-MPL cells expressing the different CALR mutants were serum- and cytokine-straved for 5 hours prior to 10 minutes stimulation with 10 ng/mL TPO. Western blot of extracts shows the phosphorylation status of JAK2, STATs, AKT and ERK with the respective antibodies. Expression of β-actin was used as a loading control and blots are representative of a typical experiment. **(B)** The cells were plated at a density of 25,000 cells/mL in the absence or the presence of increasing doses of TPO (0.005, 0.015, 0.05, 0.15, 0.5, 1.5, 5 and 10 ng/mL) for 48 hours and live cells were determined using the WST-1 proliferation assay. Dose-response curves are means of percent of viability of the maximum growth ± SEM (n=3 in triplicate). With Bonferroni's multiple comparison test p was <0.01 for all CALR mutants.

Figure 3. Type 1-like CALR mutants induce a stronger essential thrombocytosis-like phenotype *in vivo* in mice than type 2-like CALR mutants. Platelet, red blood cell (RBC) and white blood cell (WBC) counts were determined in *CALR*wt (n=5), *CALRdel52* (n=5), *CALRins5* (n=5), *CALRdel19* (n=6), *CALRdel34* (n=8) and *CALRdel46* (n=8) recipient mice, during 8 months after bone marrow transplantation (BMT). Results are expressed as mean values ± SEM. Bonferroni's multiple comparison test: *p<0.05, **p<0.001, ***p<0.001

Figure 4. BM and spleen features. (A) Spleen weight and **(B)** bone marrow (BM) cellularity (n=4-5), expressed as mean ± SEM. **(C)** Hematoxylin-eosin-safran (HES), von Willebrand factor staining (vWF) and reticulin silver stain (RET) of ten-month-old mouse BM of type 1-like *CALR* mutated mice show clusters of MKs, osteosclerosis and a progressive thickening of the reticulin network. Images were obtained using a DM2000 Leica microscope and a DFC300FX Leica camera with Leica Application Suite v.2.5,OR1 acquisition software (2.5X, 10X and 25X magnifications).

Figure 5. Amplification of hematopoietic stem cell compartment in mice. (A) Percentages of GFP^+ SLAM cells in BM. Frequency of BM **(B)** SLAM and **(C)** LSK of CALRwt and CALR mutant origins 9 months after BMT. Results are expressed as mean values ± SEM. T-test: *<0.05, **<0.01, (n=4-5). **(D)** Evolution of the percentage of GFP^+ WBC in blood, n=5-8. Bonferroni's multiple comparison test: *p<0.05, ***p<0.0001.

Figure 6. Amplification of the megakaryocytic lineage. (A) Percentages of GFP^+ MkP in BM and frequency of BM **(B)** MkP cells and **(C)** $CD41^+CD42^+$ cells of CALRwt and CALR mutant origins, after 9-month BMT (n=4-5, T-test: **<0.01, ***<0.001). **(D)** Evolution of GFP^+ platelets in blood (n=5-8, Bonferroni's multiple comparison test: **p<0.001, ***p<0.0001). **(E)** Frequencies of BM and spleen megakaryocytic progenitor (CFU-MK) colonies growing in the presence of SCF, IL-6 and with (left panel) or without TPO (right panel). Results are means ± SEM. t-test: *p<0.05, **p<0.001, ***p<0.0001, n=4-5.

A

CALR wt -----MKDKQDEEQRLKEEEEDKKRKEEEEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL

CALR ins5 -----MKDKQDEEQRLKEEEEDKKRKEEEEAED**NCRRMMRTKM**<u>**RMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA**</u>

CALR del19 -----MKDKQDEEQRLKEEEED**RRQRTRRMMRTKM**<u>**RMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA**</u>

CALR del34 -----MKDKQDEEQRLK**RRQRTRRMMRTKM**<u>**RMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA**</u>

CALR del52 -----MKDKQDEEQR**TRRMMRTKM**<u>**RMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA**</u>

CALR del46 -----MKDKQDEEQR**QRTRRMMRTKM**<u>**RMRRMRRTRRKMRRKMSPARPRTSCREACLQGWTEA**</u>

B

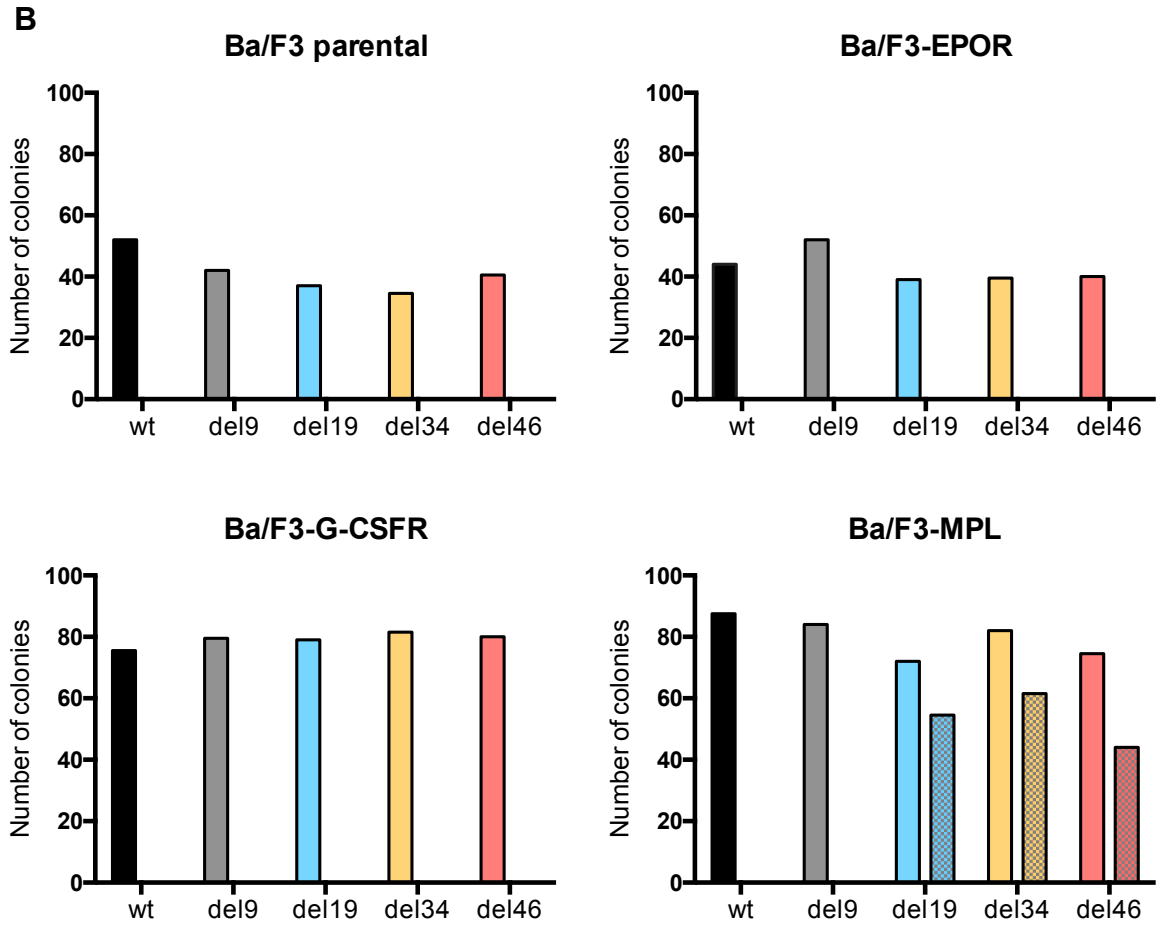

Figure 1

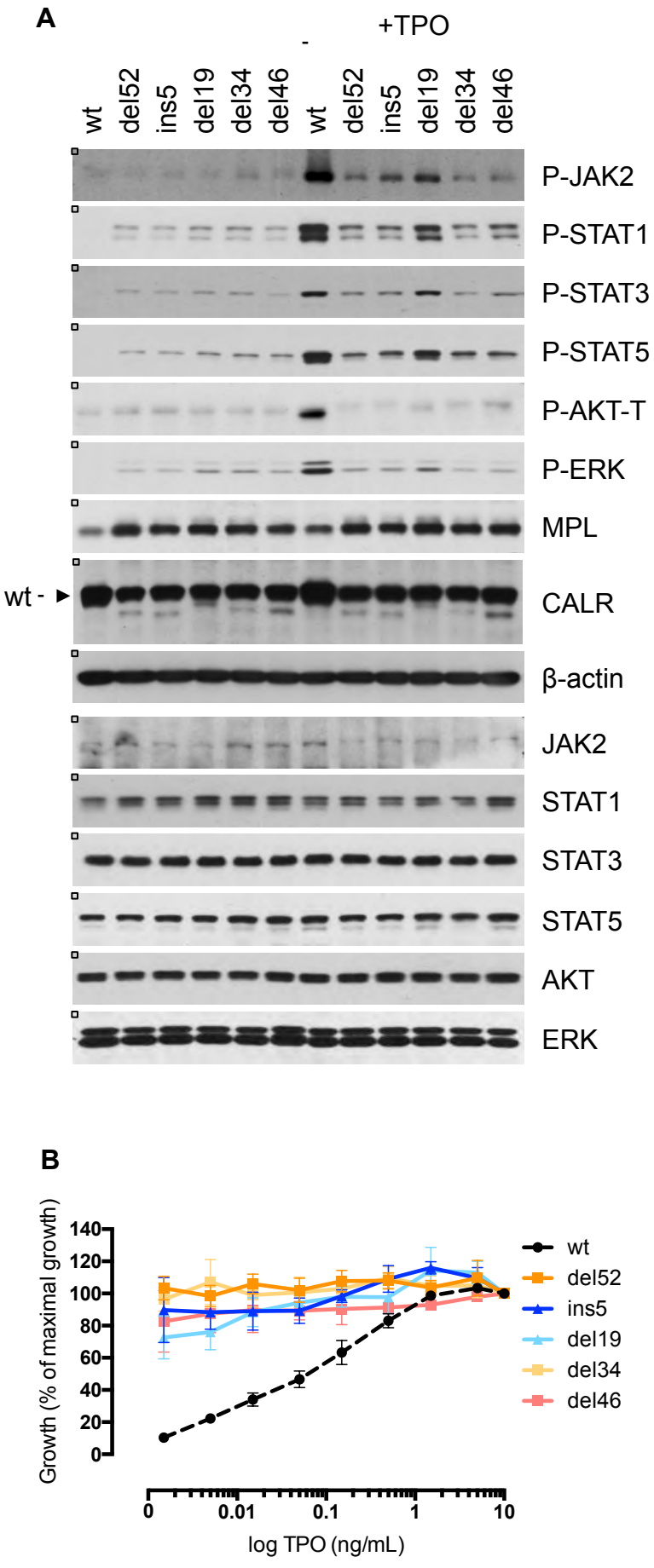
A
-
+TPO
wt
del52
ins5
del19
del34
del46
wt
del52
ins5
del19
del34
del46
P-JAK2
P-STAT1
P-STAT3
P-STAT5
P-AKT-T
P-ERK
MPL
wt
CALR
β-actin
JAK2
STAT1
STAT3
STAT5
AKT
ERK
B
Growth (% of maximal growth)
140
120
100
80
60
40
20
0
0
0.01
0.1
1
10
log TPO (ng/mL)
wt
del52
ins5
del19
del34
del46

Figure 2

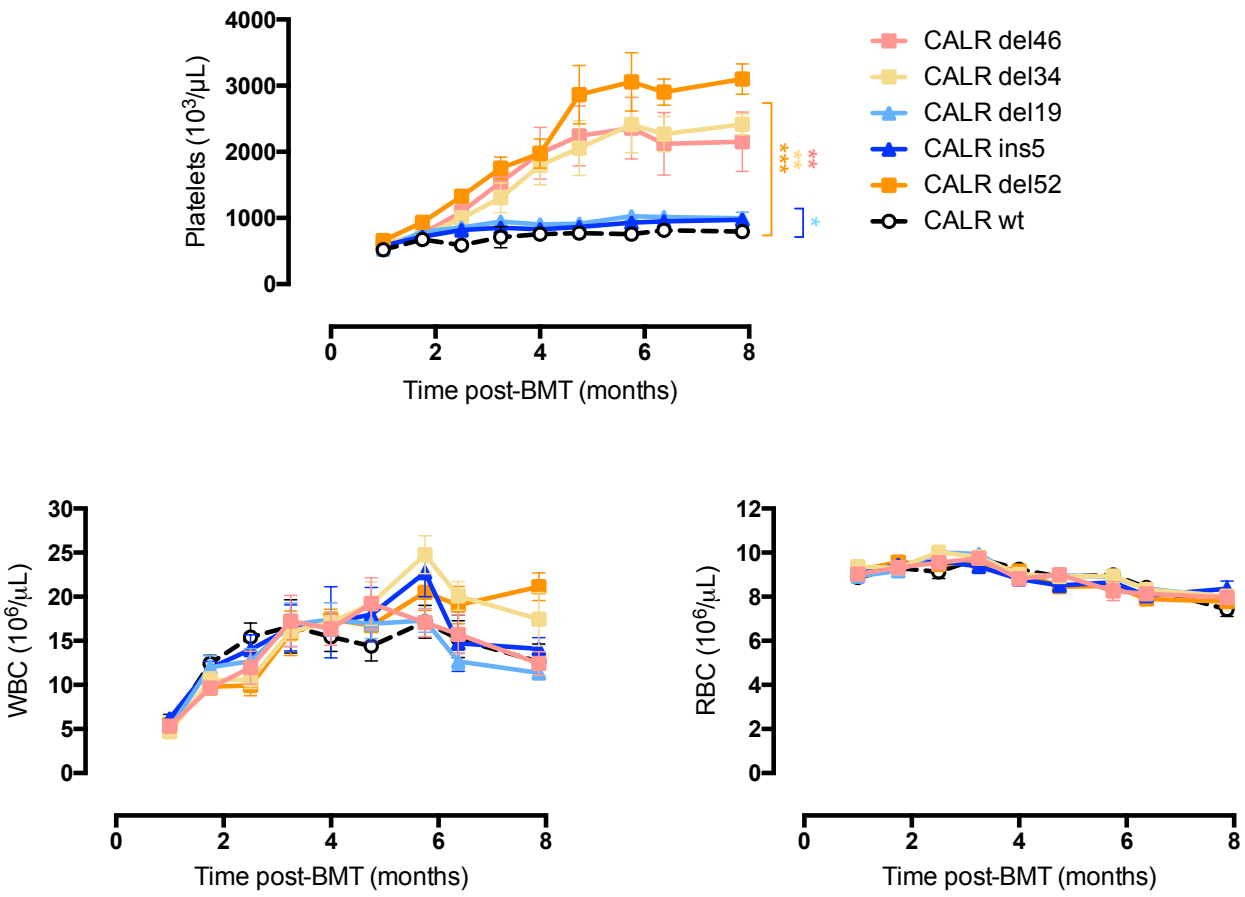
Platelets (10^3/µL)
4000
3000
2000
1000
0
0
2
4
6
8
Time post-BMT (months)
CALR del46
CALR del34
CALR del19
CALR ins5
CALR del52
CALR wt
***
**
**
*
WBC (10^6/µL)
30
25
20
15
10
5
0
Time post-BMT (months)
RBC (10^6/µL)
12
10
8
6
4
2
0
Time post-BMT (months)

Figure 3

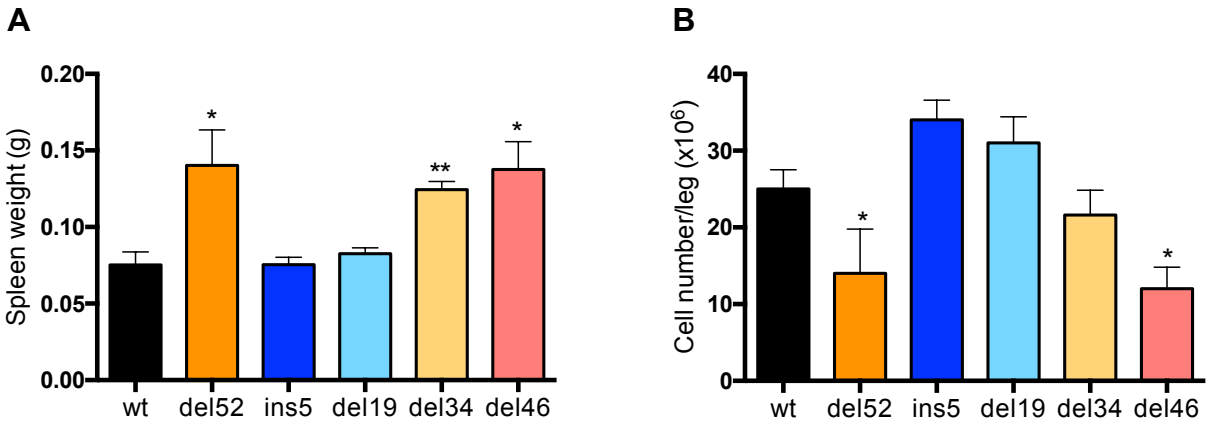
A
Spleen weight (g)
0.20
0.15
0.10
0.05
0.00
*
**
*
wt
del52
ins5
del19
del34
del46
B
Cell number/leg (x10^6)
40
30
20
10
0
*
*
wt
del52
ins5
del19
del34
del46

Figure 4

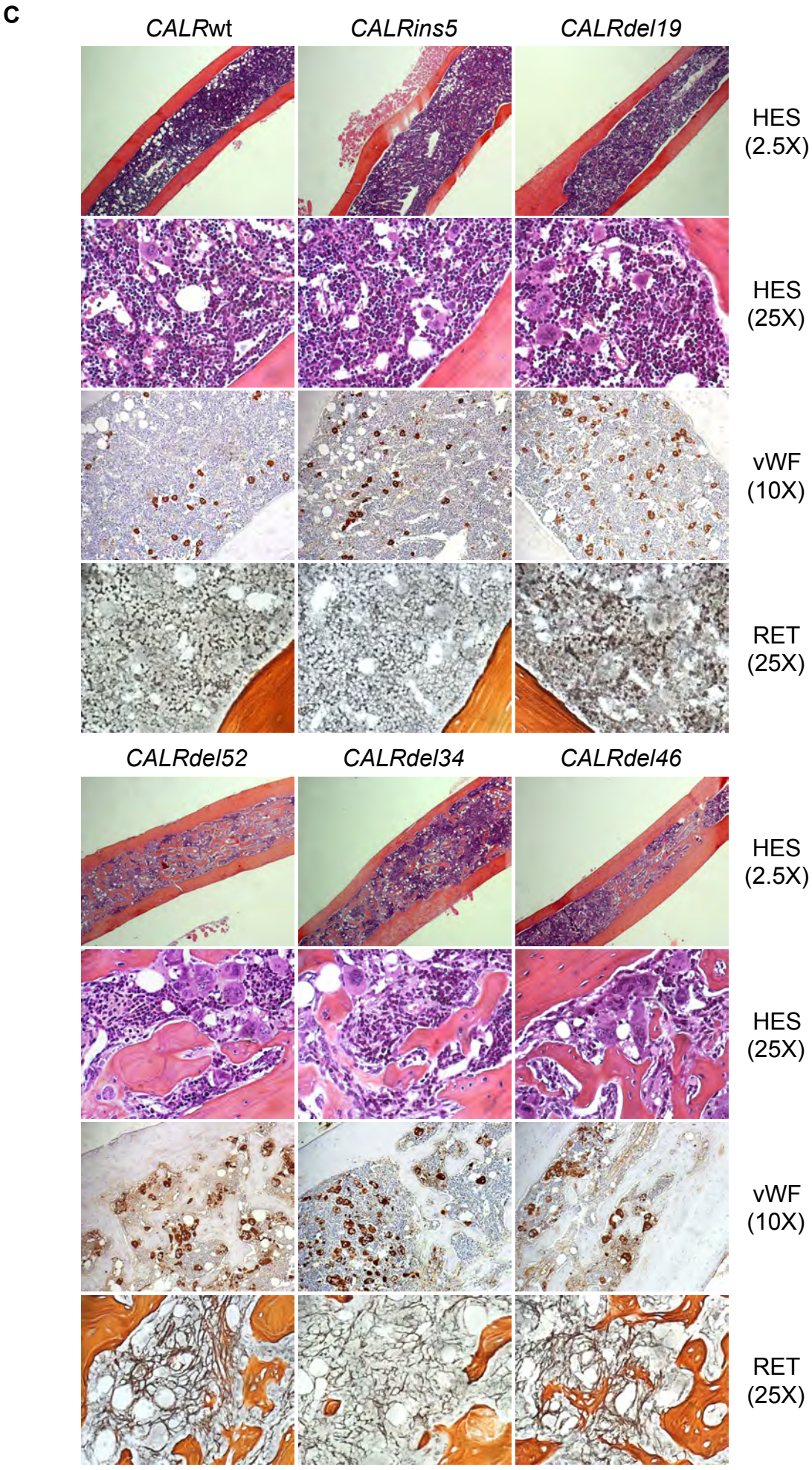
C
CALRwt
CALRins5
CALRdel19
HES (2.5X)
HES (25X)
vWF (10X)
RET (25X)
CALRdel52
CALRdel34
CALRdel46
HES (2.5X)
HES (25X)
vWF (10X)
RET (25X)

Figure 4

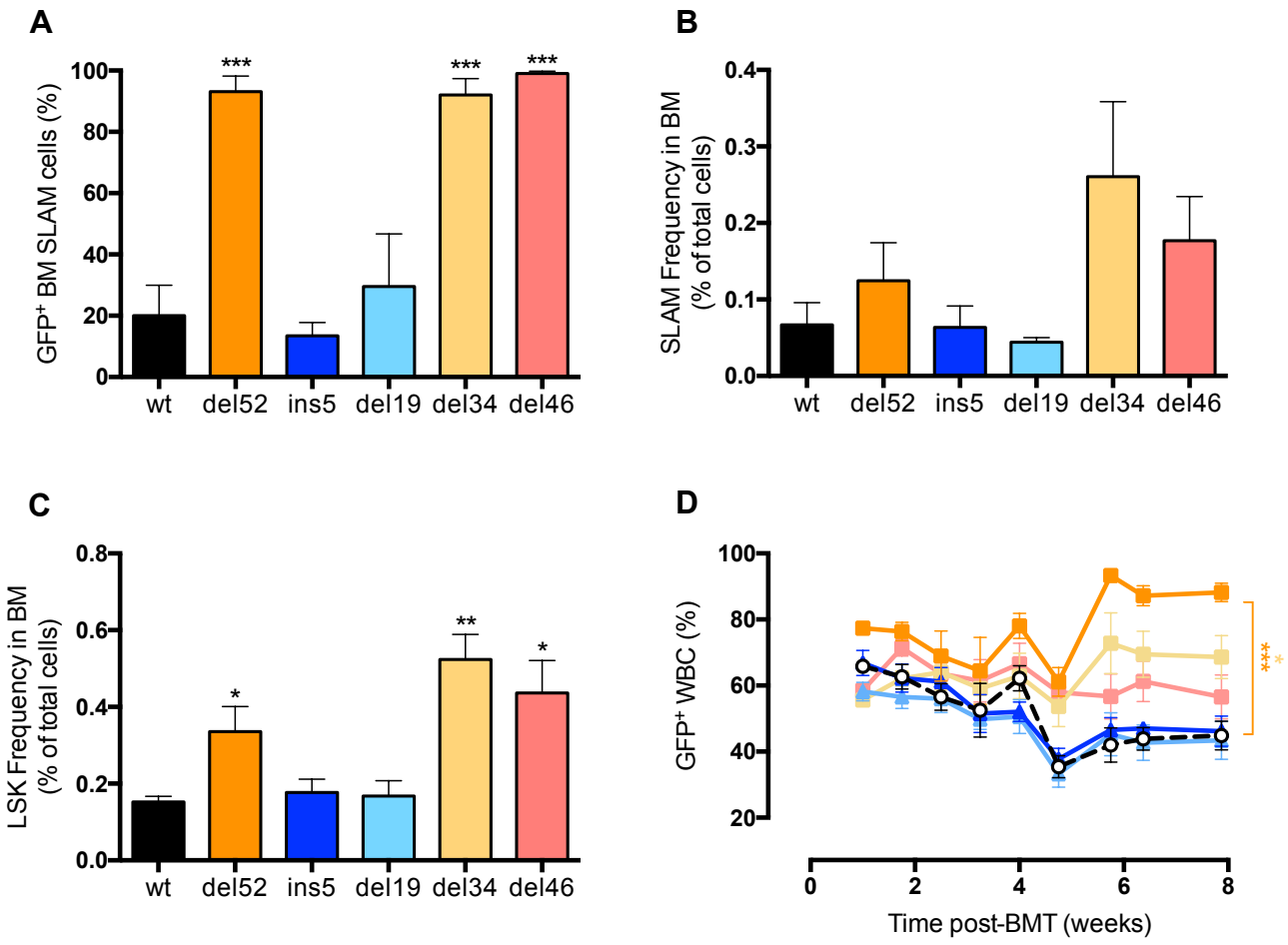
A
GFP+ BM SLAM cells (%)
100
80
60
40
20
0
***
***
***
wt
del52
ins5
del19
del34
del46
B
SLAM Frequency in BM
(% of total cells)
0.4
0.3
0.2
0.1
0.0
wt
del52
ins5
del19
del34
del46
C
LSK Frequency in BM
(% of total cells)
0.8
0.6
0.4
0.2
0.0
*
**
*
wt
del52
ins5
del19
del34
del46
D
GFP+ WBC (%)
100
80
60
40
20
0
2
4
6
8
Time post-BMT (weeks)
***
*

Figure 5

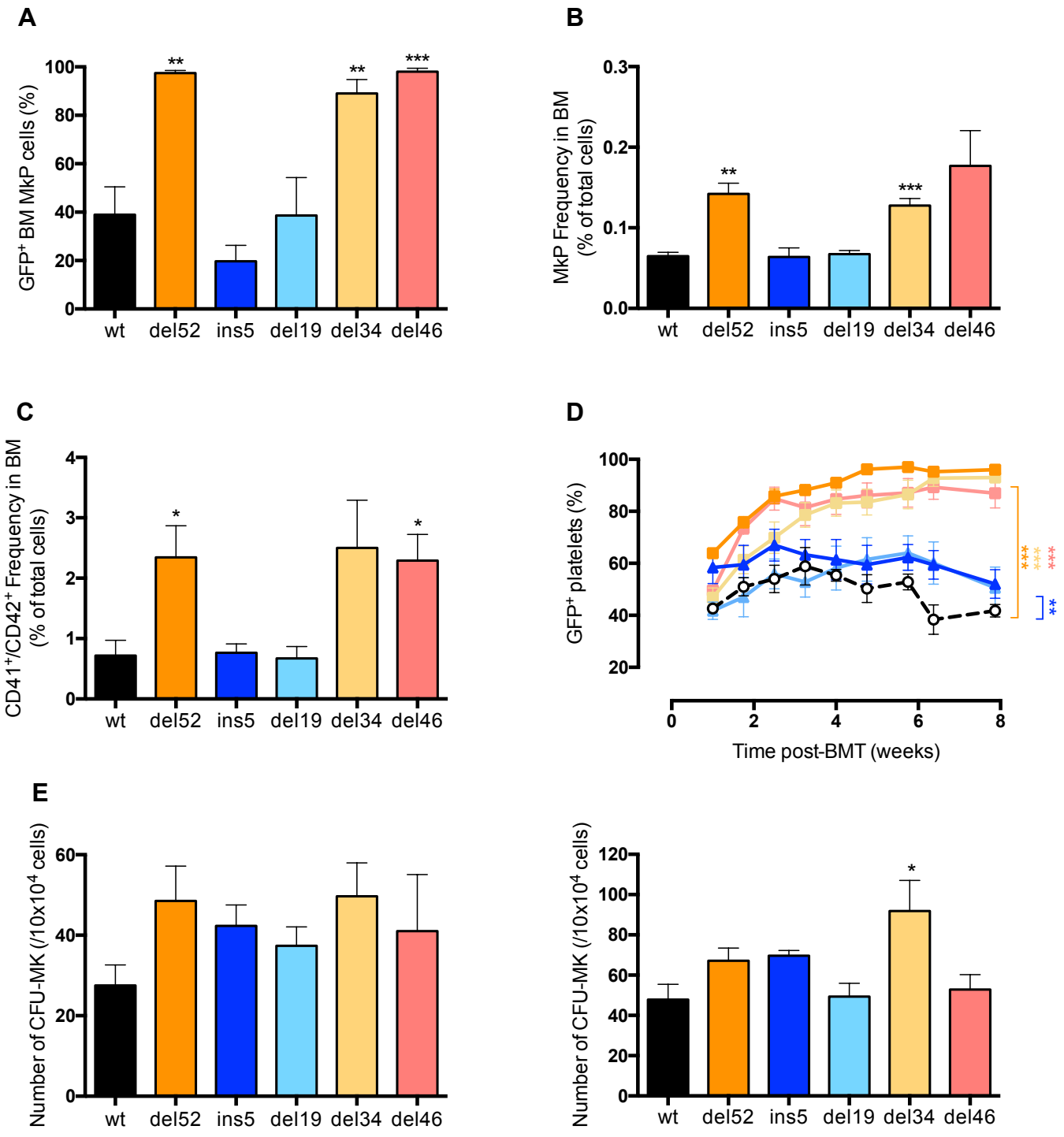
A
GFP+ BM MkP cells (%)
100
80
60
40
20
0
**
**
***
wt
del52
ins5
del19
del34
del46
B
MkP Frequency in BM
(% of total cells)
0.3
0.2
0.1
0.0
**
***
wt
del52
ins5
del19
del34
del46
C
CD41+/CD42+ Frequency in BM
(% of total cells)
4
3
2
1
0
*
*
wt
del52
ins5
del19
del34
del46
D
GFP+ platelets (%)
100
80
60
40
20
0
2
4
6
8
Time post-BMT (weeks)
***
***
***
**
E
Number of CFU-MK (/10x10^4 cells)
60
40
20
0
wt
del52
ins5
del19
del34
del46
Number of CFU-MK (/10x10^4 cells)
120
100
80
60
40
20
0
*
wt
del52
ins5
del19
del34
del46

Figure 6