

Functional Consequences of Mutations in Myeloproliferative Neoplasms

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Abstract

Driver mutations occur in Janus kinase 2 (*JAK2*), thrombopoietin receptor (*MPL*), and calreticulin (*CALR*) in BCR-ABL1 negative myeloproliferative neoplasms (MPNs). From mutations leading to one amino acid substitution in *JAK2* or *MPL*, to frameshift mutations in *CALR* resulting in a protein with a different C-terminus, all the mutated proteins lead to pathologic and persistent JAK2-STAT5 activation. The most prevalent mutation, *JAK2* V617F, is associated with the 3 entities polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF), while *CALR* and *MPL* mutations are associated only with ET and MF. Triple negative ET and MF patients may harbor noncanonical mutations in *JAK2* or *MPL*. One major fundamental question is whether the conformations of *JAK2* V617F, *MPL* W515K/L/A, or *CALR* mutants differ from those of their wild type counterparts so that a specific treatment could target the clone carrying the mutated driver and spare physiological hematopoiesis. Of great interest, a set of epigenetic mutations can co-exist with the phenotypic driver mutations in 35%–40% of MPNs. These epigenetic mutations, such as *TET2*, *EZH2*, *ASXL1*, or *DNMT3A* mutations, promote clonal hematopoiesis and increased fitness of aged hematopoietic stem cells in both clonal hematopoiesis of indeterminate potential (CHIP) and MPNs. Importantly, the main MPN driver mutation *JAK2* V617F is also associated with CHIP. Accumulation of several epigenetic and splicing mutations favors progression of MPNs to secondary acute myeloid leukemia. Another major fundamental question is how epigenetic rewiring due to these mutations interacts with persistent JAK2-STAT5 signaling. Answers to these questions are required for better therapeutic interventions aimed at preventing progression of ET and PV to MF, and transformation of these MPNs in secondary acute myeloid leukemia.

Introduction

BCR-ABL1 negative myeloproliferative neoplasms (MPNs) are diseases of the hematopoietic stem cells (HSCs), where the acquisition of somatic mutations in Janus kinase 2 (*JAK2*), thrombopoietin receptor (*MPL*, also known as *TPOR*) or calreticulin (*CALR*) genes leads to persistent activation of the JAK2-signal transducer and activator of transcription (STAT5) pathway, resulting in clonal expansion of myeloid progenitors that no longer need cytokine activation for blood formation.^{1,2} The most prevalent mutation, *JAK2* V617F, is the basis of >95% of polycythemia vera (PV) and 60% of essential thrombocythemia (ET) and primary myelofibrosis (PMF) cases.³⁻⁶ Mutations

in *MPL* (eg, W515K/L/A/R and S505N mutants) concern 5%–10% of ET and PMF cases.⁷⁻⁹ Frameshift mutations in *CALR* are associated with 20%–25% of ET, and myelofibrosis (MF) cases and are the second leading cause of MPNs.^{10,11} Irrespective of the particular MPN-inducing mutation, one key requirement in all MPNs is pathological and persistent activation of the JAK-STAT pathway via *MPL*.¹²

MPL is crucial for HSC renewal, megakaryocyte differentiation, and platelet formation,^{12,13} and is activated by several unexpected mechanisms in MPNs. Mutations of W515 of the cytosolic juxtamembrane domain RWQFP motif and the S505N mutation in the transmembrane domain of *MPL* make the receptor constitutively active (Figure 1).^{14,15} Although it seems clear at the moment that both mutations do not operate through the same mechanisms, both act via dimerization of the transmembrane domains in an active orientation.^{19,20} Mutant forms of *CALR* induce MPNs by pathological binding to the extracellular domain of *MPL* in the endoplasmic reticulum. This causes dimerization of immature receptors, transport to the cell surface, and persistent activation (Figure 2).^{21,22} The main MPN driver mutation, *JAK2* V617F, was found to activate *MPL*, promote its degradation, and alter its traffic.^{23,24} Expression of *MPL* was reported to be absolutely required for induction of an MPN phenotype by *JAK2* V617F, including PV,^{12,25} which also requires activation of the erythropoietin receptor (EPOR). Even though all MPN drivers act through constitutive activation of *MPL*, they exhibit very different clinical phenotypes, with *JAK2* V617F positive MPN occurring at an older age with a lower allele burden than *CALR* del52 positive MPN.²⁶ It is plausible that these different phenotypes correlate with distinct receptor conformations, which are known to translate into distinct signaling outcomes for EPOR and *MPL*.^{17,18,27}

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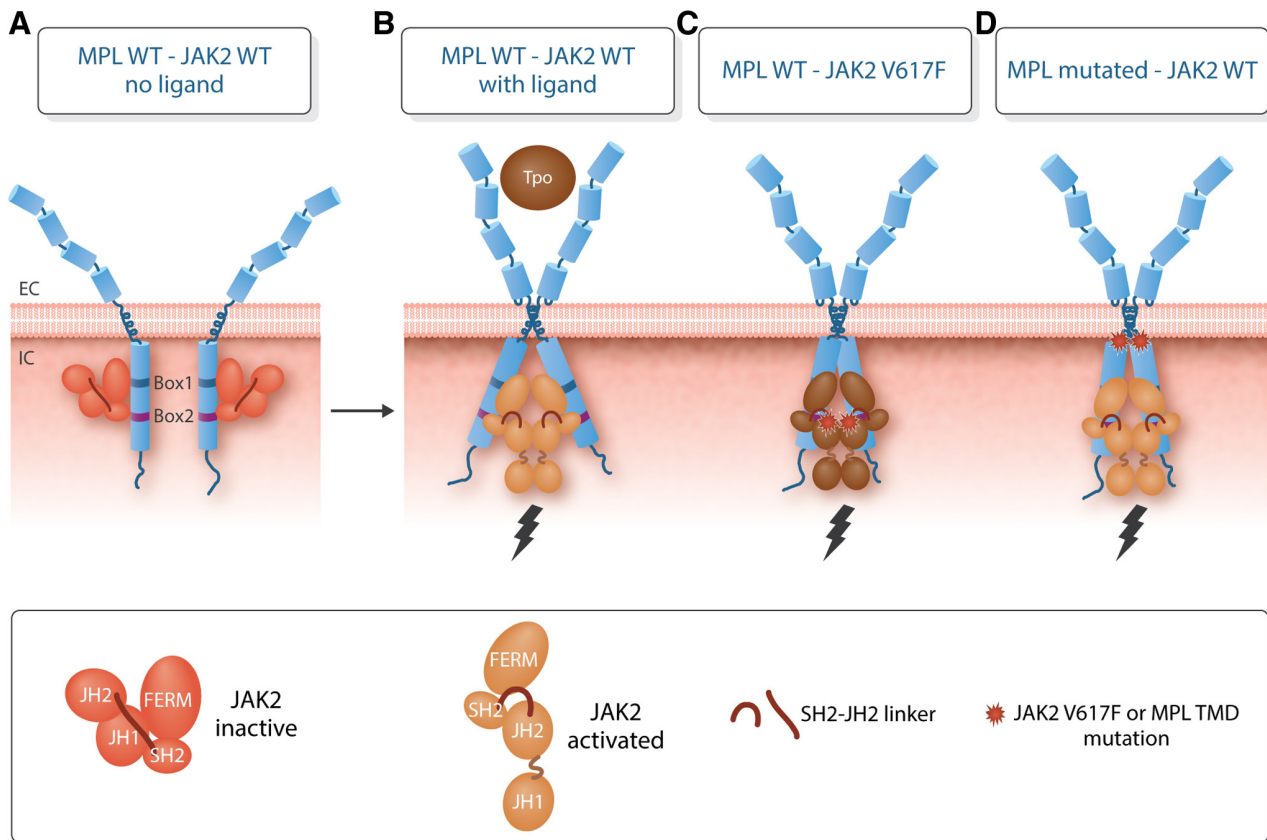


Figure 1. Physiological and pathological activation of MPL and JAK2. (A), MPL is an inactive monomer prebound to JAK2. Domains of JAK2 are represented in yellow. Box 1 and Box 2 are motifs in cytokine receptors that are important for binding of JAKs to receptors. The putative spatial relationship between different domains of JAK2 in inactive and active conformations is shown in the lower panel according to a recent model by Ayaz et al.¹⁶ (B), TPO binding to MPL induces dimerization and changes the conformation of the transmembrane and cytosolic domains so that the appended JAK2 proteins activate each other. (C), In the presence of JAK2 V617F instead of JAK2, MPL is dimerized via its cytosolic domain and in the absence of ligand. The conformation of receptor dimers appears to slightly differ from that of TPO-induced MPL dimerization with respect to possibly a closer inter-monomeric distance,¹⁷ as demonstrated for EPOR.¹⁸ (D), When activating mutations are acquired in the TMD and JMD of MPL, the dimeric conformation changes, with a lower tilt and crossing at the TMD level, leading to persistent activation of wild type JAK2. EC = extracellular; EPOR = erythropoietin receptor; FERM = 4.1, Ezrin, Radixin Moesin; IC = intracellular; JAK2 = Janus kinase 2; JH1/2 = Janus Homology 1/2; JMD = juxtamembrane domain; MPL = thrombopoietin receptor; SH2 = Src-Homology 2; TMD = transmembrane domain; TPO = thrombopoietin; TpoR = thrombopoietin receptor; WT = wild-type.

To date, no curative treatment exists for MPNs. Current treatment strategies aim at mitigating symptoms and preventing development of secondary MF. One important strategy is to reduce the over-activation of the JAK2-STAT5 pathway with the use of JAK2 inhibitors, such as ruxolitinib or fedratinib. Both show encouraging results in MPNs with and without the JAK2 V617F mutation, but their lack of selectivity leads to poor results in reduction of the mutant allele burden and to deleterious side effects.^{28–32} Recent findings on the topology of key receptors involved in MPNs suggest that alternative treatments are possible to directly target pathologic conformations of cytokine receptors.

We review current knowledge in conformational and functional effects associated with mutations in either JAK2 or MPL, how they affect signaling outcomes, and how this knowledge could be translated in more targeted therapies.

The JAK2 V617F mutation

JAK2 is a member of the family of Janus kinases, which associate to the cytoplasmic domain of several cytokine receptors lacking intrinsic kinase activity, including MPL, EPOR, the growth hormone receptor (GHR), the granulocyte colony-stimulating factor receptor (G-CSF-R), cytokine receptor like factor 2, and the interferon- γ receptor 2 (IFN γ R2). JAKs are composed of 4 structural units. The band 4.1, Ezrin, Radixin, Moesin and

Src-Homology 2 (SH2) domains serve as anchoring sites to Box 1 and Box 2 of their associated receptors (Figure 1).¹³ The Janus Homology 2 (JH2) pseudokinase domain has been associated with regulation of the C-terminal Janus Homology 1 (JH1) kinase domain.^{33,34} In the absence of ligand, JAK2 kinase activity is indeed kept silent via inhibition of the JH1 kinase domain by the JH2 pseudokinase domain likely acting in *cis*.^{33–37} Beyond its inhibitory role, JH2 is also required for cytokine-mediated activation³⁴ and stabilization of receptors dimers through *trans* JH2-JH2 interaction,³⁸ highlighting its dual role in the regulation of both activation and inhibition.

Cytokine binding to the extracellular domains of the receptors typically induces their dimerization in a productive conformation, which leads to loss of JH2-JH1 inhibition and activation of downstream signaling pathways (Figure 1). A recently proposed model captures current thoughts about how the different domains of JAK2 are positioned before and after activation (Figure 1).¹⁶ The discovery that the V617F mutation in JAK2 pseudokinase domain was the cause of a majority of PV, ET, and MF cases^{3–6} triggered strong interest in the field of MPNs. Extensive mutational and modeling studies suggested that the V617F mutant acts through 2 complementary mechanisms, both triggered by the presence of Phe at 617 and its novel interaction with F595 of helix C of the pseudokinase domain in *cis*.^{39,40}

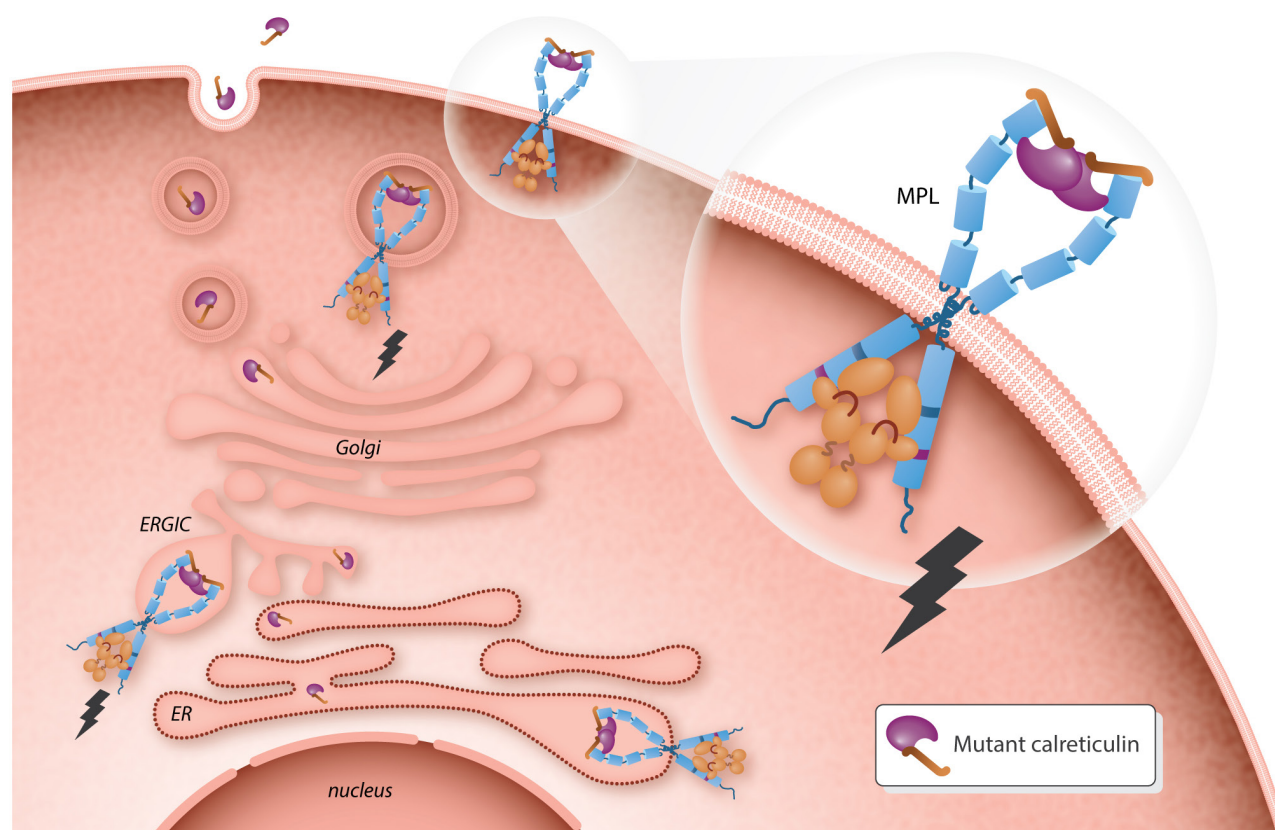


Figure 2. Expression of CALR mutants leads to complex formation with MPL in the endoplasmic reticulum and transport to the cell-surface via the secretory pathway. While JAK2 signaling is activated intracellularly, the threshold required for hematopoietic cell transformation is achieved only when the complex reaches the cell-surface. Mutant CALR proteins are also secreted. Binding and activation of MPL require the N-terminal lectin binding domain of CALR mutants and their positively charged tail. The precise relative positions of these domains of CALR mutants remains unknown and is depicted in one of several possible dispositions. The mutant CALR binds to the D1D2 regions of the extracellular domain of MPL and requires high mannose glycosylation and Asn117. CALR = calreticulin; EC = extracellular; ER = endoplasmic reticulum; ERGIC = endoplasmic reticulum golgi intermediate compartment; IC = intracellular; JAK2 = Janus kinase 2; MPL = thrombopoietin receptor; TpoR = thrombopoietin receptor.

First, the V617F mutation would trigger destabilization of the inhibitory JH2-JH1 interface in *cis*,^{39–43} this mechanism of activation being likely shared by other JAK2 oncogenic mutations lying at the JH1-JH2 inhibitory interface including R683G and T875N mutants.^{38,44} Besides destabilizing *cis* JH1-JH2 inhibition, JAK2 V617F mutant was also found to induce activation of JH1 domain via expelling a Phe residue (from the SH2-JH2 linker) that normally interacts with F595 into the Gly loop of JH1, thus presumably contributing to activation of JH1.⁴⁰ Furthermore, the V617F mutation promotes *trans* JH2-JH2 interaction when bound to some (EPOR⁴⁴ and MPL³⁸), but not to other, cytokine receptors that utilize JAK2 (GHR and IFN γ R2) and that are also not activated by the mutant kinase.^{38,40,45} This receptor specificity of the JAK2 V617F mutant highlights the key role played by the receptor in JAK2 V617F activity, which was early shown to be strictly dependent on its association with cytokine receptor dimers.^{46–48} Interestingly, the active conformation of the EPOR was proposed to be different in presence of JAK2 V617F versus EPO,⁴⁶ suggesting possible differential modulation of downstream pathways in physiological versus pathological receptor activation.⁴⁹ This possibility was given weight with advances demonstrating that both MPL⁵⁰ and EPOR⁵¹ could adopt different active conformations, which lead to differential downstream signaling patterns.^{17,18,27} One indication that conformational specificity would exist at the receptor level is the efficacy of IFN α treatment in the reduction of JAK2 V617F allele burden, but not of CALR del52 mutant clones.⁵² This specificity was recently associated with priming

of IFN α responsiveness in JAK2 V617F mutant cells through positive cross-talk with JAK1/STAT1, which are activated by JAK2 V617F, but not by CALR del52 mutants.⁵³ Interestingly, both STAT1 and the interferon regulatory factor 1 were differently activated by specific conformations of EPOR^{18,27} and MPL,¹⁷ sometimes even more by conformations different than the ones induced by their cognate ligand. While priming of IFN α response by JAK2 V617F can be explained by a number of mechanisms,⁵³ a provocative scenario would be that such priming is due to a particular modulation of EPOR, MPL, or of both receptor conformations by the mutant kinase. Validation of a presumptive specific conformation adopted by cytokine receptors when bound to JAK2 V617F would be a major breakthrough as it would open the road towards specific targeting of the mutant clone at the receptor level in a context where no specific inhibition of the mutant versus wild type JAK2 could be achieved despite intense research in the field.²⁸

Another indication that conformational specificity could exist is represented by the observation that most of secondary site mutations that restore normal function of JAK2 on the background of JAK2 V617F do not inhibit ligand-induced signaling via most dimeric and heterodimeric receptors, suggesting that a unique region/conformation of the pseudokinase domain supports activation by JAK2 V617F.^{44,45} An important exception is IFN γ receptor complex, which can no longer fully respond to ligand when such double JAK2 (or homologous JAK1) mutants are employed, suggesting that activation of the tetrameric IFN γ receptor complex may involve dimeric JAK2 interactions that

require the same conformation as JAK2 V617F.^{44,45} Yet, JAK2 V617F alone is not sufficient to fully activate the IFN γ receptor, probably because another conformational change in JAK1 is required.⁴³ Importantly, for JAK2 V617F to induce persistent activation of the kinase domain, the ATP binding domain of the pseudokinase domain must be intact and ATP is required to stabilize the fold of the JH2 of JAK2 V617F so that the new conformation activates the kinase domain.⁵⁴ An interesting avenue will be to test small molecule inhibitors that compete with ATP for their ability to disrupt the conformation required for V617F-induced activation. Yet, simply occupying the ATP binding site might suffice for stabilization of the structure of JH2 V617F. A comprehensive list of approaches currently used to test JAK2 V617F-selective inhibition strategies is depicted in Leroy et al.²⁸

MPL mutations in MPNs

Based on the GHR, Cunningham et al⁵⁵ published a landmark paper in the early 1990s where they established the operational principle of type I cytokine receptors as a cascade of activation from ligand-induced dimerization of the receptors to transphosphorylation of the JAK molecules and activation of the JAK-STAT pathway. Work on crystal structure analyses, immunofluorescence co-patching, and fluorescence resonance energy transfer assays, as well as in vitro cell models with receptor-overexpression, developed the idea that type I cytokine receptors, including MPL, were preformed dimers that changed conformation upon ligand binding, hence allowing rapid JAK trans-activation.^{56–58}

Whether EPOR (or at least the murine Epor) is a preformed dimer,⁵⁸ or a monomer that is dimerized by Epo,³⁸ as shown by single molecule tracking experiments in overexpression conditions as well as with modified receptors, remains to be determined. In a recent study,²⁷ although most EPOR chains were monomeric, an unusual behavior was determined that might suggest a tendency to dimerization. This was detected as a right shift of EC50 with Designed Ankyrin Repeat Proteins (DARPs) that induce progressively higher inter-monomeric EPOR distance, possibly reflecting a certain level of receptor preassociation via transmembrane domains³⁸ that could not be detected in the single-cell total internal reflection fluorescent microscopy approach.²⁷

For MPL it was shown that while murine c-MPL may exist in preformed dimeric complexes, human MPL was monomeric.⁵⁹ In 2020, Wilmes et al³⁸ showed by single-molecule fluorescence microscopy in the plasma membrane of living cells that spatial and spatiotemporal correlation of individual EPOR, MPL, and GHR subunits showed ligand-induced dimerization, and, of great interest, MPL oncogenic mutations induced dimerization of the receptor. Even if the debate of murine versus human MPL dimerization might not be solved yet, what seems clear is that dimerization involves its transmembrane and cytosolic juxtamembrane domains, where most mutations seen in MPN occur,⁶⁰ and that different interfaces mediate differential activities.

The juxtamembrane W515 residue in MPL was first identified as activating by Staerk et al¹⁴ in cell lines (W515A mutation), and by Pikman et al¹⁷ and Pardanani et al¹⁸ in patients with acquired ET and PMF (MPL W515L mutation). W515 has a key role in preventing activation in the absence of cytokine binding and is part of the amphipathic helical motif RWQFP, which is directly adjacent to the C-terminal end of the transmembrane domain. This motif is unique to MPL and its deletion or mutation activates the receptor in the absence of ligand,^{14,15} as do all residues at position W515 (except Trp, Cys, and Pro).⁶¹ Nuclear magnetic resonance (NMR) spectroscopy studies of transmembrane peptides suggested that mutation of W515 decreases the tilt angle relative to the lipid bilayer normal (Figure 1), bringing the dimer into an active conformation.¹⁹

Another clinically relevant transmembrane domain mutation, which activates MPL by inducing stable active receptor dimers is S505N and is found in patients with hereditary and rare sporadic cases of thrombocythemia.^{20,62–64} In addition, other rare mutations have been described in the transmembrane domain of MPL,^{65–67} and triple negative ET cases and certain hereditary thrombocytosis patients harbor rare noncanonical MPL or JAK2 mutations that also induce constitutive activation.^{68,69} A P106L mutation in the extracellular domain of MPL was shown to induce paradoxical thrombocytosis due to lower levels of MPL traffic to the surface, which would decrease thrombopoietin clearance, in turn allowing higher thrombopoietin levels to activate early progenitors that are very sensitive to this ligand.⁷⁰

In engineered constructs, it appears that several dimeric interfaces imposed on the transmembrane domain can lead to MPL activation.^{50,71} In Staerk et al,³⁰ the murine MPL had several conformations that supported cell proliferation, with differing signaling outputs and biological effects, including cell–cell adhesion and megakaryocytic differentiation. In comparison to its murine counterpart, activation of the human MPL seems limited to 3 different conformations, a mechanism that might rely on the H499 residue, which is the mouse in L492.⁷¹ The first NMR studies on the transmembrane helix of MPL found indeed that this region is not continuously helical, but rather unexpectedly contains several nonhelical residues in the sequence preceding H499, although the same region in the murine receptor is helical.⁷² Both the S505N mutation and MPL small molecule agonist, eltrombopag—which binds to H499—induce helical structure into the region, stronger dimerization, and receptor activation.⁵⁹

In 2019, Mohan et al²⁷ further demonstrated that the tilt angle of EPOR transmembrane domain, when modulated, influenced hematopoiesis through differential downstream signaling. Likewise, modulation of the tilt angle of MPL transmembrane domain would also be at stake in the inhibition of activity of canonical MPL mutations W515K/L or S505N by mutations of the W491 residue at the outer part of the transmembrane domain.⁶⁷

Mutations of MPL might induce dimerization in different interfaces or with different effects on the transmembrane or juxtamembrane domains—that might overlap, or not—with dimerization induced by JAK2 V617F³⁸ or CALR del52, which themselves might not induce the same interfaces. This would explain the plurality of MPN phenotypes, with JAK2 or CALR mutations exhibiting different clinical phenotypes,²⁶ or the fact that EPOR, which requires one specific interface for activation⁵¹ and in which no activating transmembrane or juxtamembrane domain mutations have been reported in blood diseases, would not be activated by CALR del52.²¹

Recently, strong evidence has been provided that cytokine receptors like EPOR and MPL can be topologically controlled with novel ligands that impose on nonmutated receptors different relative orientations and inter-monomeric distances, which are translated into different intracellular signaling outputs.^{17,18,27} Such novel ligands are diabodies for EPOR and MPL and DARPs for EPOR. By solving x-ray crystal structures of the different ligands, different geometries were observed that induced subtle different signaling effects, and notably a bias towards STAT1 signaling for EPOR in certain configurations.²⁷ One concept that appears to be emerging is that pathologic activation of EPOR or MPL by JAK2 V617F may require closer inter-monomeric distances than ligand-induced activation of receptors coupled to wild type JAK2.¹⁸

CALR mutations in MPNs

This class of mutations has been reviewed last year.⁷³ We will briefly review conformational issues that are linked to how frameshifting mutations in exon 9 of CALR are thought to induce MPNs, namely ET and MF.^{10,11} A solid body evidence from several groups including ours has established that

pathogenic mutants of CALR specifically activate MPL in a thrombopoietin-independent manner.^{21,74–76} This is a first example of how a chaperone can become an oncoprotein by acquiring the unique ability to bind the extracellular domain of MPL and induce transport via the Golgi apparatus to the cell surface of partially immature MPL in a dimeric and active form (Figure 2). Thus, the mutant CALRs are “rogue” chaperones.²² Activation of MPL requires both the N-terminal glycan-binding domain of CALR mutants and their positively charged tails.^{21,22} Dimerization/oligomerization of mutant CALR contributes to the activation of MPL.^{22,77,78} The interaction between the mutant CALR proteins and MPL leads to thermal stabilization of MPL and can be interrupted by a small molecule that binds to the N-glycan binding domain of CALR.⁷⁹ While intracellular complexes of MPL and CALR mutants do induce JAK-STAT signaling, this is not sufficient to transform to autonomous growth cytokine-dependent hematopoietic cells. For this, cell-surface localization of the complex is required and full activation of JAK2-STAT5/STAT3, mitogen-activated protein kinase, and phosphoinositide 3-kinase pathways appears to require cell-surface localization.²² Mutant CALR can be detected at the surface of primary MPN patient CD34+ cells.²² Noteworthy, CALR mutants are secreted,^{80,81} presumably also by cells belonging to the clone and that do not co-express MPL. The secreted form of mutant CALR was suggested to play a “rogue” cytokine role as in vitro signaling experiments indicate that cells co-expressing MPL and endogenous CALR mutant may respond to low supplementary levels of circulating mutant CALR.⁸⁰ Explanations for this phenomenon include the multimerization tendency of mutant CALRs⁷⁷ and the demonstration that cells that express endogenous mutant CALR proteins expose immaturely N-glycosylated MPL at the surface.²² In any case, in cells co-expressing MPL and mutant CALR, both receptors and JAK2 proteins form dimers to a significantly larger extent than in control cells.²¹ In cell lines, it appears that cells co-expressing MPL and mutant CALR respond less to thrombopoietin,⁷⁵ suggesting that the ligand binding sites on MPL may be sterically hindered by the bound CALR proteins. On the other hand, a mutant MPL that no longer binds thrombopoietin can be activated as well as the wild type MPL.²¹ Last but not least, in addition to MPL, one other cytokine receptor, G-CSF-R, is also activated by mutant CALRs, albeit to lower levels and insufficient to induce long-term growth of hematopoietic cells.²¹ Taken together, all available evidence indicates that most, if not all, of the phenotype induced by CALR mutants leading to MPNs is due to pathologic activation of MPL, which is corroborated by common gene expression profiles between mutated MPL and mutated CALR MPNs.⁸² In addition, the secreted mutant CALR could suppress the immune response against the MPN cells.⁸³

Ongoing work focuses on the structure of mutant CALRs compared to wild type CALR, the impact of the positively charged tail on the mutant CALR structure, and on the conformation of activated MPL in the complex.

Epigenetic alterations in MPNs

While the BCR-ABL1 negative MPN field joined the groups of pathologies for which driver mutations enormously help diagnosis and treatment, it became apparent that 35%–40% of MPNs harbor also mutations in epigenetic regulators such as *TET2*, *DNMT3A*, *IDH1/2*, *EZH2*, and *ASXL1*.⁸⁴ Thus, mutations in epigenetic regulators actually constitute the largest group of mutations in MPNs after the phenotypic drivers *JAK2*, *MPL*, and *CALR*, which lead to persistent JAK2 activation. Furthermore, some are loss of function and hence pose a very serious problem for therapy attempts. Last but not least, MPNs can also harbor mutations in genes involved in RNA splicing,

other signaling proteins, cell cycle regulators, transcription factors, and others that will not be a subject of this perspective. Suffice it to say that mutations in genes involved in RNA splicing hold the potential to generate frameshifts that could be useful targets for immunotherapy.⁸⁵

The first description of loss of function *TET2* mutations in MPNs came from the field of *JAK2* V617F-positive MPNs for which an increase in allele burden during differentiation was not seen.⁸⁶ *TET2* gene mutations/alterations were shown to exist in MPNs, myelodysplastic syndromes, and acute myeloid leukemia, therefore being present in myeloid cancers.⁸⁶ Of great interest, the *TET2* mutations are present in HSCs, which is also true for the other acquired mutations in epigenetic regulators. Given that most MPNs carry a driver mutation that activates *JAK2*, the order of acquisition of these mutations has been investigated and it was found that “order matters” in the sense that acquiring *TET2* mutations subsequent to *JAK2* V617F yields a stronger MPN with many more complications and progression, while the *TET2*-first-*JAK2* V617F-after situation yields a milder disease.⁸⁷ Furthermore, aged individuals have been identified that exhibit clonal hematopoiesis of indeterminate potential (CHIP) (defined as having >2% of peripheral blood derived from one HSC), have a normal blood formula, and exhibit in these clones mutations in either *DNMT3A*, *TET2*, *ASXL1*, *JAK2* V617F, or other genes.⁸⁸ It is thought that these mutations enhance the fitness of HSCs and that especially in aged individuals, senescence of HSCs can be counteracted by such mutations; that would give a strong advantage, hence clonal hematopoiesis. These individuals with CHIP have an increased risk to develop MPNs, myelodysplastic syndromes, or acute myeloid leukemia.⁸⁸ Very recently it has been reported that chronic infection drives *DNMT3A* loss of function, hence clonal hematopoiesis, and that this is mediated by IFN γ signaling.⁸⁹ Recent evidence suggests that there is a long delay (several decades) before *JAK2* V617F mutation occurrence and the development of the disease, and that in some cases the somatic mutation is even acquired during fetal life.^{90–93} Using mathematical modeling, it has been shown that *JAK2* V617F increases the fitness of HSCs, but in a higher order in true MPNs than in CHIP, and this occurs via an unknown mechanism.⁹⁰ Furthermore, additional epigenetic mutations such as *TET2* further increase the fitness of *JAK2* V617F HSCs.^{88,90}

At present, the reasons behind the selection of enhanced fitness in HSCs by such mutations remain unclear. While *TET2* and *IDH1/2* mutations will enhance DNA methylation, therefore possibly inducing gene repression, loss of function mutations in *EZH2* will impair polycomb repressive complex 2 and de-repress certain genes. Many patients harbor simultaneously several such mutations, therefore repression by DNA methylation may cooperate with de-repression by the absence of H3K27me3. Things are complicated by the emerging roles of methylated and hydroxy-methylated cytosines outside promoters, and novel roles of 5-hydroxymethyl cytosines in regulating transcription factor binding to promoters and transcription itself.⁹⁴ Equally unknown is how the effects of these mutations interact with the consequences of persistent JAK2-STAT5/STAT3/STAT1 signaling, which is a hallmark of MPNs. Finally, whether signaling by persistently activated JAK2 may impact epigenetic factors is unknown. These cross-talks are to be explored, but what is clear is that the presence of 2 or more somatic mutations significantly reduces overall survival in MPNs and favors progression.⁸⁴ Several clinical scores are now considering the mutations and thus help treatment guidance.

An interesting finding in patients is that treatment with ruxolitinib (*JAK2*/*JAK1* inhibitor) for MF and complicated forms of PV can lead to enrichment for *ASXL1* mutations in a significant number of patients, followed to a lower extent

by mutations in *TET2* and *EZH2* in the study by Newberry et al.⁹⁵ The reasons behind this observation, especially the recurrent enrichment/acquisition of *ASXL1* mutations, are not clear. On the other hand, from basic research studies, it is interesting to note that evidence has been provided that JAK signaling can influence heterochromatin in *Drosophila melanogaster*⁹⁶ and murine and human cells,⁹⁷ that JAK2 was shown to phosphorylate chromatin proteins excluding heterochromatin protein 1 from chromatin,⁹⁸ and that unphosphorylated STAT5 (U-STAT5) binds to the genome to sites differently than activated tyrosine phosphorylated STAT5.⁹⁹ U-STAT5 was found to bind to sites required for lineage specific transcription factor binding, impairing megakaryocyte differentiation. Activation of STAT5 removes occupancy of those sites allowing differentiation.⁹⁹ Furthermore, we have shown that persistently activated STAT5 binds to different chromatin sites than cytokine-activated STAT5⁴⁹ and that persistently activated STAT5 cooperates with p53 mutants to induce a set of genes that may contribute to the megakaryocyte phenotype in MPNs.^{100,101} Taken together, one can postulate that a deeper understanding of how JAK-STAT pathway interacts with epigenetic regulators may shed light on mechanisms of MPN pathogenesis.

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