Functional Consequences of Mutations in Myeloproliferative Neoplasms

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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), and 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. The most prevalent mutation, JAK2 V617F, is the basis of >95% of polycythemia vera (PV) and 60% of essential thrombocytopenia (ET) and primary myelofibrosis (PMF) cases. 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.

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 thrombocytopenia (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.

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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. 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 (IFNγR2). 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. 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.

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First, the V617F mutation would trigger destabilization of the inhibitory JH2-JH1 interface in cis,\textsuperscript{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.\textsuperscript{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.\textsuperscript{40}

Furthermore, the V617F mutation promotes trans JH2-JH2 interaction when bound to some (EPOR\textsuperscript{44} and MPL\textsuperscript{38}) but not to other, cytokine receptors that utilize JAK2 (GHR and IFN\textsubscript{γ}R2) and that are also not activated by the mutant kinase.\textsuperscript{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.\textsuperscript{46–48} Interestingly, the active conformation of the EPOR was proposed to be different in presence of JAK2 V617F versus EPO,\textsuperscript{44} suggesting possible differential modulation of downstream pathways in physiological versus pathological receptor activation.\textsuperscript{49} This possibility was given weight with advances demonstrating that both MPL\textsuperscript{50} and EPOR\textsuperscript{51} could adopt different active conformations, which lead to differential downstream signaling patterns.\textsuperscript{17,18,27} One indication that conformational specificity would exist at the receptor level is the efficacy of IFN\textsubscript{α} treatment in the reduction of JAK2 V617F allele burden, but not of CALR del52 mutant clones.\textsuperscript{52} This specificity was recently associated with priming of IFN\textsubscript{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.\textsuperscript{53} Interestingly, both STAT1 and the interferon regulatory factor 1 were differentially activated by specific conformations of EPOR\textsuperscript{18,27} and MPL,\textsuperscript{17} sometimes even more by conformations different than the ones induced by their cognate ligand. While priming of IFN\textsubscript{α} response by JAK2 V617F can be explained by a number of mechanisms,\textsuperscript{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.\textsuperscript{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.\textsuperscript{44,47} An important exception is IFN\textsubscript{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\textsubscript{γ} receptor complex may involve dimeric JAK2 interactions that...
require the same conformation as JAK2 V617F. Yet, JAK2 V617F alone is not sufficient to fully activate the IFNγ receptor, probably because another conformational change in JAK1 is required. 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. 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.

**MPL mutations in MPNs**

Based on the GHR, Cunningham et al 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.

Whether EPOR (or at least the murine Epor) is a preformed dimer, or a monomer that is dimerized by Epo, as shown by single molecule tracking experiments in overexpression conditions as well as with modified receptors, remains to be determined. In a recent study, 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 domains that could not be detected in the single-cell total internal reflection fluorescence microscopy approach.

For MPL it was shown that while murine c-MPL may exist in preformed dimeric complexes, human MPL was monomeric. In 2020, Wilmes et al. 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, and that different interfaces mediate differential activities.

The juxtamembrane W515 residue in MPL was first identified as activating by Staerk et al. in cell lines (W515A mutation), and by Pikman et al. and Pardanani et al. 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, as do all residues at position W515 (except Trp, Cys, and Pro). 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.

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 thrombocytopenia. In addition, other rare mutations have been described in the transmembrane domain of MPL, and triple negative ET cases and certain hereditary thrombocytopathies patients harbor rare noncanonical MPL or JAK2 mutations that also induce constitutive activation. 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.

In engineered constructs, it appears that several dimeric interfaces imposed on the transmembrane domain can lead to MPL activation. In Stäerk et al., 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. 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. 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.

In 2019, Mohan et al 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. 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 V617F or CALR del52, which themselves might not induce the same interfaces. This would explain the plurality of MPL phenotypes, with JAK2 or CALR mutations exhibiting different clinical phenotypes, or the fact that EPOR, which requires one specific interface for activation and in which no activating transmembrane or juxtamembrane mutations have been reported in blood diseases, would not be activated by CALR del52.

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. 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. 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.

**CALR mutations in MPNs**

This class of mutations has been reviewed last year. 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. A solid body evidence from several groups including ours has established that
pathogenic mutants of CALR specifically activate MPL in a thrombopoietin-independent manner. 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. Activation of MPL requires both the N-terminal glycan-binding domain of CALR mutants and their positively charged tails. 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. While intracellular complexes of MPL and CALR mutants do induce JAK-STAT signaling, this is not sufficient to transform to autonomous growth of hematopoietic cells. For this, cell-surface localization of the complex is required and full activation of JAK2-STAT3A/STAT5A signaling yields a milder disease. 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. 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. Very recently it has been reported that chronic infection drives DNMT3A loss of function, hence clonal hematopoiesis, and that this is mediated by IFNy signaling. 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. 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. Furthermore, additional epigenetic mutations such as TET2 further increase the fitness of JAK2 V617F HSCs. At present, the reasons behind the selection of enhanced fitness in HSCs by such mutations remain unclear. While JAK2 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 hydroxymethylated cytosines outside promoters, and novel roles of 5-hydroxymethyl cytosines in regulating transcription factor binding to promoters and transcription itself. 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. 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.91 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 melanogaster92 and murine and human cells,97 that JAK2 was shown to phosphorylate chromatin proteins excluding heterochromatin protein 1 from chromatin,98 and that unphosphorylated tyrosine kinase-activated STAT5 binds to different chromatin sites than cyto- 

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