Expert Review of Hematology

ISSN: 1747-4086 (Print) 1747-4094 (Online) Journal homepage: https://www.tandfonline.com/loi/ierr20

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To cite this article: William Vainchenker, Isabelle Plo, Caroline Marty, Leila N. Varghese & Stefan N. Constantinescu (2019) The role of the thrombopoietin receptor MPL in myeloproliferative neoplasms: recent findings and potential therapeutic applications, Expert Review of Hematology, 12:6, 437-448, DOI: 10.1080/17474086.2019.1617129

To link to this article: https://doi.org/10.1080/17474086.2019.1617129

Accepted author version posted online: 16 May 2019.
Published online: 22 May 2019.

EXPERT REVIEW OF HEMATOLOGY
2019, VOL. 12, NO. 6, 437–448
https://doi.org/10.1080/17474086.2019.1617129

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REVIEW

The role of the thrombopoietin receptor MPL in myeloproliferative neoplasms: recent findings and potential therapeutic applications

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ABSTRACT

Introduction: Classical Myeloproliferative Neoplasms (MPNs) include three disorders: Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF). MPNs are associated with constitutive activation of JAK2 leading to persistent cell signaling downstream of the dimeric myeloid cytokine receptors due to mutations in three genes encoding JAK2, calreticulin (CALR) and the thrombopoietin (TPO) receptor (MPL or TPOR). CALR and MPL mutants induce JAK2 activation that depends on MPL expression, thus explaining why they induce megakaryocyte pathologies including ET and PMF, but not PV. In contrast, JAK2 V617F drives all three diseases as it induces persistent signaling *via* EPOR, G-CSFR (CSF3R) and MPL.

Areas Covered: Here, we review how different pathogenic mutations of *MPL* are translated into active receptors by inducing stable dimerization. We focus on the unique role of MPL on the hematopoietic stem cell (HSC), explaining why MPL is indispensable for the development of all MPNs. Last but not least, we describe how CALR mutants are pathogenic *via* binding and activation of MPL.

Expert Opinion: Altogether, we believe that MPL is an important, but challenging, therapeutic target in MPNs that requires novel strategies to interrupt the specific conformational changes induced by each mutation or pathologic interaction without compromising the key functions of wild type MPL.

ARTICLE HISTORY
Received 30 March 2019
Accepted 7 May 2019

KEYWORDS
Myeloproliferative neoplasms; MPL/thrombopoietin receptor; JAK2; calreticulin; therapy

1. Introduction

Classical *BCR-ABL*-negative myeloproliferative neoplasms (MPNs) are clonal diseases of the hematopoietic stem cells (HSCs), which induce excessive proliferation of the three main myeloid lineages: the erythroid, granulocytic/monocytic and megakaryocytic lineages, but each at different levels according to the MPN subtype [1–3]. For this reason, they have been classified into three main disorders: Polycythemia Vera (PV) with predominant erythroid proliferation, Essential Thrombocythemia (ET) with predominant or restricted megakaryocytic (MK) hyperplasia, and primary myelofibrosis (PMF) associated with scarring of the marrow and myelofibrosis, together with granulocytic and MK hyperproliferation [4]. These three diseases represent a remarkable single pathogenic entity and a preleukemic state. They also favor hemorrhagic and thrombotic complications. In addition, there is a continuum between these three disorders: boundaries between certain ET and PV cases are difficult to establish and both ET and PV can progress to myelofibrosis, denoted as post-ET or post-PV myelofibrosis [4].

Molecular and cellular studies have established that nearly all MPNs are the consequence of an aberrant constitutive activation of JAK2 that induces signaling downstream of cytokine receptors. This signaling deregulation is related, in more than 90% of MPNs, to acquired somatic mutations at the level of HSCs in one of the three genes: *JAK2*, calreticulin (*CALR*) and the thrombopoietin receptor (*MPL*) (Figure 1). *JAK2* V617F in exon 14 is the most predominant mutation and is associated with around 90% of PVs and 50–60% of ETs and PMFs [5–8]. *JAK2* exon 12 mutations are observed in 2–3% of *JAK2* V617F-negative PVs with a phenotype close to a pure erythrocytosis, although *JAK2* exon 12 cases may progress to myelofibrosis [9,10]. *CALR* and *MPL* mutations are associated specifically with ET and PMF in 20–30% and 2–5% of the cases, respectively [11–13]. Almost all PVs are driven by *JAK2* mutations whereas 10–15% of ETs and PMFs remain without an identified somatic driver mutation [14]. The mutations in *JAK2, MPL* and *CALR* are drivers of MPNs as in mouse models all of them can induce a myeloproliferative disorder close to the human disease: a PV-like phenotype for *JAK2* V617F and *JAK2* exon 12 [15–17], an ET-like phenotype for *CALR* and *MPL* mutations as well as for *JAK2* V617F in some mouse models [11,18–20]. All mouse models may progress to myelofibrosis. The MPN driver mutations are usually mutually exclusive although in very rare cases they can co-occur, even in the same clone.

However, although found as single mutations in 50% of MPNs, these MPN driver mutations can also be found associated with numerous additional acquired mutations [14,21]. These additional mutations are not restricted to MPNs and are also observed in several other malignancies, more particularly in chronic and acute myeloid malignancies. These mutations target genes involved in epigenetic regulation (*TET2, DNMT3A, ASXL1, EZH2, IDH1/2* and others), in RNA splicing (*SRSF2, SF3B1, U2AF1, ZRSR2*), transcription factors

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Article highlights

- MPL is activated by the three driver mutations of MPNs (*JAK2* V617F, *CALR* or *MPL* mutations).
- MPL is indispensable for MPN development regardless of the MPN driver mutation.
- MPL is indispensable due to its dual function in hematopoiesis: firstly on hematopoietic stem cells, secondly on the megakaryocyte/platelet lineage.
- However, the precise mechanism of the MPL/JAK2 activation varies according to the type of MPN driver mutation.
- MPL is a potential target for therapy, but would require different approaches according to the type of MPN driver mutation.

(*TP53, CUX1, IKZF1, RUNX1, NFE2*), and in cell signaling (*NF1, NRAS, KRAS, SH2B3, CBL, PTPN11, PPM1D*). They can either precede or follow the acquisition of MPN driver mutations. They play important roles in disease phenotype modifications and disease progression and are thus important in the prognosis of the disease. For example, *ASXL1, EZH2, SRSF2, U2AF1*, and *IDH1/2* mutations are predictive of a poor prognosis [14,22]. Others are more associated with AML progression such as *TP53* and *PPM1D* or even can be late events in this progression, such as *RUNX1* and possibly *NFE2* mutations [23]. Interestingly, several of these mutations, particularly *DNMT3A* and *TET2*, are associated with the newly recognized condition denoted as clonal hematopoiesis of indeterminate potential (CHIP), strongly suggestive that they may play an important role in disease initiation [24]. Unexpectedly, although controversial [25], *JAK2* V617F is also among the most frequent mutations associated with CHIP, strongly suggesting that *JAK2* V617F is a commonly occurring mutation, but exhibits a low fitness to induce an MPN [24]. Therefore, for a disease to develop, additional events are necessary and can be environmental, such as aging and inflammation, and/or genetic such as additional somatic mutations or germ-line determinants predisposing to MPNs. Overall, the pathogenesis of MPNs cannot be restricted to the three driver mutations although they are indispensable for inducing the myeloproliferative ET, PV and MF phenotypes. This myeloproliferation is the direct consequence of an inappropriate activation of signaling pathways downstream of myeloid cytokine receptors including STATs, the PI3K, and MAPK/ERK pathways.

2. Body of the review

2.1. The MPL/JAK2 axis plays a crucial role in the development of MPNs

In hematopoietic cells, the constitutive signaling induced by endogenous levels of either of the three MPN driver mutants through JAK2 activation requires expression of a cytokine receptor [26]. In contrast, overexpression of JAK2 V617F induces ligand-independent signaling without exogenous receptor [27]. MPL plays a central role in the pathogenesis of MPNs because two driver mutants (MPL and CALR) activate only or essentially MPL [18,28–30]. JAK2 V617F exhibits an absolute requirement to bind and activate either of the three homodimeric type I cytokine receptors, including MPL, to induce a disease [26,31].

MPL belongs to the homodimeric class I receptor family, and its ligand is thrombopoietin (TPO) [32,33]. MPL plays a central role in the regulation of megakaryopoiesis by regulating platelet production in response to TPO. However, MPL also regulates the HSC and immature progenitor compartment. TPO induces the quiescence of HSCs and their survival, thus maintaining the HSC reservoir [34], but induces the proliferation of progenitors [35]. This crucial role is illustrated by the fact that loss of function of MPL or TPO in humans leads to a profound thrombocytopenia, which progresses to bone marrow failure [36,37].

From a structural point of view, MPL has three main domains (Figure 1(a)) [33,38]. The extracellular domain is composed of two consecutive cytokine receptor homology modules, each comprising two fibronectin-III-like domains. The transmembrane (TM) domain is necessary for the insertion of the receptor in the membrane through an α helix and is followed by a juxtamembrane (JM) cytosolic domain (also called the amphipathic domain), consisting of an RWQFP motif [39]. The intracellular domain is required to mediate signaling through its binding *via* box 1 and box 2 to JAK2. JAK2 is essential for the induction of downstream signaling pathways. TYK2 can also bind to MPL, but its ability to transduce a signal in the absence of JAK2 is not clear. MPL is pre-associated with JAK2 and TYK2 in the endoplasmic reticulum (ER), where the two kinases play the role of chaperone molecules and increase MPL trafficking to the cell membrane and its stability at the cell surface [40]. The MPL/JAK2 complex becomes mature through traffic in the Golgi apparatus by successive glycosylations on asparagine residues (N-glycosylation) and is expressed at the cell surface as a partially pre-dimerized receptor. It appears that MPL exists on the cell surface as a monomer or as an unstable dimer [41]. The murine receptor adopts a partial preformed dimer conformation that requires a rotation for activation, while the human receptor is maintained in a monomeric or very unstable dimeric form by the presence of a histidine at amino acid 499 at the start of the TM domain [41]. Dimerization is also prevented for both murine and human receptors by the RWQFP cytosolic JM motif, which exerts a similar function in both receptors [39]. TPO induces and stabilizes an active dimeric conformation of MPL among several potential active conformations [42,43]. These conformational changes reorient the two amphipathic domains removing the inhibition induced by W515 and bring the associated JAK2 molecules closer, which can then trans-phosphorylate [44].

2.2. MPL mutations in MPNs

MPL mutations are relatively rare in MPNs (2% and 5%) and restricted to ET and PMF. These mutations are gain of function mutations, i.e. they induce ligand-independent JAK2 activation and downstream signaling in the absence of its natural ligand, TPO (Figure 1(b)). This is quite different from some other mutations such as *MPL* P106L, which induce TPO-dependent

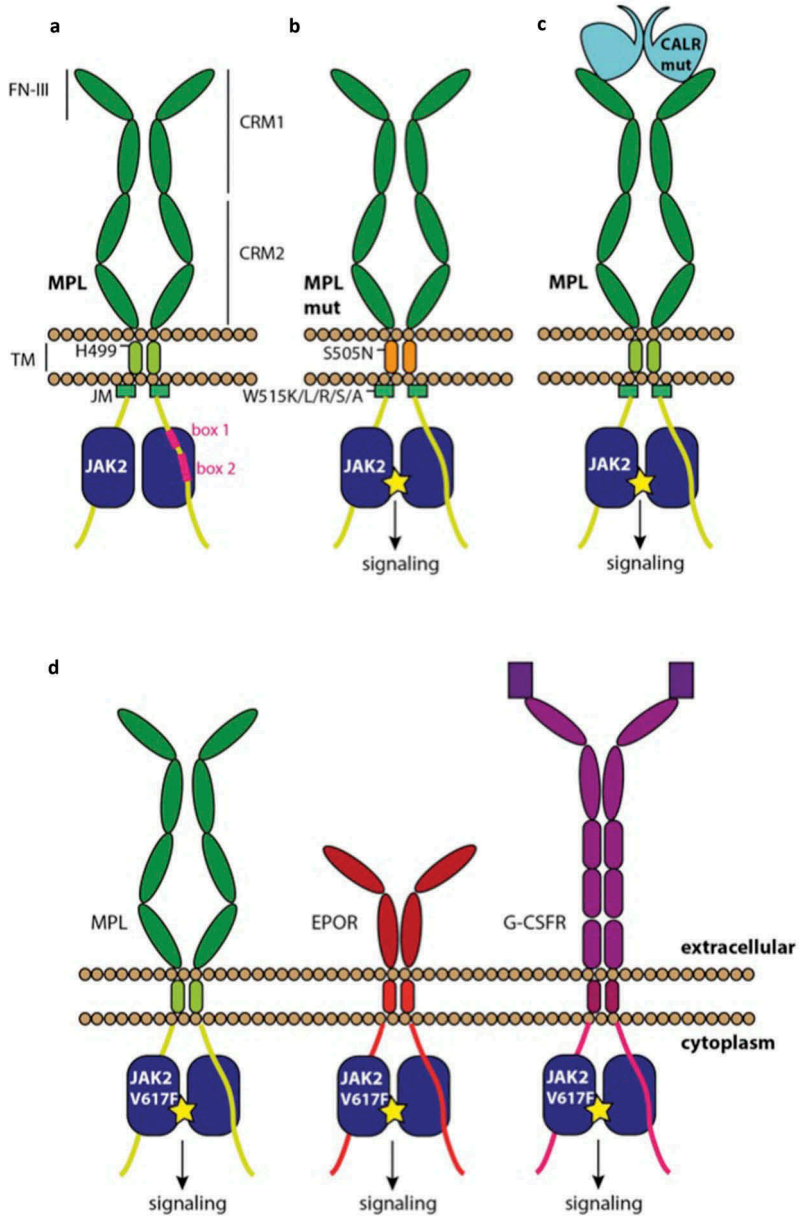

Figure 1. Activated signaling proteins coded by the three driver mutations: *MPL/TpoR* W515K/L/R/S/A, *CALR* + 1 frameshift type 1 (del52 class) and type 2 (ins5 class), and *JAK2* V617F. (a) MPL has three main functional domains: an extracellular domain, which binds the cytokine thrombopoietin, and comprises two cytokine receptor homology modules (CRM), each consisting of two fibronectin-III-like domains; a transmembrane domain, which spans the cell surface membrane; and a cytoplasmic domain containing a juxtamembrane amphipathic motif, and further into the cytosol, two box motifs, box 1 and box 2, involved in binding JAKs. (b) Mutations in the transmembrane (S505N) and juxtamembrane (W515K/L/R/S/A) domains allow mutant MPL to signal in the absence of cytokine. (c) CALR mutations result in a novel C-terminal tail which drives rogue cytokine activity via binding of mutant CALR oligomers to the extracellular domain of MPL. (d) JAK2 V617F requires the presence of one of the three homodimeric type I myeloid cytokine receptors for pathological signaling: MPL, EPOR or G-CSFR.

thrombocytosis. There are two mutational hot spots in exon 10 of *MPL*, W515, and S505, located in the amphipathic JM domain and the TM domain, respectively. There are several substitutions of W515 in MPNs, but the two most frequent are *MPL* W515L and *MPL* W515K. In fact, all substitutions of W515, except W515C and W515P lead to the activation of MPL in the absence of TPO and activate JAK2. W515 mutations are usually heterozygous, but homozygous mutations can be found in MF. The S505N mutation can also be found in MPN but is much less frequent than mutations of W515. Mutations in both W515 and S505N can be germline and are responsible for hereditary thrombocytosis [45–47]. The only described mouse models to date with W515L or W515A are retroviral and produce a thrombocytosis progressing to MF [11,48].

There are several other much rarer somatic *MPL* mutations in other domains of the receptor, such as the S204P/F mutations in the extracellular domain and the Y591F/N mutations in the cytosolic domain [49,50]. Tyrosine 591 is implicated in the negative regulation of MPL signaling [48,51], potentially as when phosphorylated it might serve as a docking site for SH2 domain-containing proteins that negatively regulate MPL, and as phosphorylation at this site is no longer possible when mutated to phenylalanine or asparagine, this negative regulation is lost [52].

2.3. Calreticulin mutants in MPNs

Mutations in exon 9, the last exon of calreticulin (*CALR*), are associated with ET and MF, and occur at a higher frequency (25%) than *MPL* mutations [12,53]. There are more than 40 mutations described, but all lead to the same +1 frame-shift in exon 9 that is responsible for the synthesis of a CALR molecule with a common methionine-rich and positively charged novel C-terminus devoid of the KDEL motif. The two most predominant mutations are a 52 bp deletion (p.L367fs*46), also called type 1, and a 5 bp insertion (p.K385fs*47), also called type 2. CALR del52 loses nearly entirely the wild-type exon 9-coded sequence and the calcium-binding sites, whereas ins5 retains around 50% of negative charges [12]. The other mutations have been classified in type 1-like and type 2-like according to the size of the remaining wild-type sequence of the exon 9. Type 1 *CALR* mutations are predominant in MF, whereas the frequency of the two types is more similar in ET. *CALR* mutations are usually heterozygous, but homozygous mutations have been observed, more particularly for type 2 mutations [12,13,39].

CALR is not a signaling molecule, but a chaperone of the ER involved in the quality control of N-glycosylated protein and in calcium storage. However, the new C-terminus changes the function of the molecule and allows the CALR mutants to tightly bind and activate MPL and JAK2 (Figure 1(c)) [28–30,54]. Notably, type 1 CALR mutants can also activate the G-CSFR, but at a low level, insufficient for inducing long-term autonomous growth of hematopoietic cell lines [28]. It is unknown if this activation found in live cells *in vitro* plays a role in the pathogenesis of MPNs, especially in the granulocytosis exhibited by certain patients, or in the high allele burdens for *CALR*-mutated patients.

Different mouse models of CALR mutants have been obtained: retroviral [18,29], transgenic with the human CALR mutant, and knock-in (KI) either of the mouse *Calr* with the human mutant C-terminus sequence or a del52 or del61 KI of the mouse sequence [55–57]. All lead to a thrombocytosis that may progress to myelofibrosis. CALR ins5 induces only a mild thrombocytosis [18], whereas del52 causes a more severe disease, which is even more significant in homozygous KI mice [18,56].

2.4. JAK2 mutations in MPNs: JAK2 V617F drives more than 70% of MPNs

JAK2 is a member of the Janus kinase family characterized by a catalytically active tyrosine kinase domain at the C-terminus, and a pseudokinase domain that prevents self-activation of the kinase domain, as well as a FERM-like domain at the N-terminus and a Src homology 2 (SH2)-like domain. JAK2 can be considered as the catalytic part of the homodimeric type I receptors, such as EPOR, MPL, and G-CSFR as it binds to them intracellularly through its FERM and SH2 domains. JAK2 is also associated with the heteromeric type I receptors of GM-CSF and IL-6 and with type II receptors, especially the IFN γ receptor [58]. Cytokines such as EPO, TPO or G-CSF bind to their cognate receptors, inducing their dimerization and/or oligomerization, which activate JAK2 by trans-phosphorylation. The activated JAK2s phosphorylate themselves and the cytosolic receptor domains on tyrosine residues, which are subsequently used as docking sites for other signaling molecules, including STATs. The V617F substitution activates JAK2 by a mechanism still not completely understood [59–65]. Cytokine receptors are indispensable for inducing constitutive signaling by JAK2 V617F at endogenous expression levels [26]. When *JAK2* V617F is introduced in an IL-3 dependent cell line such as Ba/F3, it induces cytokine hypersensitivity or independence [5], but this process is greatly facilitated by the presence of homodimeric receptors, such as EPOR, MPL, and G-CSFR (Figure 1(d)) [26]. This explains how *JAK2* V617F can induce erythroid, megakaryocytic and granulocytic proliferation and thus the three types of MPNs. In humans, PV is usually associated with the presence of *JAK2* V617F. The level of JAK2 V617F is an important determinant of the phenotype of the myeloproliferative disorders, *JAK2* V617F heterozygous clones being largely prevalent in ET and homozygous in PV [66–68]. This difference between homozygous and heterozygous mutation suggests that activation of MPL may require less JAK2 V617F than the activation of EPOR. Mouse models have confirmed that a low level of *JAK2* V617F expression induces an ET phenotype while higher levels induce a PV phenotype [19].

Mutations in exon 12, although not located in the pseudokinase domain, result in discrete changes in the conformation of JAK2. In contrast to JAK2 V617F, JAK2 exon 12 mutants exhibit a lower binding affinity for MPL than for EPOR in cell lines [69]. Accordingly, *JAK2* exon 12 mutations induce a PV phenotype in mice [15].

Importantly, MPL plays a critical non-redundant role in the development of *JAK2* V617F MPNs in a transgenic model of ET [31]. Indeed, JAK2 V617F was unable to increase the platelet count of the *mpl*$^{-/-}$ thrombocytopenic mice, as expected, but also did not expand the HSC compartment, suggesting that most effects of JAK2 V617F on HSCs are dependent on its signaling through MPL. However, more recent studies have shown that *JAK2* V617F-positive MKs regulate the HSC cell cycle and function [70]. Thus, it is still unclear whether the effects of JAK2 V617F on HSCs are cell or non-cell autonomous.

2.5. How do JAK2/MPL and CALR mutants activate the MPL-JAK2 axis?

Several lines of evidence have established that expression of MPL is crucial for the MPN phenotype, irrespectively of the driver. One exception could be JAK2 exon 12 mutants, but no validation of independence from MPL is currently available. Importantly, the precise mechanism of MPL-JAK2 activation differs between the three main driver mutations, and these differences may be relevant for the particular phenotype and progression of the disease.

2.5.1. JAK2 V617F activation of MPL

JAK2 V617F binds to the cytosolic domain of MPL activating TPO-independent signaling [44]. This leads to partial down-modulation of surface and total MPL [44,71,72], a phenomenon discovered early on by the Spivak group [71,72]. Mechanistically, MPL recycling is reduced by JAK2 V617F, which triggers proteasome-mediated degradation of MPL [44]. Inhibition of JAK2 V617F catalytic activity by JAK2 inhibitors restores cell-surface levels of MPL, re-establishing

recycling [44]. The driving force behind MPL down-modulation is the negative effect induced by MPL at high levels of activation, which appears to inhibit cell proliferation in both cell lines and MKs [44,73]. Thus, the mutated clone adjusts the levels of signaling by MPL to be able to sustain optimal cell survival and proliferation. In the absence of down-modulation, JAK2 V617F would activate levels of MPL that would induce negative signaling. The precise mechanism by which MPL induces negative signaling could involve excessive STAT or MEK/ERK signaling, and induction of p21 and of EGR1 leading to senescence [73].

In the absence of its ligand, EPOR is maintained in an inactive state and can be activated by EPO-induction of one particular dimeric interface [74]. In contrast, MPL can be activated in a multitude of manners including several point mutations and antibodies that induce different dimeric interfaces [42,43]. While arguments exist both in favor and against a predimerized state stabilized by TM domain interactions for EPOR, JAK2 V617F was demonstrated to promote close apposition of the EPOR C-terminal cytosolic domains [75]. In an unpublished work (Balligand, Leroy et al. in preparation), we show that the same is true for MPL. However, no basal level of dimerization can be detected using the nanoluciferase complementation system, NanoBit, in contrast to EPOR for which a significant basal level can be detected. For both receptors, JAK2 V617F brings the intracellular domains closer. Indeed, a recent study using diabodies targeting EPOR showed that JAK2 V617F requires closer apposition of the EPOR cytosolic domains than ligand-activated EPOR-JAK2 complexes [61]. Thus, the conformation of JAK2 V617F differs from that of wild type JAK2 by requiring closer contact between receptor monomers.

2.5.2. How do MPL mutants activate the MPL/JAK2 axis?

The two major classes of *MPL* activating mutations (S505N and W515 mutations) [11,39,46] induce persistent dimerization in active orientations for MPL, although the dimers differ. MPL S505N induces a milder phenotype *in vivo* and resembles coiled-coil MPL fusion protein [42]. In this case, dimerization is triggered by hydrogen bonds in the middle of the TM domain between the polar asparagine residues [41,45]. The W515 mutants induce a more severe phenotype *in vivo*, with a rapid myelofibrosis in the mouse retroviral model, and the dimer conformation is different from that of S505N. Dimerization is triggered *via* the loss of the tryptophan residue [76], which normally tilts the receptor [77] so that JAK2 molecules cannot phosphorylate each other. Magnetic resonance and infrared spectroscopy have shown important differences between the active conformations of S505N and W515 mutations, indicating that subtle differences in interface rotation and tilt can lead to different *in vivo* effects [41].

2.5.3. How do CALR mutants activate the MPL/JAK2 axis?

CALR mutants activate the MPL/JAK2 axis by an unusual mechanism. CALR mutants, which exhibit a frame-shifted C-terminus with positively charged residues, acquire the ability to stably bind, dimerize, stabilize and transport out of the ER dimers of MPL that exhibit two features: they carry immature high mannose sugars, such as on asparagine 117, and adopt an active dimeric orientation [28]. The binding of CALR mutants to MPL extracellular domains leads to the close apposition of MPL intracellular domains, and thus to JAK2 C-terminal domains inducing JAK2 activation (Figure 1(c)). No such effects are observed on EPOR. Using mass spectrometry, we could demonstrate that complexes of MPL extracellular domain and CALR mutants maintain nine mannoses at N117, but possibly do not need the 1-glucose, suggesting that the new CALR tail stabilizes a complex between CALR and immature MPL [78]. This differs from wild-type CALR, which only exhibits transient interactions with N-linked glycosylated proteins. The complex travels through the Golgi apparatus and arrives at the cell surface [78]. Genetic evidence suggests that cell surface localization is crucial for the ability of CALR mutants to induce autonomous growth of hematopoietic progenitors. Thus, CALR mutants behave as rogue chaperones bringing active and immature MPL molecules to the surface. CALR mutants themselves have been shown to be dimers/oligomers [79]. Finally, we have shown that CALR mutants can be detected in the plasma of patients and of the KI mice, at levels of 20–100 ng/mL. The precise cellular source of secreted CALR mutants is not known but could be myeloid cells from the clones that do not express MPL. Thus, secreted CALR proteins might play the role of a rogue cytokine (Pecquet, Balligand et al. in preparation).

Overall, JAK2 V617F, MPL, and CALR mutants all depend on the MPL-JAK2 axis for inducing MPN. Differences arise from the precise cellular compartment and relative intensity of activation of the MPL-JAK2 pathways. This includes a higher dependency of JAK2 V617F on the PI-3-kinase pathway [80,81], different degrees of down-modulation of MPL levels, and the choice of STATs. While gene expression profiling demonstrated common gene expression signatures between CALR and MPL mutant progenitors [82], *CALR*-mutated patients exhibit significantly higher allele burdens approximately 10 years earlier than *JAK2* V617F- or *MPL*-mutated patients. This suggests that factors other than MPL itself may be involved in the rapid CALR mutant clonal dominance.

2.6. The role of chromatin changes induced by driver mutations in MPN pathogenesis

The pathogenesis of MPNs does not seem to be limited to the canonical pathway of JAK2 activation and downstream signaling. A link between persistent JAK2 signaling and chromatin regulation has been proposed based on results from embryonic stem (ES) cells. Levels of chromatin-bound Heterochromatin Protein 1 alpha (HP1alpha) were decreased in *JAK2* V617F-expressing ES cells, consistent with a role for JAK2 in (direct or indirect) tyrosine phosphorylation of HP1alpha, preventing its interaction with chromatin [83]. In contrast, JAK2 inhibition repressed self-renewing genes like Nanog in ES cells, consistent with the re-establishment of the repressing function of non-phosphorylated HP1alpha [83].

JAK2 V617F and JAK2 K539L (a model for *JAK2* exon 12 mutations) also interact with and phosphorylate PRMT5, thereby preventing its ability to methylate histone substrates [84]. As a consequence, increased colony formation and erythroid differentiation occur [84].

Yet another link between JAK2 V617F and chromatin was reported when exploring the mechanisms by which *NFE2* is pathologically overexpressed in MPNs and its consequences [85]. JAK2 V617F is suggested to induce *NFE2* overexpression *via* HP1alpha inhibition, leading to high levels of JMJD1C demethylase and thus to a global decrease in H3K9me1 and H3K9me2 substrates [85]. Since down-modulating JMJD1C reduces MPN cell proliferation, JMJD1 was suggested to be an important player in MPNs [85].

Intriguingly, FLI1, a transcription factor involved in MPL expression, was identified among CALR mutant interactors [86]. It is not clear how CALR mutants arrive in the nucleus, but this potential novel effect might explain initial data where expression of CALR mutants appeared to induce or select for MPL expression, before actual activation of MPL signaling [12,87]. In addition, persistent regulation of FLI1 might induce profound effects on hematopoiesis, more particularly on megakaryopoiesis.

It is presently unknown whether these nuclear functions of JAK2 V617F and CALR mutants are indeed crucial for the MPN phenotype and the deregulated proliferation. If it is the case, it will be a limitation for therapies targeting MPL, as it seems unlikely that homodimeric type I receptors are involved in these functions.

2.7. MPL/JAK2 activation as a therapeutic target

As described above, MPL has emerged as a target of choice in therapeutic approaches for MPNs. Several *in vitro* and clinical studies have suggested that inhibition of MPL or downstream molecules could be effective. Indeed, in myelofibrosis trials, JAK2 inhibitors were efficiently used independently of the driver mutant (JAK2 V617F or MPL/CALR mutants). *JAK2* V617F-expressing cells (which co-express MPL) are synergistically inhibited by the combination of JAK2 and PI-3 kinase or mTOR inhibitors [80,81], and *CALR*-mutated cells (which also express MPL) are synergistically inhibited by JAK2 and MAPK inhibitors [28], suggesting that the relative usage of downstream pathways differs between JAK2 V617F and CALR mutant oncoproteins. More specifically, both the soluble MPL extracellular domain [28] and a peptide electrostatically complementary to the positive-charged sequence of CALR mutants prevent activation of MPL in *CALR*-mutated target cells [86,87]. Of interest, JAK2 V617F activates persistent MPL-dependent STAT3 signaling that induces transcriptional activation at the promoter of Programmed Death Ligand 1 (PD-L1) leading to enhanced PD-L1 expression at the surface of monocytes, MKs and platelets [88]. In turn, these cells reduce T-cell activation, metabolism and cell cycle, reducing T-cell responses against the mutated clone. Data in a xenotransplantation model suggest that inhibition of PD1 could be a promising strategy in MPN treatment [88].

Theoretically, there are several avenues for obtaining MPL inhibitors (Figure 2). First, antagonist antibodies that prevent TPO binding have been studied (Figure 2(a)) [89]. These prevent TPO effects and could be useful in the rare cases of TPO-overexpression. Such antibodies do not inhibit MPL activation by MPN driver mutations, but they may decrease the myeloproliferative phenotype as shown in a *Tpo* knockout mouse model [18,89]. Second, an antibody targeting the extracellular domain or the extracellular JM region (around H499, the binding site of eltrombopag) [41,90] could be inhibitory or could trigger either internalization and degradation or antibody-dependent cytotoxicity (Figure 2(b)). Third, small molecules binding to the accessible JM regions, either extracellular or intracellular, could in principle maintain the high natural tilt of the ligand-free MPL and prevent its activation (Figure 2(c)). Again, the region around H499 could be a good target as it switches conformations between a random coil and an α-helix and from inactive to active states [41]. Fourth, it could be interesting to identify small molecules binding to the intracellular region adjacent to the membrane in the vicinity of, and without overlapping with, W515 that might adopt a common structural change upon W515L/K/R/S/A mutation (Figure 2(d)) [77]. Lastly, one approach could take advantage of the differences between the interaction of JAK2 V617F or wild-type JAK2 with MPL and with EPOR, G-CSFR or other receptors (Figure 2(e)). While Box 1 is conserved, recent X-ray crystal structure data suggest unique interactions for each receptor [60,91]. Detachment of JAK2 V617F from MPL or of JAK2 from MPL mutants would interrupt the activating events leading to MPNs.

While JAK2 V617F and MPL W515 mutants are intracellular targets, the situation with CALR mutants offers better prospects (Figure 3). CALR mutants interact with the extracellular MPL domain, and the complex is transported to the cell surface [28] resulting in the exposition and secretion of the CALR mutants[92]. Thus, antibodies scavenging circulating CALR mutant (Figure 3(a)), antibodies preventing MPL-CALR mutant interactions (Figure 3(b)) and antibodies targeting surface CALR mutant to degradation (Figure 3(c)) might reduce the pathogenic clone. In addition to antibodies, vaccines or T-cell immunotherapy could be developed against the last 40 residues of the novel CALR mutant sequences (Figure 3(e)). These approaches depend on HLA presentation, and an immunosuppression with HLA1 defects [92] might be present in such MPN patients. Small molecules to destabilize the binding of oligomeric CALR mutant proteins to the extracellular domain of MPL could also be obtained (Figure 3(d)). These molecules would have to prevent the binding of the lectin domain of CALR mutants to MPL, and would have the potential to inhibit both intracellular and cell surface complexes, while antibodies would only target the latter.

There are several major challenges that account for why this has not yet been achieved in preclinical or clinical models. MPL is essential for maintaining the HSC reservoir as it regulates quiescence of long-term HSCs. Absence of TPO or MPL function leads to exhaustion of HSCs *in vivo*. Thus, a simple TPO antagonistic molecule or a strong complete inhibitor of both wild-type and mutant functions of MPL may be dangerous. On the other hand, since MPL is so easily activated by multiple mutations in multiple domains, even by antibodies against N-terminal tags, it has been very difficult to obtain a molecule able to prevent MPL activation, and especially to prevent MPL mutant activation.

3. Conclusion

The pathogenesis of MPN is related to a deregulated JAK2 activation by mutations in three genes: *JAK2, CALR*, and *MPL*.

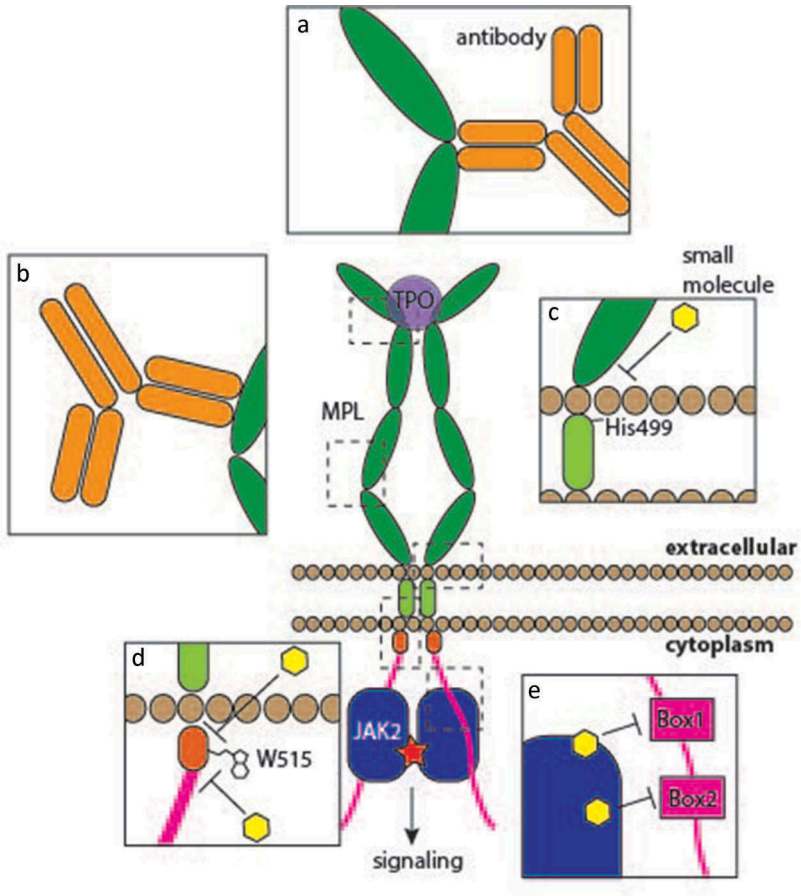

Figure 2. Potential methods of MPL inhibition in MPN. In its active state, either through TPO binding to the extracellular domain or driven by activating mutations in its membrane or juxtamembrane domains, the receptor MPL drives JAK-STAT signaling via the JAK2 associated with its cytoplasmic domain. Several methods of potentially inhibiting MPL can be envisaged. These include: (a) blocking TPO binding with antagonist antibodies; (b) inhibition, induction of degradation or toxicity via antibody targeting of the extracellular domain; (c) preventing activation of the receptor with small molecules that maintain the high natural tilt characteristic of the receptor in the absence of ligand; (d) using small molecules that bind the region of MPL immediately below the membrane, near W515, that alter the structural changes induced by activating mutations at W515; and lastly (e) small molecule inhibition of the binding of JAK2 to MPL via its box1 or box2 motifs, potentially having the advantage of specifically disrupting JAK2 and MPL interactions, whilst preserving those between JAK2 and other receptors.

Whereas the two latter activate JAK2 through MPL, JAK2 V617F activates downstream signaling *via* the three homodimeric type I receptors (EPOR, G-CSFR, and MPL). However, MPL is also indispensable for JAK2 V617F for its unique function on HSC. Thus, targeting MPL is an obvious option in MPN therapy, while remaining extremely challenging as it must not alter MPL function in normal hematopoiesis and thus must specifically target the interaction between JAK2 V617F or CALR and MPL or the MPL mutant conformation in the case of *MPL* mutations.

4. Expert opinion

Among hematological malignancies, *BCR-ABL*-negative MPNs are among the best understood. Driver mutations have been uncovered for almost 90% of such MPNs. Conceptually, these MPNs exhibit persistent pathologic JAK2 activation by multiple mechanisms, and in all cases, the TPO receptor, MPL, is key for achieving this pathogenic event. The vast majority of MPNs carries a unique acquired *JAK2* somatic mutation, *JAK2* V617F, which leads to activation of EPOR, MPL, and G-CSFR, but even in PV where EPOR is the major player for inducing erythrocytosis, expression of MPL is crucial to maintain the pool of mutated HSCs. For the rest of MPNs, MPL involvement is also key for the disease phenotype, either *via MPL* mutations or *via CALR* mutations, which activate MPL. The precise choice of STATs and ancillary pathways activated by JAK2 V617F *versus* MPL mutant and CALR mutant-MPL complexes are slightly different explaining the different phenotypes; however, a continuum exists for all *BCR-ABL*-negative MPNs.

The common event for all such MPNs is the persistent activation of the MPL-JAK2 axis. *In vitro* data using primary human cells and cell lines, as well as *in vivo* transgenic and KI mouse models of MPNs have established the crucial role of the MPL-JAK2 axis. There is also evidence that this MPL-JAK2 activation, while absolutely required, might not suffice for disease initiation. Other environmental (age, inflammation) and genetic (predisposition, somatic mutations) factors may contribute to the MPN initiation and clonal dominance. Non-canonical roles of JAK2 V617F and CALR mutants, such as the nuclear JAK2 function or regulation of Ca2+ signaling by CALR mutants, may also be involved in the pathogenesis of MPNs. Nonetheless, JAK2 and MPL appear to be the main therapeutic targets. Effective therapy would interrupt the pathogenic chain of events and reverse the phenotype, with a major decrease or eradication of the mutated clone, leading to

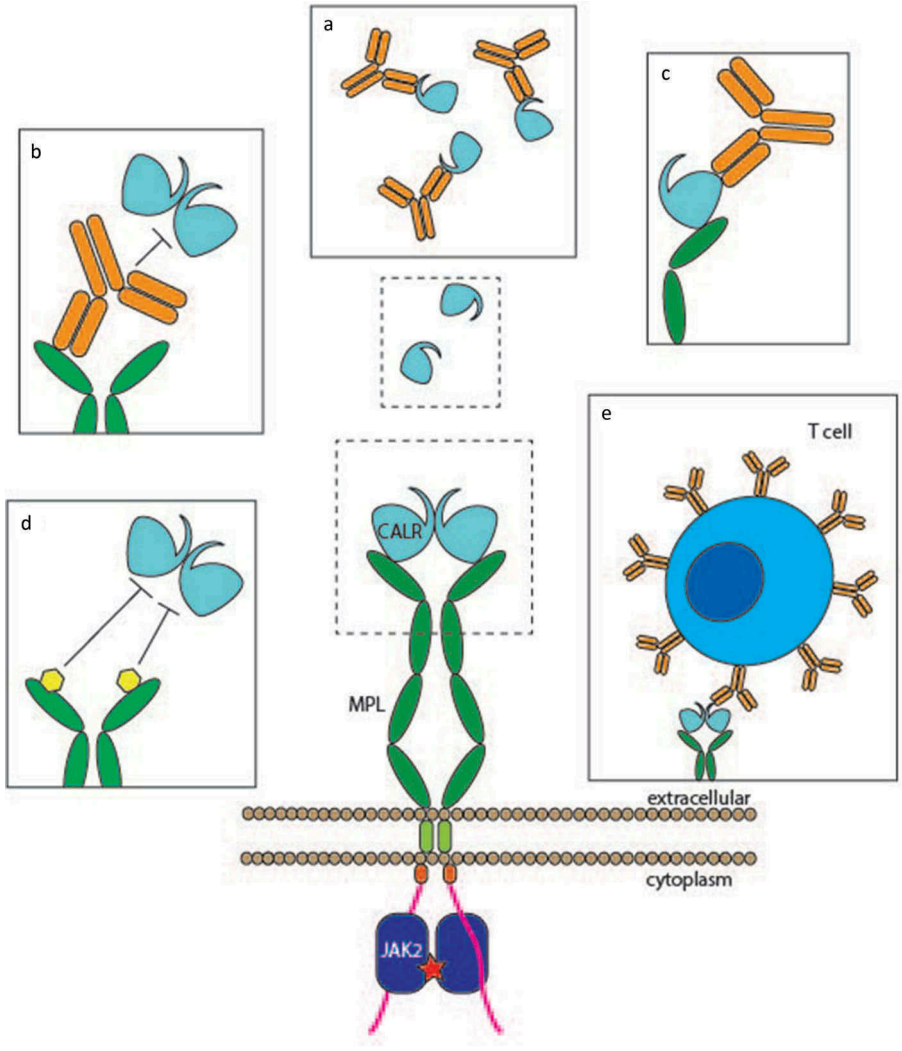

Figure 3. Targeting CALR in MPN. The interaction between CALR mutants and MPL at the cell surface allows pathogenic JAK2 signaling to occur in the cytoplasm without stimulation by TPO. This transport of the complex to the cell surface also allows mutant CALR to be secreted. Potential methods of inhibiting this pathogenic signaling by targeting CALR include: (a) the development of antibodies scavenging circulating mutant CALR; (b) antibodies preventing the interaction between MPL and mutant CALR; (c) reducing the pathogenic clone via antibodies targeting surface CALR mutant for degradation; (d) small molecules to prevent the binding of mutant CALR to MPL's extracellular domain; and (e) T cell immunotherapy or vaccines targeting the novel 40 amino acid C-terminal tail.

a cure of the disease. However, as discussed above, other factors/mutations that play synergistic effects with the driver mutations, may prevent a complete cure, although it is expected that an effective targeting of the pathogenic MPL-JAK2 axis would lead to durable anti-tumoral activity, similar to that observed with imatinib in Chronic Myeloid Leukemia. Presently the targeting of the oncogenic activation of JAK2 by ruxolitinib has shown its limitations by exerting a mild or undetectable effect on the clonal disorder. This is mainly because the inhibition of JAK2 by ruxolitinib is not specific for the MPN clones. A complete inhibition of JAK2 would lead to major toxic effects due to the absolute requirement of JAK2 for normal HSC function, erythropoiesis and megakaryopoiesis. To achieve significant clonal inhibition, specific pharmacological agents need to be obtained that would either detach mutated JAK2 from MPL (in *JAK2* V617F cases), specifically inhibit JAK2 V617F, or specifically inhibit JAK2 activation by MPL mutants or by CALR mutant MPL complexes. For the latter, the developed molecules should target and disrupt the contact interface between the extracellular domain of MPL and the CALR mutant proteins. Such an approach seems feasible, but should take into consideration manners to target intracellular interactions, and to develop specificity for mutant over wild-type proteins. Recent studies exploring in detail the structure and function of the cytosolic JM domains of cytokine receptors and JAKs [60,75,91,93] are likely to generate relevant avenues for receptor-specific inhibition of JAK activation.

The most important advance would be to succeed in specifically inhibiting JAK2 V617F. However, two studies reported that the circuit of conformational changes adopted by JAK2 V617F is not required for physiologic function *via* the majority of type I and type II cytokine receptors with one notable exception, the IFN γ receptor [75,94]. This complex contains two JAK1 and two JAK2 molecules assembled with a tetrameric complex of two IFNGR1 and IFNGR2 proteins. Strikingly, all mutations that inhibit JAK2 V617F activation inhibit the ligand-mediated receptor complex response [75].

Thus, the circuit exploited by V617F in activating JAK2 is common to the physiologic IFN γ /IFN γ receptor axis. Specific JAK2 V617F inhibitors are likely to affect immunity to a higher degree than non-specific pan-JAK2 kinase domain inhibitors, and inhibitors of IFN γ are likely to also exert specific activity against JAK2 V617F.

Targeting signaling pathways downstream of MPL-JAK2 could synergize with JAK2 inhibitors or with MPL/CALR interaction modulators. SH2-interacting STAT inhibitors [95] are emerging as novel tools to block STAT5 and STAT3 signaling, while a number of small molecules target RAS-MAPK and PI3'-kinase-Akt pathways. How blocking these pathways will impact the MPL-JAK2 axis remains to be seen.

Thus, targeting the MPL-JAK2 axis offers many opportunities, but also many challenges with respect to protein structure and inhibitor screening and design. A major limitation resides in the absence of X-ray crystal structures for the MPL extracellular domain, and for segments of JAK2 in complex with the intracellular MPL domain. If the structure and mechanics of the extracellular MPL domain is shown to resemble EPOR, then diabodies [61] or other proteins targeting and separating the extracellular domains of MPL could be considered for inhibiting MPL mutants or MPL-JAK2 V617F complexes, while other antibodies or small molecules could be considered for inhibiting signaling by CALR mutant MPL complexes.

The therapeutic target in MPNs is a complete inhibition or elimination of pathogenic MPL-JAK2 complexes. An incomplete decrease holds the potential to enhance the thrombocytosis phenotype, as was shown with the paradoxical thrombocytosis induced by partially defective MPL P106L [96] and a series of engineered MPL mutants or lower MPL expressors [97,98].

Funding

This paper was funded by Fondation les avions de Sebastien [ARC 16/21-073], Institut National de la Santé et de la Recherche Médicale, Institut National Du Cancer (PLBIO 2017), Ligue Nationale contre le Cancer (Equipe labellisée) the GR-Ex which is funded by the program "Investissement d'Avenir" and Walloon Excellence in Life Sciences and Biotechnology.

Declaration of interest

S Constantinescu is a co-founder of AlsaTech, Boston MA and MyeloPro Research and Diagnostics GmbH, Vienna, Austria. S Constantinescu acts as a consultant for Novartis, Basel, Switzerland and on speakers bureau for Shire, Amgen, and Teva. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial relationships or otherwise to disclose.

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