

Molecular determinants of pathogenesis and clinical phenotype in myeloproliferative neoplasms

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ABSTRACT

The myeloproliferative neoplasms are a heterogeneous group of clonal disorders characterized by the overproduction of mature cells in the peripheral blood, together with an increased risk of thrombosis and progression to acute myeloid leukemia. The majority of patients with Philadelphia-chromosome negative myeloproliferative neoplasms harbor somatic mutations in Janus kinase 2, leading to constitutive activation. Acquired mutations in calreticulin or myeloproliferative leukemia virus oncogene are found in a significant number of patients with essential thrombocythemia or myelofibrosis, and mutations in numerous epigenetic regulators and spliceosome components are also seen. Although the cellular and molecular consequences of many of these mutations remain unclear, it seems likely that they interact with germline and microenvironmental factors to influence disease pathogenesis. This review will focus on the determinants of specific myeloproliferative neoplasm phenotypes as well as on how an improved understanding of molecular mechanisms can inform our understanding of the disease entities themselves.

Introduction

The classical Philadelphia-negative myeloproliferative neoplasms (MPNs) are characterized by clonal expansion at a hematopoietic progenitor level with the overproduction of mature myeloid and erythroid progeny. Clinically, they share the features of bone marrow hypercellularity, increased incidence of thrombosis or hemorrhage, and an increased rate of transformation to acute myeloid leukemia, which is usually fatal. Since these are chronic conditions that normally manifest well in advance of leukemic transformation, they offer an invaluable model for studying the earliest steps of leukemogenesis, including the ways in which somatic mutations perturb stem and progenitor cell function.

Current diagnostic criteria separate Philadelphia-negative MPNs into three distinct disease entities: polycythemia vera (PV) – primarily characterized by a raised red cell mass; essential thrombocytosis (ET) – characterized by an isolated increase in platelet numbers; and idiopathic/primary myelofibrosis (MF) – in which the hematopoietic compartment is gradually replaced with collagen fibers, leading to bone marrow failure and extramedullary hematopoiesis, and which is often associated with constitutional symptoms.

Mutations affecting cytokine receptor signaling pathways

Mutations in Janus Kinase 2 (JAK2)

In 1951 William Dameshek hypothesized that rather than these being “pure” proliferations, the myeloproliferative conditions PV, ET and MF may represent differing manifestations of a single underlying process.¹ This hypothesis was borne out by the discovery of a valine to phenylalanine substitution at codon 617 (V617F, due to a G>T substitution), of the *JAK2* gene in over 95% of patients with PV and

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50-60% of those with ET or MF²⁻⁵ JAK2 is a cytoplasmic tyrosine kinase, required for signal transduction from type I cytokine receptors which include those for thrombopoietin, erythropoietin and granulocyte colony stimulating factor (G-CSF), and therefore plays a vital role in myelopoiesis.

Molecular consequences of JAK2 mutations

The V617F mutation occurs in the JH2 (or “pseudokinase”) domain (see Figure 1A) and results in constitutive activation of the JH1 kinase domain. The mechanism by which this occurs is increasingly becoming clear.⁶ In short, there is evidence that the mutation may reduce the auto-inhibitory function of the JH2 domain *via* changes in JH1-JH2 conformation⁷ and adenosine triphosphate (ATP) binding.⁸ The expression of JAK2V617F has been shown to allow for JAK2 signaling in the absence of cytokine receptor ligation,^{3,4} but the expression of type I cytokine receptors⁹ and a functional FERM domain (required for receptor binding)¹⁰ are still required for JAK2 signaling and cytokine-independent growth. Furthermore, the V617F mutation may allow for the escape from negative regulation by the suppressor of cytokine signaling 3 (SOCS3).¹¹ The increased JAK2 signaling recapitulates that seen in the physiological response to cytokine binding, namely the increased activation of signal transducer and activator of transcription (STAT) 1, 3 and 5, mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways.^{3,5,12} STAT5 in particular is thought to have a crit-

ical role in the pathogenesis of PV.^{13,14}

Mutations in exon 12, located between F533 and F547, within the linker region between the SH2 and JH2 domains (Figure 1A), are found in 1-2% of patients with PV.^{15,16} These mutations also induce constitutive activation, but to a greater degree than JAK2V617F, with greater JAK2 phosphorylation and MAPK pathway activation,¹⁵ and also result in cytokine-independent growth. The mechanism by which these mutations act is less well understood, but given their location, it is likely that they also disrupt JH1-JH2 domain interactions.

Moreover, a number of non-canonical roles have also been described for JAK2, which may be perturbed by pathogenic mutations. JAK2 has been found to localize to the nucleus, where it can phosphorylate tyrosine 41 on histone H3.¹⁷ This has been associated with the increased expression of *LMO2*, which has been implicated in leukemogenesis. JAK2V617F expression has also been associated, *in vitro*, with an increase in homologous recombination, the activation of DNA-repair mechanisms, aneuploidy and the acquisition of a mutator phenotype.¹⁸ Furthermore, there is evidence that JAK2V617F can increase the production of reactive oxygen species and reduce the apoptotic response to DNA damage by inhibiting the Bcl-xl deamidation pathway.^{14,19}

Cellular consequences of JAK2 mutations

Mutations in *JAK2* have been shown to occur in cells near the top of the hematopoietic hierarchy and can be

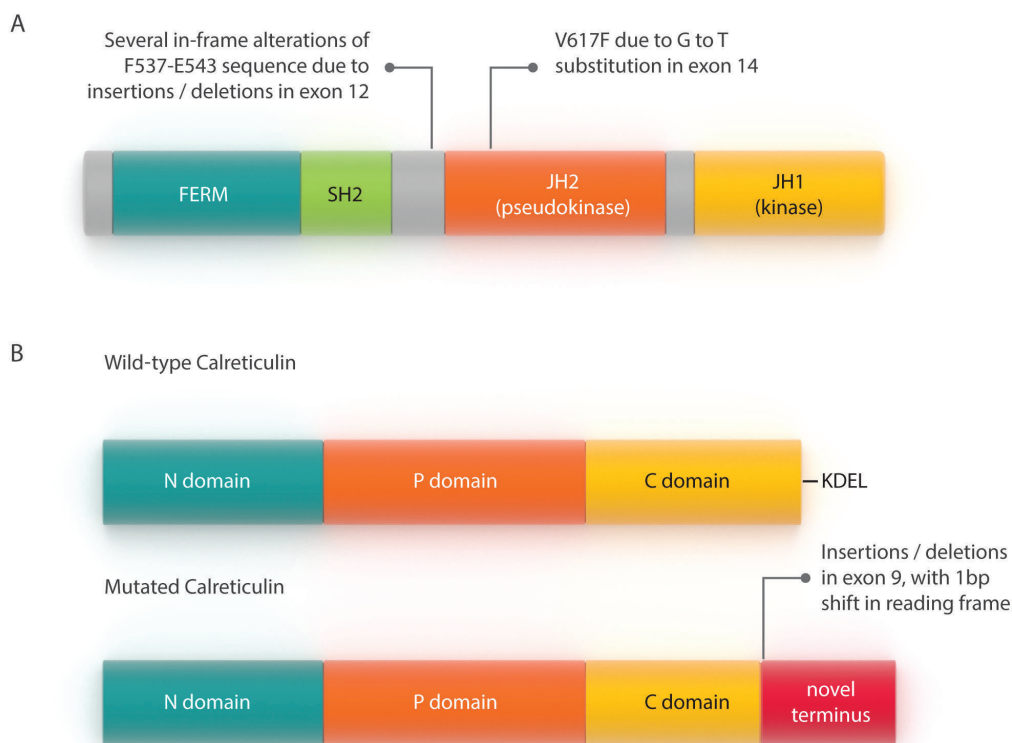


Figure 1: Panel A shows a schematic representation of the structure of the *JAK2* gene, indicating the sites of common *JAK2* mutations. Panel B shows a schematic representation of the structure of the *CALR* gene and the consequences of the common mutations seen in myeloproliferative neoplasms (MPNs). JH: janus homology; FERM: 4.1 protein, ezrin, radixin and moesin; bp: base pair; KDEL: lysine, aspartic acid, glutamic acid, leucine; SH2: Src homology 2.

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found in the hematopoietic stem cell (HSC) compartment.^{20,21} These observations are consistent with the long-term persistence of *JAK2*-mutated MPNs, and the fact that the mutation can be found in cells of the lymphoid as well as myeloid lineages in some cases.²² Several lines of evidence indicate that *JAK2V617F* does not confer an advantage at the HSC level. Xenografts of *JAK2*-mutant patient cells into immunodeficient animals suggest that *JAK2* mutations do not result in a strong self-renewal advantage;²¹ a finding that is recapitulated in several knock-in mouse models.^{23,24} Instead, *JAK2* mutant HSCs are skewed towards symmetrical differentiation with a subsequent expansion of the progenitor pool, rather than self-renewal, and do not demonstrate an advantage in competitive transplantation experiments.²⁵ These observations have led to the suggestion that *JAK2V617F* alone is insufficient to initiate disease and that additional mutations are required. This is consistent with the detection of the *JAK2V617F* mutation on its own in patients without overt myeloid malignancy.²⁶⁻²⁹ An alternative explanation for these findings is that the expansion of the progenitor (rather than the stem cell) pool is sufficient to mediate disease development, an idea that is reinforced by recent studies which demonstrate that a pool of long-term multipotent progenitors are the main drivers of adult hematopoiesis.³⁰

Finally, it is likely that there is functional heterogeneity within the *JAK2*-mutated HSC pool and across disease subtypes. For instance, there is evidence that the ability to self-renew, and therefore stably engraft, may decrease with increasing levels of *JAK2* expression,³¹ similar to the differences in stem cell behavior seen at different expression levels of *STAT5*³² and oncogenic *NRAS*.³³

Mutations in MPL and CALR

A number of hotspot missense mutations in exon 10 of myeloproliferative leukemia (*MPL*), the human homologue of the murine myeloproliferative leukemia virus oncogene (*v-MPL*), the cell surface receptor for thrombopoietin, such as M515L and M515K (and less commonly S505N), have been reported in patients with ET and MF (Figure 2A). *MPL* mutations are associated with increased *STAT3*, *STAT5*, *ERK* and *AKT* signaling and cytokine autonomous growth.^{34,35} More recently, the S204P and Y591N mutations were described in a cohort of patients with ET or MF, otherwise found to be lacking established phenotypic driver mutations. These mutations were found to have a weak gain-of-function effect, either with a degree of thrombopoietin-independent growth or signaling, or thrombopoietin hypersensitivity.^{36,37}

Mutations in calreticulin (*CALR*) are also found in approximately 25-35% of patients with ET and 35-40% of those with MF (Figure 2A). These are exclusively insertions/deletions (most commonly a 52 base pair deletion or 5 base pair insertion) in the final exon (Figure 1B), and in all cases these result in a 1 base pair shift in the reading frame.^{38,39} This points strongly to a specific gain of function involving the C-terminus of the protein. The mutual exclusivity of *JAK2*, *MPL* and *CALR* mutations point to a similar mechanism of action, as do the similarities in clinical phenotype between *CALR* and *MPL*. Furthermore, there is evidence that *CALR* mutations are also associated with increased *JAK-STAT* signaling,^{38,40} although some studies have suggested that other pathways may be of more importance.⁴¹

CALR is not known to have a direct role in cytokine signaling, hematopoiesis or cell fate decisions, and therefore the mechanism(s) by which *CALR* mutations result in megakaryocytic proliferation and an ET/MF phenotype were not initially apparent. *CALR* is known to be involved in the regulation of calcium uptake and release in the endoplasmic reticulum,⁴² and acts as a chaperone, together with calnexin and ERp57, to form part of the regulatory machinery involved in the folding and quality control of newly synthesized glycoproteins.⁴³ Differences in cytosolic calcium mobilization have been reported with the 52 base pair deletion,⁴⁴ suggesting that this may be one mechanism by which mutant *CALR* exerts its effect, and expression of the mutant protein does appear to be particularly restricted to megakaryocytes on immunohistochemical evaluation of bone marrow specimens.⁴⁵ More recently, it has been shown that *CALR* mutations can impart TPO-independence in both cell lines^{46,47} and retroviral mouse models,^{48,49} in a *MPL*- and *JAK2*-dependent manner, mimicking the effect of activating *MPL* mutations. This has been shown to be mediated by direct binding of *MPL* by the N domain of *CALR*, a phenomenon that uniquely occurs in the presence of the mutated C-terminus,^{48,49} leading to autocrine activation of *MPL*, *JAK2* dimerization and downstream *STAT5* and *ERK* phosphorylation.^{46,49}

It is therefore clear that the inappropriate activation of *JAK2* signaling is common to the three main phenotypic driver mutations (i.e., those in *CALR*, *MPL* and *JAK2* itself) and plays an important role in disease pathogenesis in each case, in keeping with the clinical efficacy of *JAK2* inhibition irrespective of the presence of the mutations in *JAK2*.⁵⁰

Other mutations in signaling pathways – LNK, CBL and RAS

Other than the aforementioned mutations in *JAK2*, *MPL* and *CALR*, a number of genes are also mutated in patients with MPNs (Figure 2B). As might be expected from the role of receptor signaling pathways in the pathogenesis of MPNs, loss-of-function mutations in negative regulators of receptor tyrosine kinases are seen, as well as mutations in members of downstream pathways.

LNK (lymphocyte specific adaptor protein, or *SH2B3*) binds both *MPL* and *JAK2* to act as a negative regulator of *JAK-STAT* signaling. *LNK* deficient mice display an MPN-like phenotype with megakaryocytic hyperplasia, cytokine hypersensitivity and splenomegaly.⁵¹ *LNK* exon 2 mutations are found in a small number of MPN patients. They are more often seen in advanced phase disease, but can also be found in patients with an erythrocytosis lacking a *JAK2* mutation, suggesting that these mutations may be sufficient to initiate disease.⁵²⁻⁵⁴

Casitas B-lineage lymphoma proto-oncogene (*CBL*) is an E3 ubiquitin ligase, which is specifically involved in the ubiquitination (and resultant degradation) of a number of receptor tyrosine kinases, as well as having a role in intracellular signal transduction. Its targets include *PDGFR*, *c-KIT*, *FLT3* and *MPL*.⁵⁵ Mutations in the RING domain, responsible for *CBL*'s ligase activity, have been described in MPNs.^{56,57} Mutations in *SOCS* proteins, another class of regulators of *JAK2* signaling, however, are only reported in a handful of patients,⁵⁸ but there is evidence that their expression may be suppressed in a subset of patients due to hypermethylation of *SOCS* pro-

motor regions.⁵⁹

Mutations increasing RAS pathway signaling are also observed in a small number of patients, but, as with CBL and LNK mutations, tend to be seen more in myelofibrosis, advanced phase/transformed disease or myeloproliferative neoplasm/myelodysplastic syndrome (MPN/MDS) overlap conditions.⁶⁰ *NRAS* mutations tend to occur in a hotspot location at codon 12. These mutations appear to result in a gain-of-function, resulting in myeloid differentiation and increased HSC self-renewal, and can induce a chronic myelomonocytic leukemia (CMML)-like disease in a mouse model.⁶¹ Similarly, negative regulators of RAS, such as *NF1*⁶² and *PTPN11*,⁶³ have also been found to be mutated in some MPN or MPN/MDS-overlap cases.

Mutations affecting other cellular processes

Mutations in epigenetic regulators

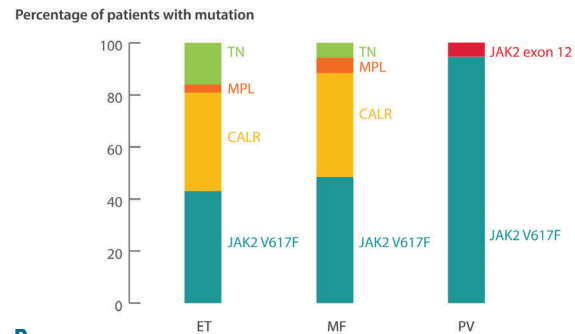
Other genes commonly mutated in MPNs are not specific to these conditions and are mutated across myeloid malignancies in general, including acute myeloid leukemia (AML) and MDS, as well as in some elderly patients without an overt myeloid malignancy.²⁶⁻²⁹ These include genes involved in epigenetic regulation and messenger RNA (mRNA) splicing (summarized in Figure 2B and Figure 3), but their role in the pathogenesis of MPNs is less well understood. The most commonly mutated of these is a member of the TET family, ten-eleven translocation 2 (*TET2*); loss-of-function mutations which are found in approximately 10% of MPNs.^{64,65} *TET2* converts 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), a process which is thought to be particularly important for gene regulation in stem cells and embryonic development. A reduction in 5-hmC is observed in *TET2* mutated patients, and is associated with increased self-renewal capacity and myeloid bias.^{66,67} The *HOXA* cluster, which is implicated in lineage commitment, is known to be regulated by *TET2*, suggesting one possible mechanism by which *TET2* mutations may result in a differentiation block.⁶⁸

Hotspot mutations in isocitrate dehydrogenase 1 and 2 (*IDH1* and *IDH2*) are described in <5% of cases.^{62,69} These enzymes catalyze the conversion of isocitrate to α -ketoglutarate, but these mutations result in the production of 2-hydroxyglutarate, which inhibits Jumonji-C domain histone demethylases. This leads to histone hypermethylation and also inhibits *TET2* activity, which in turn results in a differentiation block.^{70,71} *TET2* mutations do not appear to be associated with particular MPN subtypes, but have been found, in one study, to correlate with poorer overall survival and increased progression to AML,⁷² while *IDH* mutations are more commonly found in MF or transformed disease.⁶⁹

Loss-of-function mutations (including dominant negative missense mutations at codon 882) in DNA methyltransferase 3A (*DNMT3A*), a protein responsible for *de novo* methylation of CpG dinucleotides, are found across MPN subtypes.⁷³ As with *TET2* mutations, their exact role in MPN pathogenesis is not yet fully understood, but it is thought that the resultant epigenetic deregulation results in the upregulation of "HSC fingerprint" genes such as *GATA3* and *RUNX1* and the downregulation of differentiation factors such as Ikaros, together resulting in a differentiation block and HSC expansion.⁷⁴

Mutations of genes involved in histone methylation are

A



B

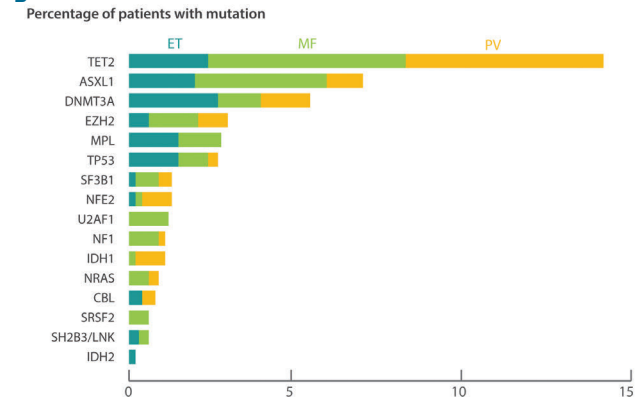


Figure 2. Panel A shows the relative frequencies of the mutually exclusive phenotypic driver mutations in *JAK2*, *CALR* and *MPL*, together with the proportions of those without mutations in these genes (triple negative – TN).^{38,39,72} Panel B shows the prevalence of additional mutations and their relative proportions across ET, PV and MF.^{39,62,72} ET: essential thrombosis; PV: polycythemia vera; MF: myelofibrosis.

overrepresented in myelofibrosis/transformed disease. *EZH2* (PcG Enhancer of Zeste Homolog 2) is the catalytic component of the polycomb repressive complex 2 (PRC2) and, together with *EED* and *SUZ12* acts to trimethylate histone H3 lysine 27 causing transcriptional repression. In contrast to *EZH2* mutations seen in lymphoma, those in MPNs tend to be loss-of-function mutations⁷⁵ that result in the derepression of a set of genes that includes a number of putative oncogenes (e.g., *LMO1* and *HOXA9*), and are associated with increased HSC self-renewal.^{76,77}

Mutations of additional sex combs like 1 (*ASXL1*) are also relatively common in MF.⁷⁸ *ASXL1*, a component of PRC1, is also known to regulate PRC2 and to have a role in the regulation of *HOX* genes. Accordingly, *ASXL1* mutations are associated with *HOXA* upregulation and the reduction in H3K27 methylation, both of which have been linked to impaired recruitment of *EZH2*.⁷⁹

In summary therefore, a number of the mutations found in MPNs affect the regulation of DNA/histone methylation in the HSC compartment and are linked to increased self-renewal and a block in differentiation; features that play a role in disease progression.

Mutations in mRNA processing machinery

Mutations in components of the spliceosome, including splicing factor 3B subunit 1 (*SF3B1*), serine/arginine-rich splicing factor 2 (*SRSF2*), U2 small nuclear RNA auxiliary

factor 1 (*U2AF1*) and zinc finger RNA binding motif and serine/arginine rich 2 (*ZRSR2*), are well described in MDS and also seen in MPNs,⁸⁰ particularly in MF or MPN/MDS overlap syndromes. The role of these mutations in disease pathogenesis is still not fully understood, but the effects of hotspot mutations of codon 95 of *SRSF2*, and codon 34 of *U2AF1* are perhaps the best explored, as knock-in mouse models have been made for each. *SRSF2* mutations appear to result specifically in skewed mRNA motif recognition (rather than loss of function), which is associated with alterations in exon usage in a number of genes. These include *EZH2* (leading to reduced expression), and bcl-6 corepressor (*BCOR*),⁸¹ which is also known to be mutated in myeloid malignancies. *U2AF1* mutations alter its 3' splice acceptor preferences leading to mis-splicing of a set of genes that includes *BCOR* and *SRSF2*.⁸² *EZH2* expression was found to be reduced in 63% of *U2AF1*- or *SRSF2*-mutated patient specimens, and this was associated with decreased H3K27 trimethylation.⁷⁷ Thus, while mutations in spliceosome components result in mis-splicing of multiple genes, there is evidence that they may also act *via* the dysregulation of PRC2 function.

Other determinants of MPN pathogenesis and phenotype

Germline variation

There are several reports of familial clustering of MPNs (where two or more family members are affected by MPNs), and there is a tendency for family members to present with the same MPN phenotypes. In fact, the relative risk of acquiring ET has been estimated to be approximately 12 times higher in first-degree relatives of MPN patients.⁸³ This strongly points to the existence of germline susceptibility factors.

Causes of familial MPNs include germline mutations of *RBBP6*,⁸⁴ and a high penetrance duplication of 14q32.2, which has been associated with overexpression of *ATG2B*, a mediator of autophagy, and *GSKIP*, a regulator of the WNT/ β -catenin pathway.⁸⁵ It appears that these changes operate *via* independent pathways: *RBBP6* mutations affect the p53 pathway and thereby influence the response to apoptotic stimuli and the risk of developing further mutations, while *ATG2B* and *GSKIP* overexpression promote megakaryopoiesis *via* increased thrombopoietin sensitivity.

A number of more common, but lower penetrance germline variants have been associated with MPN development, including single nucleotide polymorphisms (SNPs) present in, or close to, telomerase reverse transcriptase (*TERT*)⁸⁶ and "MDS1 and EVI1 complex locus" (*MECOM*).⁸⁷

Two *JAK2* haplotypes (46 and 1) are found to be in linkage disequilibrium (with the exception of one SNP). Whilst the combined haplotype (termed 46/1, or GGCC in reference to the defining alleles) is found in 24% of the population, it is found in up to 56% of patients with MPNs, with an odds ratio of 3 to 4.^{88,89} Together with *TERT* and *MECOM*-associated SNPs, the 46/1 haplotype is estimated to account for 55% of the population attributable risk of developing an MPN.⁸⁷

Furthermore, the *JAK2*V617F mutation preferentially occurs on the 46/1 allele. One possibility for this association is that the 46/1 haplotype is more prone to mutation

and therefore more likely to give rise to *JAK2*V617F and exon 12 mutations. However, perhaps a more likely possibility is that the occurrence of mutant *JAK2* in the context of the *JAK2* 46/1 haplotype confers an additional clonal or phenotypic advantage – the 'fertile ground' hypothesis. Further support for a clonal advantage for *JAK2* 46/1 is the fact that it is also seen more frequently in patients with *MPL* mutations.⁹⁰

One final SNP, in the intergenic region between *HBS1L* and *MYB* (rs9376092) has been found to be enriched in *MPL*- and *CALR*-mutated MPNs, and more frequently in *JAK2*-mutated ET patients.⁸⁷ With the exception of the 14q32.2 duplication mentioned above, which was found to be predominantly associated with an ET phenotype, the *HBS1L*-*MYB* SNP appears to be one of the few germline variants that is associated with a particular MPN phenotype.

Role of the microenvironment in MPN pathogenesis and the development of fibrosis

The importance of cell extrinsic factors, and in particular the bone marrow microenvironment, in the pathogenesis of MPNs is exemplified by the fact that the deletion of *Mib1* (causing dysregulated Notch signaling)⁹¹ or of retinoic acid receptor γ ,⁹² in non-hematopoietic cells alone was sufficient to induce a myeloproliferative phenotype in mouse models. One possible mechanism by which the microenvironment could support the development of MPNs may be through the secretion of soluble factors, such as tumor necrosis factor α (TNF α), interleukin-6 (IL-6), fibroblast growth factor (FGF) or interferon- γ -inducible protein 10 (IP-10). Such cytokines are produced by the bone marrow stroma and have been shown to promote the growth of MPN clones, while, in some cases inhibiting the growth of wild-type clones.^{93,94} Overexpression of NF- κ B, which has been described in patients with uniparental disomy (UPD) of chromosome 9, has also been suggested to result in resistance to transforming growth factor- β (TGF- β), which has been demonstrated to have inhibitory effects on the hematopoiesis and on myeloid cell lines.^{95,96} The secretion of proteases which disrupt the stromal cell derived factor-1/chemokine receptor 4 (CXCR4) axis, as well as the downregulation of CXCR4 itself by tumor cells, have both been associated with greater mobilization of HSCs, and these mechanisms may contribute to extramedullary hematopoiesis and potentially mediate the loss of HSC quiescence.⁹⁷

Whilst there are numerous mechanisms by which the microenvironment can affect MPN clones, neoplastic cells can also subvert their niche. Clonal megakaryocytes and monocytes themselves secrete a number of cytokines, which include FGF, interleukin-8, TGF- β and vascular endothelial growth factor, that stimulate angiogenesis and drive fibroblast differentiation and recruitment, leading to bone marrow fibrosis.^{98,99} The secretion of other cytokines by the clone, such as TNF α , may create an autocrine/paracrine loop, promoting the growth of the tumor clone while inhibiting normal hematopoiesis. The overexpression of these pro-inflammatory cytokines is particularly well described in MF; however, there is a considerable overlap between the cytokine profile seen in MF and those seen in PV and ET, suggesting the possibility of a biological spectrum.^{100,101} In mouse models, *JAK2*-mutated clones have also been found to secrete lipocalin-2, which has been shown to suppress normal hematopoiesis *via* paracrine oxidative DNA damage, and may also drive

the development of additional mutations in the tumor clone.¹⁰² In addition, there is also evidence that, through direct cell-cell interactions and the secretion of soluble mediators, such as TPO, CC chemokine ligand 3 (CCL3) and interleukin-1 β , the mutant clone can remodel the bone marrow niche to create an environment more permissive for its expansion, *via* the depletion of sympathetic nerve fibers and nestin-positive mesenchymal cells¹⁰³ and the expansion of osteoblast lineage cells.¹⁰⁴

Determinants of MPN phenotype – towards an integrated model?

The MPNs represent a heterogeneous group of diseases with phenotypes that include isolated thrombocytosis, the expansion of all three myeloid lineages, and pancytopenia. In the majority of cases, abnormalities in cytokine signaling pathways are a common factor, and most commonly lead to increased JAK-STAT signaling. It is increasingly clear that the traditional division into three MPN subtypes does not necessarily reflect the underlying biological complexity of these conditions, and that the resultant clinical phenotype is a function of genetic factors intrinsic to the neoplastic clone, the overall clonal architecture, host factors (including genetic background), and factors relating to crosstalk between the neoplastic clone and its microenvironment.

Cell intrinsic factors – somatic mutations, transcription profiles and gene dosage

At the cell intrinsic level, mutations in phenotypic driver mutations (*JAK2*, *CALR* and *MPL*) subvert physiological EpoR and TpoR signaling, leading to erythrocytosis and megakaryopoiesis, respectively. It is clear that this is a major factor in determining phenotype. Most strikingly, mutations in *MPL* are never found in cases with PV (Figure 2A), and this is also the case for *CALR* mutations, which, as discussed above, are likely to act *via* interaction with *MPL*.

How the same mutation in *JAK2* can occur in three different MPN phenotypes is a central question within the MPN field. One possible mechanism may relate to the level of gene ‘dosage’, as several lines of evidence suggest that increased *JAK2* signaling leads to more of a polycythemic phenotype. Firstly, *JAK2* exon 12 mutations are found exclusively in PV, and have been shown to result in greater STAT5 phosphorylation than V617F mutations.¹⁵ Secondly, UPD of chromosome 9, leading to *JAK2V617F* homozygosity, has been associated with greater erythropoietin independence in hematopoietic progenitors^{105,106} and is found in approximately one-third of patients with PV, but was not initially reported in ET.⁹⁶ The hypothesis that increasing *JAK2V617F* dosage may skew towards erythrocytosis is supported by the correlation between the size of homozygous clones and hemoglobin concentrations in patients,¹⁰⁷ as well as by knock-in mouse models, where the ratio of mutant to wild-type *JAK2* correlates with the degree of erythrocytosis,^{24,108} and by an induced pluripotent stem cell model.¹⁰⁹ Furthermore, even within the context of ET, greater *JAK2V617F* allele burdens are associated with a higher degree of erythrocytosis and leukocytosis.¹¹⁰

However, some PV patients do not carry a homozygous *JAK2V617F* subclone,¹¹¹ and *JAK2V617F*-homozygous subclones have been detected in patients with ET^{107,110-112} (although these were generally small (<10%), in contrast

to those in PV where they tended to represent the dominant MPN clone). Together, these findings demonstrate that *JAK2V617F* homozygosity is neither necessary nor sufficient for PV. It is possible that mutations in other, as yet unidentified, genes may play a similar role to homozygosity in these *JAK2V617F*-heterozygous PV patients.

There is also reason to believe that UPD of 9p may have effects beyond inducing loss of heterozygosity of *JAK2V617F*, as evidenced in patients where it occurs prior to the acquisition of a *JAK2* mutation.¹¹³ This suggests that 9p UPD itself may independently carry a competitive advantage (whether due to selection of a particular *JAK2* haplotype or alterations in other genes on 9p, such as *NFIB*⁹⁷) that is unrelated to *JAK2V617F* dosage. Finally, *JAK2V617F*-heterozygous cells from patients with ET are characterized by greater STAT3¹¹⁴ and STAT1¹² phosphorylation as well as upregulation of interferon- γ regulated genes, when compared to those from patients with PV. This again demonstrates that mechanisms beyond *JAK2V617F* dosage alone contribute to the determination of the PV or ET phenotype.

Differences in phenotypic driver mutations may additionally account for some of the clinical heterogeneity seen within the individual MPN subtypes. As mentioned above, the presence of *JAK2* homozygosity is associated with higher hemoglobin levels in *JAK2*-mutated ET and PV as well as with a greater incidence of aquagenic pruritus and splenomegaly.^{107,110} *JAK2* homozygosity was also associated with a greater risk of progression to MF and of thrombosis in patients with ET.¹¹⁰ *CALR*-mutated patients with ET tend to have higher platelet counts, but lower leukocyte counts and hemoglobin levels and lower rates of thrombosis than those with *JAK2* mutations.^{38,39,115,116} *CALR* mutations are also associated with higher platelet and lower leukocyte counts, and are independent predictors for improved overall survival in patients with MF.^{117,118} There is some evidence that the type of *CALR* mutation itself may also result in differential phenotypes.^{44,119}

TET2 and *DNMT3A* mutations are commonly seen across all three MPN phenotypes and are not specifically associated with MF or MDS/MPN overlap syndromes, in contrast to mutations in other epigenetic regulators. Their role in disease biology and phenotype is therefore not entirely clear. Recent evidence suggests that the context in which these mutations are acquired can influence the proliferative potential of a given clone in a cell-intrinsic manner.¹²⁰ Namely, the acquisition of a *JAK2* mutation on a wild-type background resulted in a proliferative advantage, but this was not the case on the background of an earlier *TET2* mutation. Furthermore, *TET2* and *JAK2*-mutated (double mutant) HSCs/progenitors from *JAK2*-first patients were able to generate more progenitors than those from *TET2*-first patients.

Some additional mutations seen in MPNs, however, do appear to be enriched in certain subtypes, as indicated in Figure 2B, and may additionally have an impact on the survival or risk of leukemic transformation. Mutations in components of the spliceosome (including *SRSF2*, *U2AF1* and *SF3B1*) are strongly correlated with an MF (or MPN/MDS overlap) phenotype, as are mutations in *ASXL1* and *EZH2*, which are prevalent in MDS and *de novo* AML and associated with leukemic transformation and a worse overall survival in MPNs.^{59,62,75,121} This may be related to the deregulation of PRC2 and derepression of stem cell signature genes, causing increased stem cell self renewal,

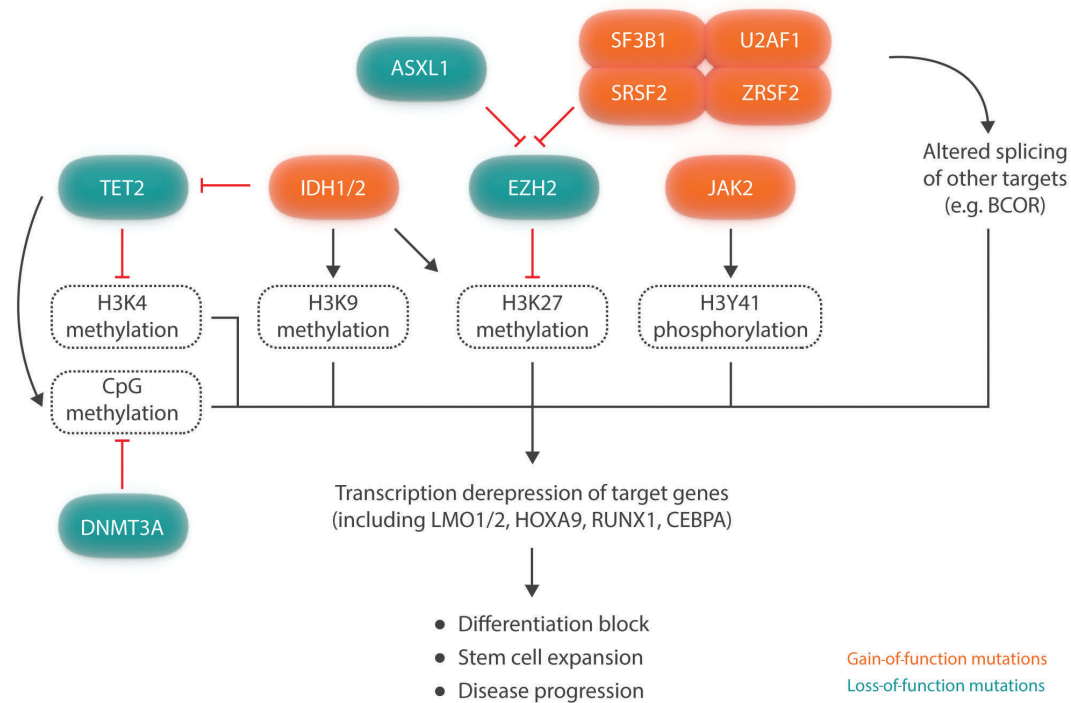


Figure 3. Summary of epigenetic regulatory pathways affected by known mutations. In addition to the “phenotypic driver” mutations in *JAK2*, *CALR* and *MPL*, a number of other mutations are described, affecting epigenetic regulation and the spliceosome. These pathways are interlinked in a number of ways, and in common, appear to lead to the dysregulation in genes involved in stem cell fate decision choices. These in turn may be associated with expansion of the hematopoietic stem cell (HSC) compartment with differentiation block, dysplasia and accelerated disease. CpG: cytosine-phosphate-guanine dinucleotide.

as discussed above^{77,79,81} (Figure 3). Finally, mutations in *NRAS* and *CBL* are also more frequently seen in patients with MF, atypical chronic myeloid leukemia and chronic myelomonocytic leukemia, and are associated with poorer overall survival.⁵⁷

Heterogeneity in clonal architecture and the significance of order of mutation acquisition

Another important source of phenotypic diversity across MPNs, even amongst those that share phenotypic driver mutations, is heterogeneity in clonal architecture.¹²² Given that *JAK2* mutations themselves may not promote a clonal advantage, and may even result in an impairment of HSC self-renewal, as discussed previously, there may be selective pressure on the *JAK2* clone leading to the selection of particular subclones (e.g., those carrying concurrent mutations in epigenetic modifier genes). This may be further enhanced by differential sensitivities to, and production of, secreted soluble mediators, such as TNF α and TGF β . The overall disease phenotype is likely to be a function of the relative proportions of all the subclones, as they will differ in terms of their functional properties, for example in terms of differentiation or self-renewal potential and soluble mediator secretion profiles. Furthermore, subclones may be present which, while not directly contributing to the disease phenotype, exert an effect *via* constraints on the growth of other clones.

One factor that may play a role in determining clonal architecture is the order in which somatic mutations are acquired. The importance of the order of mutation acquisition has been demonstrated in *JAK2*-mutated MPNs

harboring concurrent *TET2* or *DNMT3A* mutations, where mutation order has been shown to have an impact on disease phenotype,^{120,123} thrombotic risk, age at presentation and response to treatment.¹²⁰ Patients in whom the *JAK2* mutation occurred first have larger “double mutant” subclones (harboring both *JAK2* and either *TET2* or *DNMT3A* mutations) as well as larger *JAK2*-mutated homozygous clones. In the case of *TET2* mutations, these patients show expansion of erythroid progenitors, present at a younger age, and are at a greater risk of thrombosis.¹²⁰ In contrast, *TET2*-first or *DNMT3A*-first patients are characterized by a dominant “single mutant” subclone (i.e., harboring the *TET2/DNMT3A* mutation only), in keeping with the greater self-renewal capacity seen with *TET2/DNMT3A* mutations compared to those in *JAK2*. These patients were more likely to have ET, which may in part be related to constraints on the expansion of the *JAK2*-mutated clone and on the development of homozygosity. Therefore, as well as the cell-intrinsic effect of mutation order on clonogenic potential discussed in the previous section, it is likely that the order in which mutations occur will influence the composition of the stem cell niche in which later subclones will arise and reside, potentially introducing constraints on their potential to expand.

As well as competition between clones causing constraints on relative clone sizes, it is also feasible that separate clones may act cooperatively. This has been shown in solid tumors such as glioblastoma, where cytokine secretion by a relatively minor subclone can drive the expansion of a more dominant tumor clone.¹²⁴

Role of non-tumor factors

Germline variants have also been shown to influence MPN phenotype. One example previously discussed is that of rs9376092, an intronic SNP which affects the expression of *MYB*, and is found more frequently in *JAK2*-mutated ET than PV patients. This is in keeping with a knockdown mouse model where low *MYB* levels were sufficient to induce an ET-like phenotype.¹²⁵ In addition, germline copy number variations on chromosome 14, which are associated predominantly with an ET phenotype, appear to result in increased sensitivity to TPO and cytokine-independent growth, even in the absence of a phenotypic driver mutation.⁸⁵

Other non-genetic host factors may also influence MPN phenotype. For example, iron deficiency may constrain erythropoiesis and promote thrombocytosis, skewing towards an ET phenotype. The greater incidence of ET in pre-menopausal women is consistent with such an effect, and may also suggest an additional role for estrogens in skewing towards megakaryopoiesis rather than erythropoiesis. Finally, given the role of pro-inflammatory cytokines, and other microenvironmental changes, in promoting the growth of MPN clones and the fibrotic process as discussed previously, it is conceivable that concurrent inflammatory conditions, age or sex-related changes in the bone marrow microenvironment, or even the host's microbiome may influence the resultant MPN phenotype.¹²⁶

Overall, it is clear that the evolution of a MPN is a dynamic process involving complex interactions between subclones and the bone marrow microenvironment, which in turn drive changes in the tumor itself (such as the acquisition of new mutations, genetic copy number or epigenetic changes, or mobilization of cells into a separate niche) as well as changes in the bone marrow environment (such as sympathetic neuropathy, osteoclast expansion, fibroblast recruitment/differentiation and differential soluble mediator secretion).

Final remarks and questions

A number of common threads run through this discussion of MPN pathogenesis. First, it is clear that MPNs are diseases of cytokine signaling pathways. This is evident from the prevalence of mutations in cytokine signaling pathways, not only in *JAK2* and *MPL*, but also in loss-of-function mutations of negative regulators of JAK-STAT signaling. There is increasing evidence that *CALR* mutations also act on these pathways. Furthermore, cytokine secretion by the bone marrow stroma and by the tumor clone itself appears to play a role in disease persistence and progression.

Secondly, additional mutations, such as those in epigenetic modifiers (specifically *ASXL1* and *EZH2*) and spliceosomal components, are likely to modulate the disease phenotype *via* the derepression of genes regulating stem cell quiescence and self-renewal. This in turn may contribute to the development of accelerated phase disease, bone marrow fibrosis and leukemic transformation, and be associated with worse overall survival.

Thirdly, it is clear that not only is the presence of somatic mutations important in disease pathogenesis, but also that the genetic background upon which they occur has functional and clinical relevance. This applies to both the

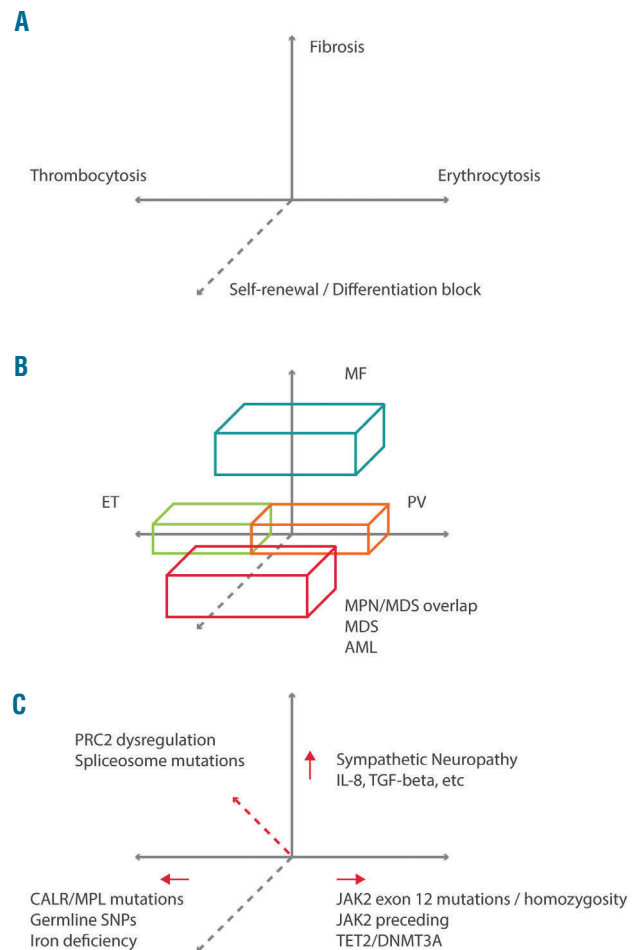


Figure 4. An integrated model of determinants of MPN phenotype. Different germline, acquired or microenvironmental factors (vectors represented in 4C) can influence differentiation/self-renewal fate choices, polarization of differentiation towards erythropoiesis or megakaryopoiesis or stimulate fibrosis (the main processes determining MPN phenotype, represented in 4A). Some may operate on more than one process, for example *ASXL1* mutations may promote both self-renewal and fibrosis. These then result in a continuum of different phenotypes and account for the heterogeneity seen within the MPNs, despite the small number of phenotypic driver mutations. A representation of where different myeloid disorders sit within this framework is provided in Figure 4B. MF: myelofibrosis; PV: polycythemia vera; ET: essential thrombocytosis; MPN: myeloproliferative neoplasms; MDS: myelodysplastic syndrome; AML: acute myeloid leukemia; PRC2: polycomb repressive complex 2; SNPs: single nucleotide polymorphisms; IL-8: interleukin-8; TGF- β : transforming growth factor- β .

germline background (for example, the *HBS1L-MYB* SNP and 46/1 haplotype) and to that of other somatically acquired mutations, such as those affecting *TET2* and *DNMT3A*.

Finally, the fact that changes in clonal architecture, mutation profile and microenvironment can occur in a given patient over time with a resultant change in clinical phenotype (e.g., transformation of ET to PV, or chronic phase disease to accelerated phase or acute leukemia), further supports the idea that ET, PV and MF are not distinct biological entities but rather sit in a biological continuum where clinical phenotype is determined by three main factors: (1) the relative degrees of erythropoiesis compared to megakaryopoiesis, (2) the degree of differentiation *versus* stem cell/progenitor expansion, and (3) the degree of fibrosis.

A simplified representation of this is shown in Figure 4. Under this schema, for example, *JAK2*-mutated ET may be an intermediate on the spectrum between *CALR/MPL*-mutated ET and *JAK2*-mutated PV, as is reflected in clinical parameters such as hemoglobin concentration, platelet counts and the risk of venous thrombosis.^{115,116,127,128} This model is also consistent with the concept that “primary” MF reflects progression from a preceding (but previously undiagnosed) MPN. Similarly, it has been suggested that prefibrotic MF may represent a transitional point between ET and MF.¹²⁹

Our understanding of the biological complexity under-

pinning MPNs and how this relates to clinical parameters and outcomes continues to improve. The ongoing challenge will be to integrate our understanding of these molecular processes into everyday clinical practice in order to allow for better refinement of our diagnostic classifications as well as improved prognostication and therapeutics.

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