Signaling alterations in myeloproliferative neoplasms

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A B S T R A C T

Acquired phenotypic driver mutations in human myeloproliferative neoplasms (MPNs) are represented by JAK2 V617F, rare exon 12 JAK2 mutations, MPL- thrombopoietin receptor mutations, rare mutations in negative regulators CBL or LNK and, most recently, calreticulin mutations. Collectively these mutations are seen in approximately 90% of MPNs. With an interesting specificity of MPL and CALR mutations for essential thrombocythemia (ET) and myelofbrosis (MF), the hallmark of these diseases is pathological activation of JAK2 with persistent signaling via STAT5. For the JAK2 V617F-induced MPN phenotype, STAT5 is obligatory, STAT1 promotes thrombocytosis, while STAT3 limits thrombocytosis and promotes inflammation. The phosphatidylinositol-3'-kinase (PI3K) and MAP-kinase pathways are also pathologically activated in MPNs. While sporadic MPNs are clonal, germ-line activating JAK2 mutations have been identified that induce polyclonal thrombocytosis and signal via MPL and STAT1. Mutant CALR MPNs appear earlier in age and exhibit higher allele burdens than JAK2V617F-positive MPNs, suggesting different effects on stem cells. A fraction of MPNs harbor mutations in TET2, EZH2, DNMT3A or ASXL1 that are shared with myelodysplasia and acute leukemia. These mutations may play a role in initiation but also progression of the disease, while p53 mutations are the most frequent alterations in secondary myeloid leukemia.

Learning goals

At the conclusion of this activity, participants should know that:

- the vast majority of MPNs are driven by pathological JAK2 activation and JAK-STAT signaling, with persistent STAT5 activation being required for MPN development;
- ATP competitive type I inhibition of JAK2 can alleviate symptoms and reduce splenomegaly in myelofibrosis and exert benefic effects in PV resistant to therapy, but more specific mutant JAK2 or pathway inhibitors, along with targeting mechanisms of chromatin regulation might be required for curative effects;
- loss of function mutations in epigenetic regulators TET2, EZH2, DNMT3A or ASXL1 are shared with MDS and AML. These mutations are seen at low frequency in PV and ET but are common in PMF, being associated with clonal dominance and progression, especially when several mutations co-exist;
- the most frequent genetic alteration in progression of MPNs to secondary acute leukemia is bi-allelic mutations in p53.

Structure-function aspects of phenotypic driver mutants and inhibition by JAK2 inhibitors

JAK2 V617F

The somatic acquired unique JAK2 V617F mutation is associated with over 70% of MPNs, namely 98% of polycythemia vera (PV) and over 60% of essential thrombocythemia (ET) and myelofibrosis (MF) patients.[1-4] The V617F mutation is located in the pseudokinase domain of JAK2 (Figure 1), and leads to activation of the kinase domain. This activation process requires the full-length JAK2 (also the amino-terminal FERM and SH2-like domains). The V617F mutation forms an aromatic stacking interaction with F595 in helix C of the pseudokinase domain,[5] and this interaction is critical for the constitutive activation of JAK2 kinase domain. The V617F mutation has as structural effect the prolongation and stabilization of the alpha helix C of the pseudokinase domain, which changes the conformation of this domain.[6] This in turn is sensed by the kinase domain and leads to constitutive activation. The X-ray structures of the JAK2 kinase and pseudokinase domains,[6] modeling,[7] as well as the structures of JAK1 and TYK2 pseudokinase and kinase domains[8] provide evidence that aromatic interactions promoted by V617F are crucial for activation and that cytokine receptors might interact with JAK SH2-like domains in a novel way using their Box 2 regions (where charged and hydrophobic amino acids are present). While the mechanisms by which several activating JAK2 mutations other than V617 function have been elucidated by showing that they disrupt the precise interface by which the pseudokinase domain functionally inhibits the kinase domain, the precise mechanism of kinase domain[7] activation remains

unknown. The discovery of JAK2 V617F prompted a major effort to identify JAK2 inhibitors that could be used as treatment in MPNs. Several ATP-competitive JAK2 inhibitors are being studied in the clinic, and one, ruxolitinib, has been approved for the treatment of intermediate- and high-risk myelofibrosis,[9] and most recently for treatment of refractory PV. They are type I inhibitors as they block ATP binding only for the active conformation of JAK2 kinase domain.[10] Type II inhibitors which bind the inactive JAK2 kinase domain conformation and prevent activation are predicted to be more effective.[10] Indeed the resistance of JAK2 V617F cell type I JAK2 inhibition[11] can be prevented by a type II inhibitor.[12] Current type I JAK2 inhibitors do not discriminate between wild-type and normal JAK2, and also inhibit normal hematopoiesis. Their use is limited by this toxicity. Two phase III clinical trials in myelofibrosis have demonstrated positive effects on reducing spleen size and reducing constitutional symptoms in MF, which led to approval of ruxolitinib for use in intermediate 2 and high-risk myelofibrosis.[9,13] A phase III clinical trial in hydroxyurea-resistant or intolerant PV also showed positive results, leading to approval of ruxolitinib for this indication.[14] While JAK2 inhibition alleviates symptoms and decreases spleen size in MF, the allele burdens and marrow fibrosis are not significantly decreased. Thus, these inhibitors are not curative and their effects cannot be compared with imatinib in chronic myeloid leukemia. Several factors can explain these findings: i) these inhibitors are type I ATP competitors and can bind and inhibit only enzymes in their active conformation, which can be regained after inhibitor consumption (if half-life is short, like in the case of ruxolitinib); ii) inhibition of wild-type JAK2 is toxic and induces side-effects that limit dose-escalation; iii) events other than JAK2 activation are involved in selection of clonal proliferation, more particularly in MF. In that sense it is very interesting that JAK2 V617F homozygosity drives a phenotypic switch from ET to PV, but is insufficient to sustain disease.[15] One of the knock-in JAK2 V617F mouse models shows that JAK2 V617F mutated stem cells develop a severe HSC defect, and thus other determinants need to sustain clonal expansion.[15] Analysis of single colonies from PV and ET patients showed that homozygosity occurs in both conditions, but in PV, expansion of such subclones appears to occur by non-genetic stochastic mechanisms.[16] Thus, other factors might be involved in JAK2-driven disease that would need to be targeted as well for efficient inhibition. Current JAK2 inhibitors were shown to inhibit and reduce

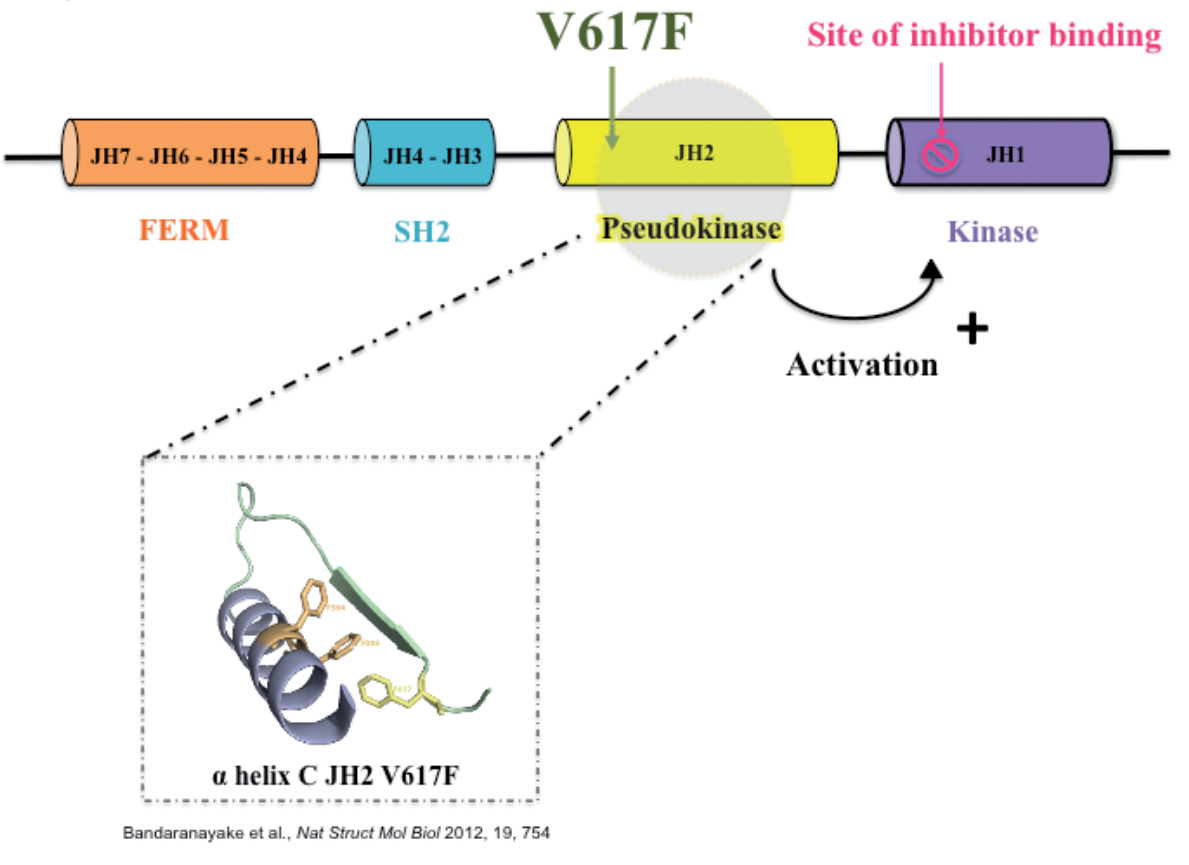

Figure 1. Domain structure of Janus kinase 2, location of the activating V617F mutation and the target of inhibition for type I ATP competitive inhibitors utilized in the clinic. (A) The kinase domain (JAK homology, JH1), the pseudokinase domain (JH2), the Src Homology 2 (SH2)-like domain, and the FERM (protein four point 1, ezrin, radixin, moesin) homology domain. The V617F mutation is located in JH2 and leads to activation of JH1. (B) The conformational change induced by the V617F occurs at the level of the helix C of JH2, which is prolonged and stabilized by aromatic stacking interactions. This leads to activation of JH1.

allele burdens of patients with positive chronic eosinophilic leukemia induced by t(8;9)(p22;p24)/PCM1-JAK2 fusion proteins that contain only the kinase domain of JAK2.[17] However, in the PCM-JAK2 and BCR-JAK2 malignancies, this complete remission induced by ruxolitinib is only short term.[18]

TpoR (MPL) W515 mutants

TpoR contains a unique amphipathic domain R/KWQFP that separates its transmembrane and cytosolic sequences (Figure 2).[19] This motif prevents activation of signaling via the cytosolic Janus kinase JAK2 in the absence of ligand Tpo. Within the motif it is the Trp residues, W515 that plays a major role in this physiological inhibition.[19] Around 5%-10% of MF and ET patients that do not harbor JAK2 V617F mutation or calreticulin mutations exhibit TpoR W515 activating mutations.[20-23] Mutation of W515 to alanine, leucine, lysine, arginine, or even to closely related residues phenylalanine or tyrosine leads to receptor activation.[24] In mouse bone marrow transplantation models, these induce a very rapid and severe disease, with spleen and marrow fibrosis.[20,23] One cytosolic tyrosine residue Y626 which couples to activation of MAP-kinase ERK1,2 and to STAT5/3 is essential for pathological signaling by TpoR W515A.[23] Mutagenesis and biophysical assays showed that W515 prevents dimerization of the transmembrane domains of the human TpoR, imposing an inclination (tilt) that is non-productive for parallel dimerization and close apposition of TM domains. W515 faces the lipid, at the exterior of the TpoR dimeric interface and ligand addition or mutation allow rotation of the W515 into the interface, productive transmembrane dimerization and activation of signaling via JAK2.[24]

Calreticulin mutants

Around 17%-22% of ET and MF patients harbor mutations in exon 9 of the calreticulin gene (CALR) represented by insertions and deletions that lead to a unique frameshift.[25,26] All the over 60 mutants described to date contain the same novel sequence at the C-terminus, as a consequence of this unique frameshift, suggesting that this sequence is important for the pathogenic effects. More than 85% of CALR mutated MPN are associated with two major classes of mutants, deletion of 52 bp (del 52; also referred to as Class I) or insertion of 5 bp (ins 5; also referred to as Class II).[25,26] CALR mutants are largely associated only with ET and myelofibrosis and not with PV. [25,26] Two PV patients were recently reported who are negative for JAK2 V617F or exon 12 JAK2 mutations, but carry del 52 CALR mutation in granulocytes and BFU-E.[27] While other mutations cannot be excluded, this finding deserves further investigation. Another special case is represented by rare atypical MPN where CALR mutation can be acquired before BCR-ABL with a thrombocytosis resistant to inhibition of BCR-ABL by small molecule tyrosine kinase inhibitors.[28]

In general, ET patients with CALR mutants appear to be younger, there is male predominance, and interestingly for ET, the allele burden is higher than that of ET patients with JAK2 V617F (40%-50% *vs*. 15%). These data suggest that signaling at the hematopoietic stem cell or at an early progenitor level appears to be stronger by CALR mutants than for JAK2 V617F, hence the younger age of disease

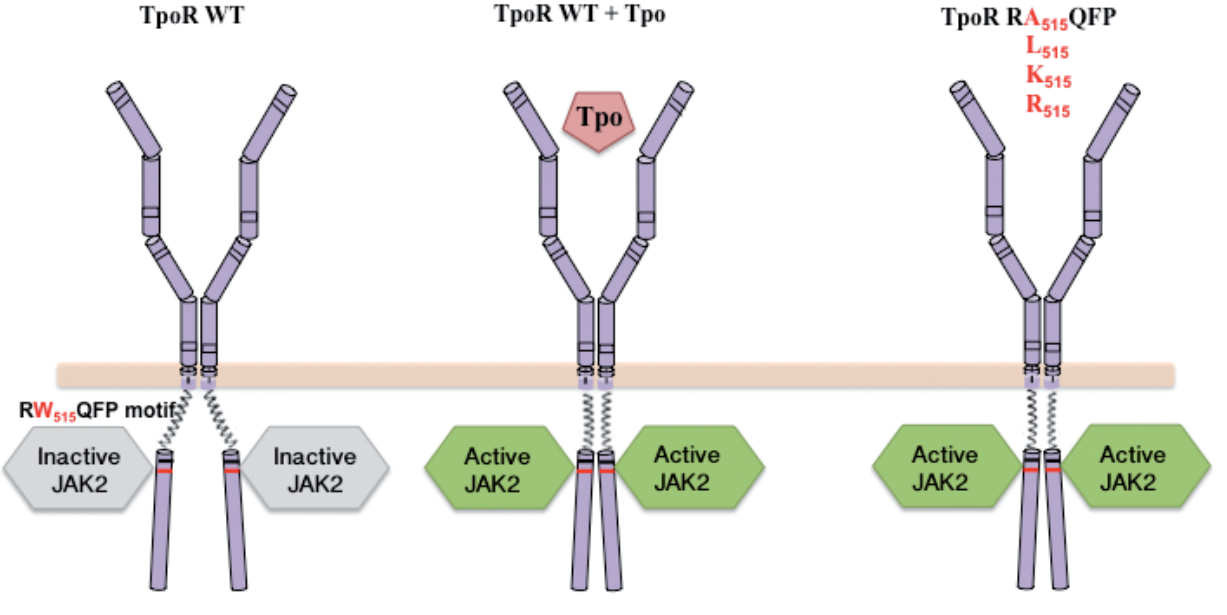

Figure 2. The thrombopoietin receptor (TpoR, MPL) is coupled to Janus kinase 2 (JAK2) but in the absence of Tpo ligand, due to an amphipathic RWQFP motif, the receptor remains inactive. Mutation of W515 in the motif (to L, K, A, R and others) leads to constitutive activation of the receptor which leads to activation of the wild-type JAK2 and downstream STAT1/3/5 and MAP-kinase and PI-3'-kinase persistent signaling.

and higher allele burden. A collaborative study on 1027 patients revealed differences in the ET phenotype between del 52 and ins 5 CALR mutated patients, with the former being associated with male sex, while the latter with younger age and higher platelet counts.[29] Such differences between the two main CALR mutant classes extend also to a better myelofibrosis prognosis for del52 CALR mutated patients only.[30] In contrast to the del 52 CALR mutant, the ins 5 CALR is mostly associated with ET.[31] del 52 CALR-mutated ET patients are predominantly male and exhibit higher platelet counts and lower hemoglobin and leukocyte counts when compared with JAK2- and TpoR-mutated patients.[32]

The mechanism by which CALR mutants induce MPN is under investigation. Bone marrow transplantation experiments showed that CALR del 52 mutation induces a thrombocytosis phenotype reminiscent of ET, evolving to myelofibrosis and that it is transplantable.[33] CALR is a chaperone involved in folding of proteins in the endoplasmic reticulum (ER) and in calcium homeostasis and is normally retained in the ER as it contains an ER retention signal.[34] This sequence is not present in the mutant CALRs, and there is controversy as to its precise intracellular localization, in one study ER localization being shown to be changed and in another not. Ba/F3 cells transformed by CALR mutants to autonomous growth exhibit constitutive JAK2 and STAT5 activation that can be inhibited by JAK2 inhibitors[25] (Figure 3). These data suggest that CALR mutants do indeed activate the JAK2-STAT pathway in myeloid progenitors. These findings are supported by recent reports that JAK2 inhibitors are effective in myelofibrosis/MPNs with CALR mutants.[35] On the other hand, studies in the MARIMO cell line, which is a leukemia transformed cell line occurring on cells from an ET patient with del61 CALR mutation, indicated that JAK-STAT pathway might not be involved.[36] However, this cell line is derived from a patient that has been treated with busulfan,[37] and the leukemia cells may be independent of the signaling pathways initially responsible for the ET. An integrated genomic analysis illustrated the central role of the JAK-STAT pathway activation in MPNs including those associated with CALR mutations.[38]

One important aspect is that the positive effects of JAK2 inhibition are exerted on all MPN patients with myelofibrosis irrespective of the presence of JAK2 V617F mutation. This is likely due to the fact that the other MPNs induced by TpoR W515 mutants (W515L/K/A/R and others) and by CALR mutants also exhibit pathological JAK2 activation, in these cases pathological activation of the wild-type JAK2.

Signaling alterations downstream JAK2 V617F in sporadic MPNs

Signaling via STATs

Since its discovery, it has been demonstrated that expression of JAK2 V617F in cells that express dimeric cytokine receptors, EpoR, TpoR and G-CSFR leads to persistent activation of STAT5, STAT3, STAT1, phosphatidyinositol-3'-kinase (PI-3'K) and Ak.[1,39]

Two mouse models have established that STAT5 is essential for MPN establishment.[40,41] The roles of STAT3 and STAT1 remained unclear until recently when STAT3 and STAT1 knockout mice were crossed with JAK2 V617F knock-in or transgenic mice. These studies showed that, contrary to the expectation that STAT3 mediates myelofibrosis and severe evolution, STAT3 knock-out led to a more severe thrombocytosis and phenotype.[42] The same result was obtained for the active MPLW515L mutant when STAT3 was deleted from the hematopoietic system.[43] In contrast, knocking-out STAT3 from non-hematopoietic cells alleviated disease by reducing inflammation. Overall it appears that STAT3 opposes thrombocytosis in the hematopoietic system and promotes inflammation via non-hematopoietic cytokine production and function.

STAT1 was initially shown to be specifically activated in erythroid colonies of ET patients, but not of PV patients,[44] in agreement with its stimulating activity on platelet production. Indeed, the thrombocytosis phenotype is reduced in transgenic JAK2 V617F mice by deleting STAT1, with decreased megakaryopoiesis and increased erythropoiesis.[45] Interestingly, potential compensation effects of other STATs could occur, given that STAT1, STAT3 and STAT5, all interact with chromatin via the same GAS (gamma interferon activated sequences) with

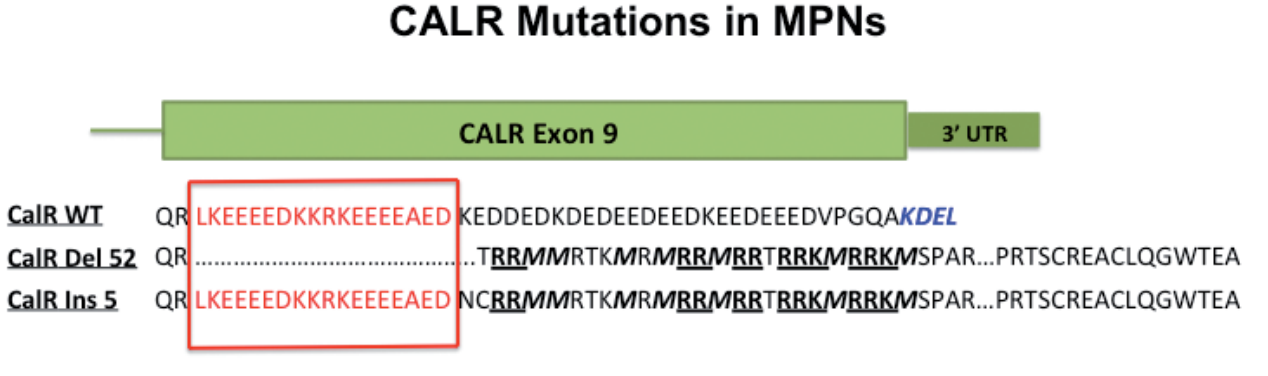

Figure 3. The C-terminus of exon 9 of calreticulin (*CALR*) gene is targeted for insertions and deletions in MPNs. The most prevalent mutations are a deletion of 52 bp or an insertion of 5 bp in exon 9. These changes lead to a frameshift resulting in translation of a novel sequence that is rich is basic residues and lacks the KDEL retention signal for the endoplasmic reticulum.

consensus motifs being TCC-NNN/N-GAA. Thus, deletion of STAT1 might enhance function of STAT3, which opposes thrombocytosis. It will be of great interest to study the role of STAT1, STAT3 and STAT5 using genetic models also for the rare exon 12 JAK2 mutants, which are responsible for 2%-3% of PV cases,[46] and which seem to penetrate with more difficulty into the granulocytic lineage. More recently, it was reported that also STAT2 can be activated by Tpo and TpoR.[47] STAT2 forms complexes with STAT1 and IRF9 (p48), denoted ISGF3 (interferon stimulated gene factor 3), which is also the complex activated by type I interferon and mediates antiviral and antiproliferative activities.[48] While Tpo activates STAT5, STAT3 and STAT1 in all cells that express TpoR, activation of STAT2 complexes requires high JAK2 levels, which is characteristic of megakaryocytes in late development. It remains to be seen what contribution STAT2 activation in such cells brings to the phenotype induced by JAK2 V617F, but one attractive hypothesis is that the negative effects of Tpo on proliferation in late megakaryocytes might be due to this pathway.[49,50] It was recently proposed that JAK2 and TpoR expression levels regulate megakaryocytic proliferation *versus* differentiation in both normal and pathological conditions, and that lower TpoR levels, like low doses of JAK2 inhibitors, could induce paradoxical thrombocytosis by altering this balance.[51] Specifically, it appears that low TpoR levels at the end of megakaryocyte differentiation, or low JAK2 levels (even no JAK2 expression) do not impact platelet production but prevent clearance by platelets of Tpo, which is then increased in circulation and stimulates early megakaryocyte progenitors to proliferate. In addition, TpoR at late stages of megakaryocyte differentiation is inhibitory and induces senescence,[49] thus explaining the paradoxical effects of lower TpoR or JAK2 levels on platelet production.

Regulation of megakaryocyte hyperplasia and differentiation defects in myelofibrosis has been shown to involve several miRNA circuits. A network consisting of several microRNAs (mi-RNAs) with oncogenic potential has been identified, especially miR-155-5p targeting JARID2 is involved in the myelofibrosis-associated defects in the megakaryocytic cell lineage.[52] Downmodulation of JARID2, which is a histone demethylase, leads to increased formation of $CD41^+$ precursors.[52] Interestingly, depending on the cell type, miR-155 is induced by interferons (type I and II), and regulates type I interferon production.[53] At the same time, pathological expression of several genes that are targeted by pathological complexes between constitutive active STAT5 and p53 (LPP, VEGFC, LEP and others) was reported.[54] The Lipoma Preferred Partner is the host gene of miR-28, which downmodulates TpoR and induces defects in differentiation.[55] Overall, pathological signaling in MPNs leads to altered gene expression that affects the megakaryocytic lineage.

Signaling via PI-3'-kinase and Akt/mTOR

Evidence for an important role in PI-3'-kinase activation came from studies combining in cell lines and preclinical models the association of JAK2 inhibitors with several serine-threonine kinase inhibitors. In such studies, a strong synergy was detected when JAK2 inhibitors were associated with pan type I PI-3'-kinase inhibitors and PI-3'-kinase/mTOR inhibitors.[56,57] These data are in agreement with the positive effects of mTOR inhibitors in myelofibrosis. Detailed experiments suggest that PI-3'-kinase activation is the major pathway driving survival and proliferation downstream JAK2 V617F, and that incomplete inhibition would still allow sufficient PI-3'-kinase activation for proliferation. The role of the PI-3'-kinase pathway in the phenotype induced by other mutants such as CALR remain to be determined.

Activating JAK2 mutations in hereditary thrombocytosis

Sporadic MPNs are clonal diseases, and it has recently been shown in elegant *in vivo* experiments that MPNs can be initiated in mice from a single stem cell expressing JAK2 V617F.[62]

In contrast to sporadic MPNs, which are clonal, hereditary thrombocytosis cases were recently reported where the thrombocytosis is polyclonal. Several families have been described where hereditary thrombocytosis is associated with weakly activating mutations in JAK2. These patients have polyclonal disease, namely polyclonal thrombocytosis. One of the families harbors the JAK2 V617I mutations, previously described as being a weak variant of JAK2 V617F. Others harbor R564Q also in the pseudokinase domain,[58] or the double mutant R938Q/S755R or R867Q in the kinase domain.[59] Interestingly, certain mutants like R938Q/S755R exhibit longer stability and resistance to JAK2 inhibitors. Overall these JAK2 mutants appear to preferentially activate TpoR signaling and to induce hypersensitivity to Tpo of megakaryocytic colonies. For the R938Q/S755R or R867Q, when expressed as in patients as heterozygous mutants, they preferentially activate TpoR signaling via STAT1.[59]

Interestingly, another germ-line mutant (JAK2 E846D) was reported that appears to be associated with hereditary polyclonal erythrocytosis, and activates EpoR signaling via JAK2 and STAT5.[60] However, it remains to be seen whether this mutant alone can induce the observed phenotype. Taken together, these studies on families with germline JAK2 mutants indicate that such mutants induce polyclonal disease, without evolution to clonal MPN or leukemia. The JAK2 mutants induce significantly weaker signaling than JAK2 V617F and somewhat preferentially via one receptor. It will be of great interest to continue studying these families.

Signaling alterations to chromatin and novel links

MPN clones can harbor the JAK2 V617F mutation and loss of function TET2 mutations.[61] Loss of TET2 was shown in *in vivo* model systems to aggravate the JAK2 V617F-induced disease, to promote prolonged leukocytosis, splenomegaly, extra medullary hematopoiesis, and decreasing survival. While transplantation of JAK2 V617F single-mutant cells along with JAK2 wild-type cells showed a disadvantage for JAK2 V617F cells, JAK2 V617F and loss of TET2 developed and maintained MPNs, suggesting that TET2 loss increases fitness of HSCs mutated for JAK2.[62] Loss of TET2 functions as a disease accelerator and sustainer in combination with JAK2 V617F (Figure 4).[62] Interestingly, the order of acquisition of JAK2 V617F and TET2 is important. When

the JAK2 mutation is acquired first, a greater likelihood of presenting with PV than with ET was reported.[63] Prior mutation of TET2 is associated with a reduced ability of JAK2 V617F to up-regulate proliferation genes.[63] The order in which JAK2 and TET2 mutations are acquired may also reflect differences in age for these two classes of patients, with TET2 patients being older and having a senescent HSC phenotype that is partially rescued by TET2 deletion and who then acquired the JAK2 V617F mutation.[62]

In addition to TET2 loss, inactivating mutations in EZH2, ASXL1, DNMT3A and IDH1/2 are associated with MPNs, especially with MF and are shared with myelodysplasia and AML. These mutations are thought to contribute to clonal expansion especially in the context of aging and senescence of HSCs and they contribute to progression especially when several such mutations are found in the same patient.[64] Another perspective would be that patients who exhibit many such mutations are experiencing a mixed MDS-MPN disease, given that the driver mutations such as JAK2 V617F can alone induce a true MPN disease from single cells on its own,[65] which is probably also the case for TpoR/MPL and CALR mutants.

How do all these mutants promote clonal expansion is not clear; TET2 loss would reduce 5-hydroxy-methyl cytosine levels which is predicted to allow maintenance of repressed DNA sequences. Loss of EZH2 and ASXL1 is predicted to reduce the repressing function of the polycomb repression complex 2 and thus to turn on genes. Perhaps TET2 loss leads to hyper-methylation and repression of the genes that code for repressors of expression of the same targets as those regulated by EZH2/ASXL1. Identification of such modulators, and how JAK-STAT pathway regulates them, or how such pathologically expressed genes regulate/potentiate the JAK-STAT pathway will be of utmost importance for understanding MPN pathogenesis.

Such epigenetic alterations might be treatable with histone deacetylase (HDAC) inhibitors that would also force expression of pathologically repressed genes. JAK1/2 and

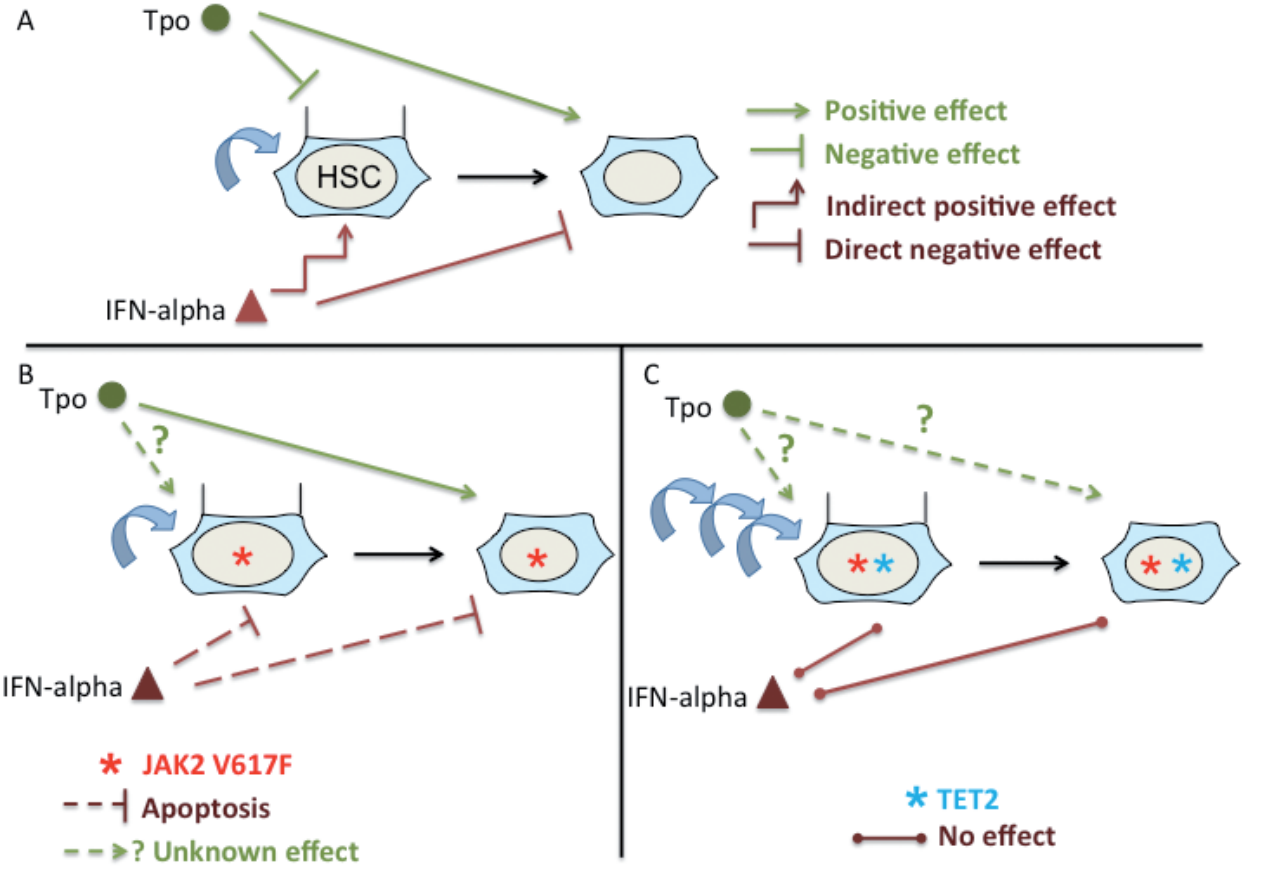

Figure 4. Hematopoietic stem cells (HSCs) are the cells that acquire the activating mutations in myeloproliferative neoplasms (MPN) and represent the disease reservoir. (A) Normal HSCs are maintained quiescent by Tpo and induced to cycle by IFN alpha via a direct and an indirect mechanism involving effects of IFN on stroma or other cells. (B) Acquisition of JAK2 V617F and signaling by this activated kinase results in cycling of HSCs and in a higher sensitivity of these cells to the pro-apoptotic and exhausting effects of IFN. The effect of Tpo on these cells is unknown as Tpo levels in MPN are variable. (C) Loss of TET2 enhances the fitness and self-renewal of HSCs and such clone does not respond to IFN alpha. In the order: TET2 first; JAK2 V617F second, IFN alpha may be less active in exhausting these cells due to the effects of TET2 loss on the properties of HSC, although in one study such a clone responded to IFN treatment.[66] It is not known whether the response to IFN-alpha may also depend of the order of first TET2 and JAK2V617F second. Stronger proliferative programs are established in the JAK2 V617F first TET2 second situation (not shown). The precise genes that are pathologically regulated by TET2 loss are unknown.

pan-HDAC inhibitor combination therapy was reported to yield improved efficacy in pre-clinical mouse models of JAK2V617F-driven disease.[67] *In vivo*, the combination exhibited a more profound effect on splenomegaly, as well as on bone marrow and spleen histology, compared with either agent alone.[68] It will be important to determine the key genes targeted by the combination, as panobinostat will affect non-specifically deacetylase-repressed genes, but also deacetylation of non-histone genes involved in cytoskeletion reorganization and signaling.

JAK2 V617F promotes defects in DNA replication, namely replication fork stalling with disease-restricted impairment of the intra-S checkpoint response specifically in MPN for erythroblasts from PV and not from ET patients.[69] This depends on the PI-3'-kinase pathway and leads to increased DNA damage when p53 is inhibited. The cause of the restriction of this defect to PV is still unknown.

An unexpected connection is represented by common germ-line variation at the TERT locus being linked to familial clustering of myeloproliferative neoplasms.[69] A clinical result using TERT inhibitors also showed efficacy against the MPN clone of a TERT inhibitor. In addition, recent data in mouse models demonstrate that this TERT inhibitor has a major effect on the leukemic stem cell in AML. The C allele of the rs2736100 SNP in TERT was identified as an MPN susceptibility factor in an Icelandic population, and also in a large cohort of Italian sporadic MPN patients.[69] Indeed, this SNP is also enriched in familial MPN, but shows a low penetrance and is shown to have an additive effect on disease risk with the 46/1 JAK2 GGCC predisposition haplotype.[69] However, it is still unclear whether these alleles impair hematopoiesis, or confer hypersensitivity to JAK-STAT activation, so that appearance of a mutation is positively selected in the presence of such alleles. In principle both scenarios are possible, since in both cases an activating JAK would lead to enrichment of mutated clones. Since constitutional genetic variation at TERT is associated with a wide range of malignancies and not just MPN, a mutagenic process cannot be ruled out.

Signaling alterations associated with progression to secondary acute leukemia

Recent studies using targeted sequencing to compare sequential samples from chronic phase MPN *versus* secondary acute myeloid leukemia showed that p53 mutations or functional inhibition are the most frequent associated event with leukemia transformation,[70] while this is not the case for *de novo* AML. Patients harboring a heterozygous inactivating mutation in p53 remain in chronic phase, while leukemia transformation coincides with acquisition of a second mutation, therefore inactivating p53 in a bi-allelic manner.[64] In the case of JAK2 V617F-positive MPNs, p53 mutations lead to clonal dominance of the JAK2 V617F/p53 mutated leukemic cells.[71] There is some evidence that p53 mutations may occur early in the chronic phase of the disease but are selected with time, as in therapy-related leukemia.[72,73] In mouse models, combining JAK2 V617F expression with p53 deficiency leads to a fully penetrant AML[71] and post-MPN AML cells are sensitive to JAK2 inhibition and inhibitors of heat shock protein 90 as well as decitabine.[71] Interestingly, constitutively activated STAT5 in MPNs was shown to co-operate with p53, both wild-type and mutant, and induce pathological gene expression.[54] It remains to be seen whether such events contribute to MPN phenotype or progression. In addition, the presence of two or more somatic mutations significantly increased the risk of progression to acute myeloid leukemia.[64] Interestingly, the association of p53 mutations in the progression to sAML appears to be dependent on JAK2 V617F mutational status, with p53 mutations not being associated with JAK2 wild-type and CALR mutated patients.[71]

Effects of interferon and of estrogen on signaling alterations in MPNs

At present, the only therapeutic strategy that can lead to molecular remission is represented by type I interferon treatment. Hematologic remission and strong decrease in JAK2 V617F allele burden occur in a minority of patients, especially PV, with more modest effects on ET and myelofibrosis. The kinetics of type I IFN effects suggests that it targets the mutated HSCs that plays a role of cancer stem cell, and under IFN treatment, these cells become exhausted and eventually cannot sustain the disease. Studies in knock-in models of JAK2 V617F and competitive bone marrow reconstitution assays support this mechanism.[74,75] However, the precise molecular bases for why mutated HSCs are exhausted by IFN are not known. One hypothesis is that mutated HSCs are addicted to the JAK-STAT pathway and IFN induces negative regulators such as SOCS1.[76] Another is that JAK2 signaling primes for higher levels of anti-apoptotic effects induced by IFN. An anti-proliferative effect of IFN on committed myeloid progenitors could promote pathological cycling for HSCs and exhaustion, especially under continued presence of IFN, as was recently demonstrated.[77] Finally, indirect effects of IFN on stroma[77] can also contribute to exhaustion of mutated stem cells. In any case, signaling alterations induced by pathological JAK2 activation via JAK2 mutation or via calreticulin mutations induces hypersensitivity of mutated stem cells to long-term IFN treatment. Why the majority of MPN patients are resistant to IFN effects, in contrast to MPNs driven by activated JAK2 in mouse models, might be explained by additional events, possibly those that induce clonal expansion. Along those lines, epigenetic mutations, such as loss of TET2, EZH2, ASXL1 and others, appear to confer resistance to IFN, but more studies are required before identifying the precise genes involved in sensitivity and resistance.

Recently, different effects were reported for estrogen receptor activation in the context of hematopoietic stem and progenitor cells, and of JAK2 V617F positivity. Activation of Estrogen Receptor alpha (ER-alpha) induces apoptosis in short-term HSCs and multipotent progenitors. In contrast, its activation induces proliferation of long-term HSCs, associated with defects in expression profiles of self-renewal genes and reversible reconstitution defects.[78] Importantly, activation of ER-alpha by tamoxifen prevented the establishment of an MPN phenotype by JAK2 V617F transgenic mice and led to apoptosis of

human JAK2 V617F-positive HSCs in a xenograft model.[77] These data might have implications for understanding differences in prevalence of MPNs between females and males, and open new avenues for treatment.

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