Chapter 2
Genetics of the Myeloproliferative Neoplasms
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Introduction

The myeloproliferative neoplasms (MPNs) are clonal disorders of hematopoiesis characterized by the production of mature appearing cells within the bloodstream. The MPNs were initially grouped together by William Dameshek in 1951, who noted the pathogenetic and clinical similarity of the different MPN [1]. However, it was not until the last 5 years that researchers have begun to elucidate the genetic basis for this group of diseases. Beginning with the 2005 discovery of the JAK2-V617F mutation, different MPNs have been the source of multiple gene discovery efforts [2–5]. This has led to the exciting discovery of a series of mutations in patients with MPN in known genes as well as in novel genes not previously known to be involved in pathogenesis of human disease.

In many ways, the history of genetic discoveries in the MPNs has mirrored the history of genomic technology development [6]. Early studies of the genetic underpinnings of MPNs relied on seminal X-inactivation studies, which demonstrated the clonal, stem cell origin of the different MPNs. Later, completion of the Human Genome Project as well as greater access to Sanger sequencing promoted candidate-based sequencing approaches, which were partially responsible for the discovery of activating mutations in JAK2 and MPL [2, 3, 7–10]. Sequencing of the human genome provided a high-resolution genome-wide map of common SNPs, which, in turn, promoted greater analysis of single nucleotide polymorphisms (SNPs) in the international HapMap project and the SNP database (dbSNP). These efforts allowed the identification of a germline SNP, which confers heritable risk of MPN development and the first specific genetic evidence of a germline cause for MPNs [11–13]. More recently, the use of array-based technologies, including SNP arrays and comparative genomic hybridization arrays (aCGH), has led to the identification of somatic mutations in TET2 and ASXL1 in MPNs [14–18].
In this review, we discuss the somatic as well as germline genetic aberrations that contribute to the pathogenesis of the different MPNs. Despite the insights into the genetic basis of these diseases that has emerged in recent years, it has become increasingly clear that there are yet additional unidentified genetic events, which contribute to MPN development. To this end, we also review recent data on the role of additional genetic and epigenetic abnormalities in MPN pathogenesis, including the role of micro-RNAs, epigenetic regulation, and post-translational modifications that may promote or influence the MPN phenotype.

Somatic Mutations in MPN Pathogenesis

Early Evidence for Clonal Disorder

The classic MPNs include the disorders such as polycythemia vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (MF). The earliest insights into the genetic causes for the MPNs were made in 1976–1981, when a series of studies demonstrated that all three classic MPNs represented clonal disorders derived from a genetically aberrant hematopoietic progenitor cell [19–21]. Each of these studies took advantage of the ability to identify polymorphisms in the X chromosome gene glucose-6-phosphate dehydrogenase (G-6-PD) within female patients heterozygous for the gene. By demonstrating that the granulocytes, platelets, and red blood cells from patients with PV, MF, and then ET contained only one of the two possible G-6-PD alleles, while all other tissues of the body contained both G-6-PD alleles, the clonal nature of these disorders was proven. Moreover, several patients in these initial studies harbored additional karyotypic abnormalities, which were present only in the myeloid compartment and not in other tissues of the body as further evidence for the existence of clonal somatic abnormalities present in MPNs [19–21]. Despite further extensive X-chromosome inactivation studies as well as efforts to identify somatic genetic abnormalities in MPNs based on gross cytogenetic abnormalities, no specific gene involved in MPN pathogenesis was identified until over 20 years later.

JAK2 and MPL Mutations: Constitutive Tyrosine Kinase Activation in MPN Pathogenesis

JAK2 Mutations in MPNs

The initial specific mutation identified in MPN pathogenesis was the JAK2V617F mutation. The JAK2V617F mutation is a somatic guanine to thymidine substitution, which results in a substitution of valine for phenylalanine at codon 617 of JAK2. JAK2 encodes a cytoplasmic nonreceptor tyrosine kinase, which is downstream of both type I and type II cytokine receptors [2–5]. The JAK2V617F mutation is present in the vast majority of patients with PV, most patients with ET and MF and a minority of patients with other myeloid disorders (Table 2.1).

The JAK2V617F mutation was discovered independently by four different groups in 2005 and their findings were all published in April to March of 2005 [2–5]. James et al. identified the JAK2V617F mutation based on the elegant demonstration that chemical or short interfering RNA (siRNA)
mediated inhibition of JAK2 in primary patient samples led to abolishment of endogenous erythroid colony (EEC) formation in PV samples [3]. Given this finding, they hypothesized that mutations resulting in aberrant JAK2 activity may be present in MPNs and sequenced JAK2 leading to the discovery of the frequent somatic mutation of JAK2 in patients with MPN. Baxter and colleagues utilized a candidate gene sequencing approach to identify the JAK2V617F mutation [2]. On the basis of the hypothesis that the classic MPN might bear a mutation in a tyrosine kinase such as that found in the related disorder chronic myelogenous leukemia (CML), Levine et al. utilized high-throughput DNA sequencing of tyrosine kinases in MPNs to identify the JAK2V617F mutation [5]. In contrast, Kralovics et al. utilized prior knowledge that ~30% of patients with MPN harbored a region of loss of heterozygosity (LOH) on chromosome 9p [4]. They then hypothesized that JAK2 may be implicated in MPN patients, based on its location in the minimally duplicated region at 9p. This led to sequencing of JAK2 in patients with MPN and the discovery of the JAK2V617F mutation.

All of these four initial publications reported that the JAK2V617F mutation was most commonly found in PV (81–99% of cases) followed by ET (41–72%) and MF (39–57%) and could be present as a heterozygous or homozygous mutation. Moreover, they each demonstrated that homozygous JAK2V617F mutation rise as a result of duplication of the mutant allele by mitotic recombination [also referred to as uniparental disomy (UPD)]. For reasons that are still unclear, the frequency of JAK2V617F homozygosity differs amongst the MPN with homozygosity for JAK2V617F occurring in ~30% of MPN patients compared with only ~2–4% of patients with ET [2–5].

A subset of the initial four publications documenting the JAK2V617F mutation also provided some functional insight into the JAK2V617F mutation. Levine et al. and James et al. both demonstrated that the JAK2V617F mutation results in constitutive tyrosine kinase phosphorylation and showed that expression of JAK2V617F, but not wild-type JAK2, in Ba/F3 cells coexpressing the erythropoietin receptor resulted in transformation to factor-independent growth of Ba/F3 cells [3, 5]. Importantly, James et al. also reported that expression of the JAK2V617F allele in vivo via retroviral murine retroviral bone marrow transplantation (BMT) into C57 Bl/6 mice resulted in significant erythrocytosis.

### Table 2.1 Frequency of the JAK2V617F allele in myeloid disorders.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Frequency</th>
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<tbody>
<tr>
<td>Polycythemia vera</td>
<td>81–99%</td>
</tr>
<tr>
<td>Essential thrombocytosis</td>
<td>41–72%</td>
</tr>
<tr>
<td>Primary myelofibrosis</td>
<td>39–57%</td>
</tr>
<tr>
<td>Chronic myelomonocytic leukemia</td>
<td>3–9%</td>
</tr>
<tr>
<td>Myelodysplasia</td>
<td>3–5%</td>
</tr>
<tr>
<td>Acute myeloid leukemia</td>
<td>&lt;5%</td>
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</tbody>
</table>

*JAK2V617F mutations are enriched in the MDS subtype, RARS-T (refractory anemia with ringed sideroblasts and thrombocytosis) and occur in 40–50% of RARS-T patients

*JAK2V617F mutations are most common in AML evolved from a preceding MPN and account for 30–50% of such secondary AML patients*
Although the initial discovery of an activating mutation in a kinase downstream of myeloid cytokine receptors in PV, ET, and MF was a major insight into MPN pathogenesis, and a number of questions immediately arose with the discovery of $JAK2V617F$. First question was how a single recurrent mutation could be responsible for the development of three phenotypically disparate disorders. At least three publications of the phenotype of mice undergoing retroviral bone BMT with the $JAK2V617F$ allele demonstrated that the genetic background of recipient mice clearly affected the hematopoietic phenotype (Table 2.2) [22–25]. For instance, transplantation of the $JAK2V617F$ allele into Balb/C mice resulted in polycythemia and leukocytosis, while transplantation into C57 Bl/6 mice most commonly results in isolated polycythemia with modest changes in the white blood cell count. This provided the earliest genetic evidence that the genetic background upon which the $JAK2V617F$ mutation is introduced may influence the disease phenotype. Moreover, transplantation of $JAK2V617F$ into either background did not result in thrombocytosis in mice suggesting that additional inherited or acquired genetic events might be necessary for development of ET and/or MF.

An additional question that arose immediately after the initial discovery of $JAK2V617F$ relates to the pathogenetic relevance of gene dosage. Specifically, given that some patients who are heterozygous vs. others who are homozygous for the $JAK2V617F$ allele, it is important to ascertain whether there are functional differences based on mutational gene dosage. As stated earlier, the initial genetic studies identified homozygous $JAK2V617F$ mutations more often in PV than in ET, suggesting homozygous $JAK2V617F$-mutant progenitors might be biased toward the erythroid lineage. Given this finding, Tiedt et al. generated conditional $JAK2V617F$ transgenic mouse models to investigate the effects of varying wild-type $JAK2$ to $JAK2V617F$ ratios in MPN phenotype (Table 2.2) [26]. The resultant cross of their $JAK2V617F$ transgenic mice with mice expressing Cre-recombinase under the control of the hematopoiesis specific Vav promoter (VavCre;FF1) led to the expression of $JAK2$-$V617F$, which was lower than the endogenous wild-type $JAK2$. These VavCre;FF1 mice developed thrombocytosis and neutrophilia most closely resembling human ET. In contrast, conditional expression of the $JAK2V617F$ transgene using the interferon-inducible MxCre resulted in the expression of $JAK2V617F$ approximately equal to wild-type $JAK2$, which resulted in polycythemia, leukocytosis, and variable thrombocytosis most reminiscent of human PV. As discussed earlier, retroviral BMT assays result in much higher levels of $JAK2V617F$ and a PV-like phenotype without thrombocytosis. In total, the results of these genetic experiments suggest that the phenotype of MPN progenitor cells are affected by the level of $JAK2V617F$ expression such that lower $JAK2V617F$:wild-type $JAK2$ levels result in megakaryocytic expansion and thrombocytosis, while higher $JAK2V617F$:wild-type $JAK2$ levels result in a more marked erythrocytosis over thrombocytosis. A second study reporting creation of $JAK2V617F$ transgenic mice under the Vav promoter confirmed that low-level $JAK2V617F$ expression results in thrombocytosis (Table 2.2) [27]. Despite the utility of the current retroviral transplant and transgenic models, more accurate genetic “knock-in” models in which $JAK2V617F$ is expressed from the endogenous promoter will further elucidate differences in heterozygous vs. homozygous states of the $JAK2V617F$ mutation on hematopoiesis. Moreover, it remains unclear whether the differences between cells homozygous and heterozygous for $JAK2V617F$ result from increased expression of the mutant $JAK2$ kinase, from the absence...
Table 2.2 Murine models of JAK2V617F-positive MPN.

<table>
<thead>
<tr>
<th>Disease model</th>
<th>Strain</th>
<th>Expression of JAK2V617F:JAK2 wildtype</th>
<th>Polycythemia</th>
<th>Leukocytosis</th>
<th>Megakaryocytic hyperplasia</th>
<th>Thrombocytosis</th>
<th>Myelofibrosis</th>
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<tr>
<td>Retroviral BMT</td>
<td>BalbC</td>
<td>&gt;&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Retroviral BMT</td>
<td>C57/Bl6</td>
<td>&gt;&gt;</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Transgenic</td>
<td>VavCre;FF1</td>
<td>&lt;</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Transgenic</td>
<td>MxCre;FF1</td>
<td>=</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Transgenic Line B</td>
<td>C57/Bl6</td>
<td>&lt;</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Transgenic Line A</td>
<td>C57/Bl6</td>
<td>&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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Evidence that host genetic variation and JAK2V617F gene dosage influence MPN phenotype

aBone marrow transplantation (BMT)

bIn one study, a subset of mice with reduced JAK2V617F expression in a murine BMT developed transient thrombocytosis
of expression of wild-type JAK2, or a combination of both factors and whether the differences in signaling between cells homozygous and heterozygous for JAK2V617F are quantitative and/or qualitative in nature.

The genetic studies of the effect of JAK2V617F gene dosage on hematopoeisis raised the question of what stage in the hematopoietic hierarchy does the JAK2 mutation arises. Although the clonal nature of MPNs had been known for more than 20 years, it had not been previously known whether the JAK2V617F mutation arises at the level of a self-renewing hematopoietic stem cell (HSC), in nonself renewing multipotent progenitors, or even in myeloid-erythroid progenitors (MEP). This question was addressed by Jamieson et al., who utilized progenitor FACS analysis and methylcellulose cultures to identify that the JAK2V617F mutation occurred in cells with an HSC surface immunophenotype (cells that are CD34+ CD38– CD90+ Lin–) [28]. Moreover, it was noted that HSCs from patients with PV had increased numbers of common myeloid progenitors as well as a skewed differentiation potential toward an erythroid fate. Later work from the same group elucidated that enforced expression of JAK2V617F specifically led to upregulation of the erythroid transcription factor GATA-1 and decreased expression of the myeloid transcription factor PU.1 [29]. Interestingly, this was shown in both primary patient samples as well as by transduction of JAK2V617F into umbilical cord blood stem/progenitor cells. Use of these transduced cells further demonstrated that expression of JAK2V617F at the stem/progenitor cell level led to skewed differentiation toward an erythroid lineage.

Despite these insights into the effects of JAK2V617F on signaling, hematopoeisis, and gene dosage effects of the mutation, the exact mechanism by which the valine to phenylalanine substitution at residue 617 leads to aberrant signaling of JAK2 has yet to be fully elucidated. The Jak kinases have seven homologous domains (JH1–7), which includes the catalytic kinase domain (JH1) and a catalytically inactive pseudokinase domain (JH2). It is hypothesized that the JH2 domain serves autoinhibitory function and that the valine-to-phenylalanine substitution at codon 617 might abrogate autoinhibition and result in constitutive tyrosine kinase activity. Although this hypothesis seems logical, yet it has to be proven functionally [30]. Moreover, it has been shown that substitution of other residues (tryptophan, methionine, isoleucine, and leucine) instead of phenylalanine at position 617 leads to the same constitutive activation of JAK2. Despite this the valine-to-phenylalanine substitution is the exclusive mutation identified at codon 617 in humans with MPNs implying that this particular amino acid substitution may be critical to myeloid transformation [31]. Moreover, it has recently been shown that the JAK2V617F mutation can be acquired multiple times in multiple different clones on different strands of DNA in patients with polyclonal ET.

ET, unlike PV and MF, is frequently found as an oligoclonal or polyclonal disease based on X-chromosome inactivation patterns [8]. Using a technique of allele-specific restriction enzyme digestion to determine whether the heterozygous JAK2 mutation could be detected as occurring on different strands of DNA in cells from the same individual, Lambert et al. demonstrated that several ET patients had clones with the JAK2V617F mutation occurring on different strands of DNA [32]. This again raises the question of why the particular 617F residue is so critical, so much such that it has been acquired several times independently in different hematopoietic stem/progenitor cells within the same individual. Lastly, a recurring mutation in the pseudokinase domains
of JAK2 has also recently been discovered in patients with Down Syndrome acute lymphoblastic leukemia (ALL) with a recurring mutation at codon R683 in 18–25% of cases (Fig. 2.1) [33]. Why mutations at R683 result in lymphoid transformation and V617F mutations result in MPNs is not understood.

Given that a small number of PV patients and a significant proportion of ET and MF patients do not have evidence of the JAK2V617F mutation, a number of candidate gene resequencing efforts in MPNs were performed following the discovery of the JAK2V617F mutation. This led to the discovery of a series of mutations in the thrombopoietin receptor gene MPL in 2006 as well as a series of mutations in exon 12 of JAK2 in 2007 [9, 10].

JAK2 exon 12 mutations were discovered by Scott et al. through systematic resequencing of all coding exons of JAK1, JAK2, JAK3, TYK2, STAT5A, and STAT5B in JAK2V617F-negative PV patients [10]. Four novel somatic mutations in exon 12 of JAK2 were identified including the K539L allele as well as three additional alleles that were small deletions or insertions at codons 538–543 (Fig. 2.1). These four mutations were functionally validated to lead

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**Fig. 2.1** Schematic representation of the most frequent point mutations in JAK2 found in myeloproliferative neoplasms (a) and the acute leukemias (b). In addition the TEL-, PCM1-, and STRN3-JAK2 fusions involved in various blood cancers are shown in (c), (d), and (e), respectively. The TEL-JAK2 fusions involve the entire JH1 kinase domain with various lengths of the JH2 pseudokinase domain. The PCM1-JAK2 fusions all involve the JH1 and JH2 domains and variable amounts of the SH2 domain. A recent translocation between exon 9 of the striatin gene STRN3 to exon 18 of JAK2 (e) was recently discovered by transcriptome sequencing in ALL.
to constitutive activation of JAK2 through transformation of Ba/F3 cells to factor independent growth. The JAK2K539L allele was also shown to result in erythrocytosis and a PV phenotype in a murine BMT assay.

Since the original description of JAK2 exon 12 mutations, several additional mutations in exon 12 of JAK2 have also been described including the H538Q allele [34]. Interestingly, clinical analysis of patients bearing the JAK2 exon 12 mutations suggested that exon 12 mutations are most commonly associated with a phenotype of isolated erythrocytosis without associated leukocytosis or thrombocytosis [10]. This provides further evidence that different mutations in JAK2 may result in varying clinical phenotypes via mechanisms that are not currently identified.

In addition to the activating point mutations in JAK2 in MPNs, a number of chromosomal translocations involving JAK2 have been found in leukemias and rarely in classic MPNs. A fusion of JAK2 to TEL (translocation ETS leukemia or ETV6) was the first described JAK2 somatic alteration identified in human malignancy [35]. All described TEL-JAK2 fusion proteins include the JH1 kinase domain of JAK2, and occasional translocations have also been found to include the JH2 pseudokinase domain [35, 36] (Fig. 2.1). The TEL-JAK2 fusion results in constitutive kinase activity of JAK2, which is transforming in Ba/F3 assay and in vivo models. The second most frequently reported JAK2 translocation fuses JAK2 to PCM1 [37–40]. The PCM1 gene encodes a centrosomal protein, pericentriolar material 1. Unlike the TEL-JAK2 fusion, all PCM1-JAK2 fusions identified to date contain both the kinase and pseudokinase domains of JAK2. Lastly, a recent translocation between JAK2 and the striatin gene was recently discovered in a patient with ALL, using next generation transcriptome sequencing [41].

In the original description of JAK2 exon 12 mutations, it was thought that exon 12 mutations occur exclusively in patients who do not bear the JAK2V617F mutation. However, it has been more recently shown that rare PV patients may actually bear both JAK2V617F as well as exon 12 mutations. In a report by Li et al., it was identified that a single patient had both the JAK2V617F mutation and an exon 12 mutation [34]. Clonal analysis demonstrated that the JAK2V617F and JAK2 exon 12 mutations had occurred in independent clones suggesting that the two mutations possibly have overlapping effects on cell behavior and/or indicate the presence of a MPN predisposition allele. Moreover, in the same report, clonal analysis of several patients with the JAK2V617F mutation revealed the presence of endogenous erythroid colonies, which were negative for any mutation of JAK2. Several other authors have corroborated this last finding, strongly suggesting the possibility of transforming mutations in MPN patients, which precede acquisition of mutations in JAK2 and MPL and illustrate the clonal heterogeneity present within MPN patients.

Somatic mutations in MPL were originally discovered by Pikman et al. during candidate gene resequencing of EPO-R, MPL, and GCSF-R [9]. A series of somatic mutations in MPL have now been identified including MPLW515L, MPLW515K, MPL505N, MPLA506T, MPLA519T (Fig. 2.2). MPL mutations may occur in as many as 8% of ET and MF patients, although the actual frequency of MPL mutations in MPN patients has not been as extensively studied as the prevalence of JAK2V617F [42]. As with JAK2 exon 12 mutations, mutations in MPL were originally thought to occur only in patients without the JAK2V617F
mutation; however, several reports have identified MPN patients with concurrent JAK2 mutations as well as MPL mutations [43]. Interestingly, whenever clonality studies have been performed on such patients, it is clear that when MPL mutations occur concurrently with JAK2V617F mutations, the MPL and JAK2 mutations occur exclusively in independent clones, suggesting overlapping effects of these mutations on cell growth/phenotype [43]. This is in contrast with cytogenetic alterations, including deletion of 20q and gains of portions of chromosome 8 and 9, which have been documented to occur concurrently with MPL/JAK2 mutations in MPN clones [43].

As with the JAK2 mutations described earlier, MPL mutations have been clearly shown to be transforming in Ba/F3 assays [9]. In addition, murine BMT assays with MPLW515K/L result in a completely penetrant MPN with marked thrombocytosis, splenomegaly, and fibrosis of the bone marrow recapitulating human ET and MF.

TET2 and ASXL1 Mutations in MPNs: Epigenetic Events in MPN Pathogenesis?

Evidence for Additional Mutations Beyond JAK2 and MPL

Although the discovery of mutations in JAK2 and MPL were extremely enlightening, several lines of evidence made it clear that mutations in genes other than JAK2 and MPL must be present in the MPNs. First was the aforementioned question of how a single mutation in JAK2, which appeared to be sufficient for MPN pathogenesis, could result in the development of three phenotypically variable diseases. One attractive hypothesis to this question was that additionally acquired or inherited genetic modifiers outside of JAK2 or MPL could be present and modify the MPN phenotype resulting in the JAK2V617F mutation.
Evidence for the presence of additional genetic events in MPNs came from the fact that despite the discovery of \textit{JAK2} and \textit{MPL} mutations, a significant proportion of ET and MF patients still had no identifiable known mutations. Moreover, clonal analysis of patients with \textit{JAK2/MPL} mutations consistently demonstrated the presence of occasional patients with \textit{JAK2} wildtype EEC – clear evidence that an additional aberration responsible for erythropoietin-independent growth may be present in some MPN patients [44]. Clonality analysis of patients with both a cytogenetic abnormality in conjunction with the \textit{JAK2V617F} mutation also revealed that occasional patients could be identified, where cytogenetically abnormal clones with and without the \textit{JAK2V617F} mutation could be identified [43]. This was first described in a patient with loss of the long arm of chromosome 20 (20q-) in addition to the \textit{JAK2V617F} mutation and suggested that loss of a gene on 20q might be responsible for the cytokine-independent growth of \textit{JAK2} wild-type clones (Fig. 2.3).

Since the discovery of the \textit{JAK2V617F} mutation in 2005, a number of reports have consistently reported that leukemic blasts of acute myeloid leukemia (AML) derived from a \textit{JAK2V617F} MPN are frequently \textit{JAK2} wild-type [45, 46]. This suggests that the MPN and AML clones could arise from two different progenitor cells or that an ancestral clone bearing an abnormality preceding the \textit{JAK2V617F} mutation could be present giving rise to both the MPN and the AML.

Lastly, the discovery of \textit{JAK2} and \textit{MPL} mutations led to intense scrutiny of possible genetic causes for familial cases of MPNs. Although the germline \textit{MPLS505N} mutation was identified in familial ET kindred in Japan [47], thus far no heritable mutations in \textit{JAK2} have been described. Given that some heritable genetic abnormality must be present to account for \textit{JAK2/MPL} wild-type familial MPN cases, additional novel mutations in MPN pathogenesis have been speculated to exist for some time.

**Somatic Mutations in TET2**

Mutations in the gene \textit{TET2} (ten-eleven translocation two) were originally published in papers by Delhommeau et al. and Langemeijer et al. in 2009 and identified mutations in \textit{TET2} in MPNs, myelodysplastic syndromes (MDS), and AML (Table 2.3 and Fig. 2.4) [16, 18]. However, the earliest description
Table 2.3 Frequency of TET2 mutations in myeloid disorders.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Frequency</th>
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<tbody>
<tr>
<td>Polycythemia vera</td>
<td>9.8–16%</td>
</tr>
<tr>
<td>Essential thrombocytosis</td>
<td>4.4–5%</td>
</tr>
<tr>
<td>Primary myelofibrosis</td>
<td>7.7–17%</td>
</tr>
<tr>
<td>Systemic mastocytosis</td>
<td>20–30%</td>
</tr>
<tr>
<td>Chronic myelomonocytic leukemia</td>
<td>20–58%</td>
</tr>
<tr>
<td>Myelodysplasia</td>
<td>10–26%</td>
</tr>
<tr>
<td>De novo acute myeloid leukemia</td>
<td>12–24%</td>
</tr>
<tr>
<td>Secondary acute myeloid leukemia</td>
<td>15–25%</td>
</tr>
</tbody>
</table>

Note: No formal study of TET2 mutations in CML has been published but evaluation of a small number of BCR-ABL positive accelerated and blast-crisis phase CML and ALL has documented existence of TET2 mutations in DNA from patients with BCR-ABL positive disease.

Fig. 2.4 Schematic representation of TET2 and mutations found in 354 MPN (a), 144 MDS (b), and 46 CMML (c), samples. Mutations in TET2 have been found in every exon and occur as missense (down arrowheads), nonsense (up arrowheads), and frameshift mutations (diamonds). This diagram also illustrates that the frequency of TET2 mutations as well as the frequency of bi- and tri-allelic TET2 mutations (which are color-coded) are more common in MDS and CMML than in classic MPNs. Adapted from Abdel-Wahab et al. [14].
of the TET2 mutation was made public, based on the observations in MPN patients in a late-breaking abstract by Delhommeau et al., at the December 2008 meeting of the American Society of Hematology. It was noted in this abstract that the majority of JAK2V617F mutant PV patients (~85%) reveal expansion of CD34+CD38+ committed progenitor cells over CD34+CD38- multipotent progenitors in ex vivo liquid cultures. In contrast, a minority (~15%) of JAK2V617F mutant PV patients reveal expansion of the more immature multipotent progenitor cells (CD34+ CD38-) over CD34+CD38+ cells. Hypothesizing that a novel genetic abnormality might be responsible for this immunophenotypic difference in the 2 patient subsets, the authors performed SNP arrays (Affymetrix 500K) and array CGH (Agilent 244K) on a small number of these patient samples. They remarkably found that 3 of 5 JAK2V617F mutant PV patients with expansion of CD34+CD38- cells had evidence of LOH at the genomic locus 4q24. One of these 5 patients had a deletion of a 325 kB region of DNA at 4q24- with the only gene present in this region being TET2. This then led to sequencing of TET2 in these patient samples and identification of TET2 mutations in MPNs.

Prior to the discovery of TET2 mutations, karyotypic abnormalities at 4q24 had been described in patients with MDS and AML by Viguie et al. [48] In this report, four patients with 4q24 rearrangements (t(3;4)(q26;q24), t(4;5)(q24;p16), t(4;7)(q24;q21), del(4)(q23;24)) were described, and the karyotypic abnormality was present in both myeloid and lymphoid lineages. Moreover, one patient who developed concomitant lymphoma also had the 4q24 rearrangement in the malignant lymphoid cells. Consistent with this observation, both Delhommeau and Langemeijer found evidence of 4q24 LOH in MDS and AML [16, 18].

Sequencing of TET2 has led to the identification TET2 mutations in every myeloid disorder investigated to date (Table 2.3) [14, 16, 18, 49–53]. Mutations in TET2 have been found in all coding regions and can appear as missense, nonsense, or frameshift mutations (Fig. 2.3). In addition, mutations in TET2 are not uncommonly biallelic (i.e., involving both copies of TET2). Taken together, these data suggest that mutations in TET2 represent loss-of-function mutations.

TET2 is a member of the TET family of genes, the first member of which to be described was TET1. TET1 (ten-eleven translocation 1), located on chromosome 10, was originally identified in cases of adult and pediatric AML as a translocation with the gene MLL (located on chromosome 11) [54]. Although TET1 was the original gene member identified in hematologic malignancies, no sequence alterations in TET1 or TET3 have been identified to date [14]. In a landmark publication in 2009, the function of TET1 was first described (Fig. 2.5) [55]. Tahiliani et al. were interested in the identification of human enzymes, which modify bases of nucleic acids as a means to understand how catalytic modifications of DNA bases affect the genetic code. As such, they undertook a bioinformatics approach to identify human homologs of the trypanosome proteins JBP1 and JBP2, which are known to oxidize the 5-methyl group of thymine [56]. Such enzymes were not previously known to exist in higher organisms. Surprisingly, they found that the TET family of genes was human homologues of these trypanosome enzymes. Further characterization of TET1 revealed that it is a 2-oxoglutarate- and iron(II)-dependent dioxygenase, which serves to oxidize the 5-methyl group of cytosine leading to the formation of 5-hydroxymethylcytosine. The exact function of this modification is not yet known but it is hypothesized to affect gene expression
by (1) serving as an intermediate step to promote demethylation of DNA, (2) affecting recruitment of methyl-binding proteins to DNA, (3) recruiting proteins to DNA, which specifically recognize the hydroxy-methylcytosine mark.

It has been shown that mutations in $TET2$ lead to decreased $TET2$ gene expression in granulocytes of diseased patients [18]. Although it is predicted that $TET2$ and $TET3$ share the catalytic domain of $TET1$, $TET2$ and $TET3$ have not been formally shown to share the catalytic activity of $TET1$ to oxidize 5-methyl group of cytosines. This question is critical as both $TET1$ and $TET2$ appear to be expressed in myeloid cells and it is known whether decreased $TET2$ expression and/or function by mutation can be compensated by expression of $TET1$.

The fact that $TET2$ is expressed at the level of the CD34+ stem/progenitor cell and that $TET2$ mutations are found in all myeloid disorders to date suggests that $TET2$ mutations might be an early event in MPN pathogenesis [16, 18, 50]. Initial clonality studies of a small cohort of $JAK2V617F$ mutant MPN patients with a $TET2$ mutation revealed that $TET2$-mutant/$JAK2$-mutant and $TET2$-mutant/$JAK2$-wildtype clones could be identified but not $TET2$-wildtype/$JAK2$-mutant clones – clear support of the hypothesis that $TET2$ mutations occur as a “pre-$JAK2$” event [16]. Subsequent studies, however, have no repeated cases where $TET2$ mutations are clearly acquired after $JAK2$ mutations, suggesting that mutations in $TET2$ may not represent the earliest genetic aberration in all patients with $TET2$-mutant MPNs.

Although it is clear that $TET2$ mutations can arise at the level of the stem/progenitor cell, the effect of $TET2$ mutations on hematopoietic function and transformation is not yet known. The accumulating genomic data have proven that $TET2$ alterations do not seem to associate with any particular phenotype of

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**Fig. 2.5** Recently discovered enzymatic function of $TET1$ to mediate hydroxymethylation of 5-methylcytosine. $TET2$ and $TET3$ are also predicted to contain the 2-oxoglutarate and Fe(II)-dependent enzymatic domain seen in $TET1$ but their enzymatic function has not been formally demonstrated.
myeloid disease [14, 51–53]. Moreover, TET2 mutations can occur in concert with JAK2, KIT, MLL, Flt3, and CEBPa mutations but are not enriched in patients with these recurrent somatic alterations. One single report describes a possibly significant association of TET2 mutation with NPM1 alterations in AML but this finding has not been reproduced [57].

Preliminary evidence of the functional importance of TET2 myelopoiesis comes from xenograft studies in the initial reports of TET2 mutations by Delhommeau and colleagues [16]. They noted that injection of JAK2V617F-positive CD34 cells from MPN subjects with \((n = 2)\) versus without TET2 mutations \((n = 3)\) into NOD-SCID mice revealed a more efficient engraftment of TET2 mutant cells over TET2 wildtype CD34+ cells. Moreover, the resulting hematopoiesis was skewed toward increased frequency of myeloid progenitors over lymphoid progenitors. Further experiments in more genetically uniform human and murine HSPCs are needed to confirm and expand this data given that unknown additional genetic factors beyond TET2 and JAK2 alleles in these 5 samples may have influenced the results.

The effect of TET2 alterations on clinical outcome in myeloid malignancies has been the subject of much debate. Thus far TET2 mutations have not been shown to influence survival or prognosis in any MPN (including PV, ET, and systemic mastocytosis) [51–53]. Moreover, TET2 mutations do not appear to influence the number or type of karyotypic abnormalities found in MPN patients [58]. At the same time, TET2 mutations have been shown to cause a negative effect on overall survival in AML [14, 18]. In two separate reports, TET2 mutations were found to lead to significantly decreased overall survival in AML, which was independent of other prognostic factors including age, cytogenetic status, and other genetic alterations (Flt3, NPM1, MLL, CEBPa). This is in striking contrast with a report of a similar number of patients with MDS from the Francophone Study Group, which reported an overall survival of 76.9% in TET2 mutant MDS patients (95% CI, 49.2–91.3%) vs. 18.3% (95% CI, 4.2–41.1%) in TET2 wild-type MDS patients \((p=0.005)\) [59]. In a multivariate analysis including age, IPSS, TET2 mutational status, and transfusions, the absence of a TET2 mutation was associated with a 4.1-fold increased risk of death (95% CI, 1.4–12-fold, \(p=0.009\)) suggesting that TET2 mutations were an independent favorable prognostic factor. Clinical analysis of larger numbers of patients with MDS and larger number of more genetically uniform and uniformly treated AML patients will be needed to clarify these findings.

It has been hypothesized for some time from empirical clinical observations that alteration of DNA methylation must be important in the pathogenesis of myeloid malignancy as the use of DNA methyltransferase inhibitors, particularly in MDS, has been an important therapeutic development. The possibility exists that TET2 mutations at the level of the stem/progenitor cell might result in increased likelihood of hematopoietic transformation and aberrant myelopoiesis due to alterations in DNA methylation. How mutations in TET2 might influence response to DNA methyltransferase inhibitors such as decitabine or 5-azacitidine is currently unknown. Moreover, if alterations in TET2 precede JAK2 mutations and promote neoplasia at the level of the stem/progenitor cell, it is not clear how TET2 mutations might affect response to treatment of MPN patients with JAK2 inhibitors.
Somatic Mutations in ASXL1

Shortly after the discovery of TET2 mutations in myeloid cancers, somatic mutations were identified in another putative epigenetic modifier in myeloid malignancies, Additional Sex Combs Like 1 (ASXL1). Mutations in ASXL1 were originally identified in aCGH studies of MDS samples [17]. Gelsi-Boyer et al. performed Agilent 244K CGH arrays on several patients with MDS and noted deletions in one patient at 20q11. In this particular patient, the 20q deletions involved only two possible genes – ASXL1 and DNMT3B. Sequencing efforts of both genes followed and mutations in ASXL1 were found in 4/35 patients with MDS (11%). Further sequencing of ASXL1 by this group and others led to reports of ASXL1 mutations at a similar frequency and spectrum of myeloid malignancies as TET2 mutations (Table 2.4) [15, 17, 60].

ASXL1 is one of the three mammalian homologs of the Additional sex combs gene in Drosophila. The genes are named for the fact that deletion of the Additional sex combs gene in Drosophila leads to homeotic transformations. This occurs because ASXL1 members appear to regulate the expression of both Polycomb group (PcG) and Trithorax group (TxG) proteins in Drosophila and mammals [61, 62]. The ASXL family members can simultaneously promote and/or repress PcG and TxG members, and the exact mechanism of ASX/PcG/TxG interactions is not well-understood. ASXL1 was more recently found to be a coactivator of retinoic acid-mediated transcriptional activity in a yeast-two hybrid screen [61]. All three ASXL family members are characterized by an amino-terminal homology domain, two proximal interaction domains for nuclear receptors, and a C-terminal plant homeodomain (PHD) (Fig. 2.6) [61–63]. The PHD domain has been recognized in more than 100 human proteins (including MLL, JARID1, and TIF1) and appears to be a motif necessary for binding to modified histones. The exact motif at which the PHD domain of ASXL1 binds is not known but two commonly recognized motifs, which PHD domains have been shown to recognize, include histone H3 tri-methylated on lysine 4 (H3K4me3) and histone H3 tri-methylated lysine 9 (H3K9me3).

The accumulating genetic data on ASXL1 has suggested that ASXL1 mutations are predominantly found as nonsense and frameshift alterations in exon 12 (Fig. 2.5) [15, 17, 60]. The mutations found have all been heterozygous, can be found at the level of CD34+ cells, and may overlap with TET2 and JAK2 mutations in MPNs (Table 2.4). Of note, none of the published genetic reports have included paired normal tissue and as a result many potential missense mutations

<table>
<thead>
<tr>
<th>Disease</th>
<th>Frequency</th>
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<tbody>
<tr>
<td>Classic MPNb</td>
<td>-7.8%a</td>
</tr>
<tr>
<td>Chronic myelomonocytic leukemia</td>
<td>11–36.4%</td>
</tr>
<tr>
<td>Myelodysplasia</td>
<td>17–43%</td>
</tr>
<tr>
<td>Acute myeloid leukemia</td>
<td>-17.8%a</td>
</tr>
</tbody>
</table>

aNone of the published studies of ASXL1 mutations in patient samples have included paired normal tissue. As such, possible missense mutations have been censored from analysis
bClassic MPN includes the disease PV, ET, and MF. No large genetic studies of ASXL1 mutations in MPN patients have been performed to elucidate the percentage of ASXL1 mutations separately in each of the three classic MPNs
in ASXL1 have been censored from the literature. Nonetheless, the finding of recurrent nonsense and frameshift mutations at this particular location of ASXL1 is striking and suggests that the mutations in ASXL1 may serve to truncate the PHD domain of ASXL1 while leaving the nuclear interacting domains intact (Fig. 2.5).

The initial excitement over the finding of widespread and recurrent ASXL1 mutations in myeloid malignancies may have been quelled by the recent reports of a somatic ASXL1 knockout mouse, which had minimal hematopoietic defects [62, 63]. This model, created by Fisher et al. placed a PGK promoter-drive neomycin expression cassette into exon 5 of ASXL1 interrupting the reading frame of ASXL1 (this allele is referred to as Asxl1tm1BC) and leading to truncation of nuclear interacting domains as well as the PHD domain. Mice homozygous for this allele (Asxl1tm1BC/tm1BC) had incompletely penetrant perinatal lethality; however, neither did they develop any overt hematologic malignancy nor did they observe defects in number or function of multipotent progenitors. Potential reasons for the fact that no clear hematopoietic phenotype was seen in the Asxl1tm1BC/tm1BC mice include the possibility that (1) other ASXL genes exist, which have overlapping expression and redundant function, and (2) the ASXL1 mutations found in humans are actually gain-of-function mutations poorly modeled by a mice lacking ASXL1 expression altogether. Of note, ASXL2 is expressed in hematopoietic tissue and no mutations have been found in ASXL2 in myeloid malignancies to date, leading credeence to the possibility of ASXL2 compensation in a state of total ASXL1 loss.

Thus far no studies have examined whether mutations in ASXL1 are associated with any particular outcome. Moreover, it is not known whether mutations in ASXL1 affect response to chromatin-modifying therapies such as histone deacetylase inhibitors.

**Genetics of Leukemic Transformation of MPNs**

As stated earlier, it has been known since the discovery of the JAK2V617F mutation that JAK2-wildtype AML may arise from a preceding JAK2V617F MPN [45, 46]. The largest series to date reported by Theocharides et al. identified
JAK2V617F mutations in 17/27 samples at MPN diagnosis, yet 9 of these 17 JAK2-mutant patients presented with JAK2V617F-negative blasts during transformation to sAML. Since then, a number of studies have investigated potential genes, which might be altered at leukemic transformation of MPNs [45, 46, 49, 64]. The majority of these studies have been hampered by the relatively low number of samples and the lack of paired material from the MPN and leukemic state. Nonetheless, aggregate data from these studies have implicated mutations in p53, TET2, NRAS, RUNX1, and CBL at leukemic transformation. Reports from four groups have identified that a sizeable percentage of patients acquire TET2 mutations in the leukemic state when not originally present in the MPN state [45, 46, 49, 64]. In contrast, analysis of paired samples revealed that ASXL1 mutations seem to be present in both members of the pair and are not enriched in the leukemic state compared with the MPN phase of disease [65]. The functional relevance of this finding is not yet clear given that the molecular function of TET2 and ASXL1 mutations in hematopoiesis is not yet understood. Lastly, one recent report identified deletions in Ikaros in a small number of patients at the time of leukemic transformation of MPNs [66]. This is a particularly intriguing finding as deletions of Ikaros appear to be a near-obligate requirement in lymphoid leukemia transformation of CML (but not myeloid leukemic transformation of CML) [67].

The Possibility of Additional Mutated Genes in MPNs

Despite the discovery of mutations in four genes (JAK2, MPL, TET2, and ASXL1) in patients with MPN over the last 5 years, there is a significant proportion of patients with ET and PV without evidence of mutations in any of the aforementioned genes. This has motivated continued interest in gene discovery efforts in MPNs, and there are several intriguing possible genetic alterations being actively investigated. Longest suspected are the genes located in regions that are occasionally involved in gross cytogenetic alterations in MPN patients. The most common cytogenetic alteration in MPN is the deletion of the long arm of chromosome 20 (20q-). 20q- is found in 10% of PV patients, 4% of MDS patients, and 1–2% of AML patients [68]. It has been previously demonstrated that 20q-/JAK2 wildtype EEC clones may be grown from 20q-/JAK2V617F mutant MPN patients bolstering the hypothesis that a novel tumor suppressor sufficient for EPO-independent growth may exist on 20q in MPNs.

Careful mapping of the commonly deleted region (CDR) in all three diseases has led to identification of a deleted region, which overlaps in all three conditions (termed as the “Myeloid CDR”) [68]. The “myeloid CDR” spans 2 Mb and contains 16 known genes. Out of these 16 genes, 6 are expressed within normal CD34+ cells and as such these 6 genes have been the subject of intense investigation (Fig. 2.3). So far, mutations have not been reported in any of these 6 genes, and thus it is assumed that the gene involved in 20q-myeloid malignancies is a tumor suppressor gene affected by haploinsufficiency. A systematic RNA-interference strategy to identify the gene target in the 20q-CDR, as was done in 5q-MDS [69], has not been performed to date. Moreover, potentially important micro-RNAs within the noncoding region of 20q-, as with miR-145 in 5q-MDS [70], have not been identified.

In addition to the possibility of novel tumor suppressor gene on 20q, at least 4 new genes have been recently identified in myeloid malignancies related to
MPNs. This includes the genes \textit{UTX} and \textit{CBL} in MDS and CMML as well as \textit{IDH1} and \textit{IDH2} in AML [65, 71–76]. Thus far, no large-scale sequencing efforts of any of these genes have been published in the classic MPNs and the possibility that one or more of these genes may be mutated in some proportion of MPNs exists.

\textbf{Inherited Genetic Events in MPN Pathogenesis}

\textit{Evidence for Germline Mutations in MPN}

The existence of families with MPNs has long suggested the presence of germline susceptibility genes, which predispose to MPN development. As mentioned earlier, mutations in \textit{JAK2} were quickly evaluated and discarded as a potential cause for familial MPN as (1) no germline mutations in \textit{JAK2} have been identified, and (2) there is repeated evidence of \textit{JAK2} wild-type clones in members of familial MPN kindreds. There have been several studies of kindreds with familial MPNs (Table 2.5) [44, 77–80]. Familial MPNs are loosely defined as families where at least two relatives within the pedigree have an MPN. This must be distinguished from inherited disorders with Mendelian transmission and single hematopoietic lineage transmission (this includes the conditions known as “Hereditary Erythrocytosis” and “Hereditary Thrombocytosis.”).

The single largest report of the familial aggregation of MPNs comes from a population-based study in Sweden, which examined the risk of MPN development in relative of citizens diagnosed with an MPN, was reported [81]. This study found that first-degree relatives of MPN patients have a three-to-sevenfold increased risk of developing an MPN compared with members of the population without a family history of MPN. This risk was specific to PV (RR = 5.7; 95% confidence interval (CI) 3.5–9.1), ET (RR = 7.4; CI 3.7–14.8), and MPN NOS (RR = 7.5; CI 2.7–20.8) but not CML where the relative risk was 1.9 with a \( p \)-value of 0.09. From the accumulated studies of families with MPNs, it seems that in most kindreds the inheritance pattern appears to be consistent with an autosomal dominant trait with decreased penetrance (Table 2.5). However, no clear cytogenetic or genetic locus was associated with germline predisposition toward MPN until the discovery of a risk SNP within \textit{JAK2} itself in 2009.

\textbf{The Germline JAK2 Haplotype}

In 2009, three independent reports were published simultaneously of a germline haplotype block (commonly referred to as “46/1”) that includes \textit{JAK2} itself and is associated with development of both the \textit{JAK2V617F} mutation and MPN. This haplotype block is markedly enriched in MPN patients and tagged by a SNP (rs10974944) located within an intron between exons 12 and 13 of \textit{JAK2} [11–13]. All these three studies demonstrated that heterozygotes for this haplotype were significantly more likely to acquire the \textit{JAK2V617F} mutation in \textit{cis} with the predisposition SNP allele than on the other chromosome. Genotypes at rs10974944 exist as homozygous for the major allele (CC), heterozygous (GC), or homozygous for the minor allele (GG). The frequency of the GG/GC genotypes is much higher in MPN compared with matched controls (OR = 3.1, \( P = 4.1 \times 10^{-20} \)) consistent with the G allele functioning as a dominant MPN predisposition allele and increasing the likelihood of developing an MPN to three-to-fourfold [11–13].
<table>
<thead>
<tr>
<th>Reference</th>
<th># Families</th>
<th># Patients</th>
<th>Inheritance pattern</th>
<th>Findings</th>
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</thead>
<tbody>
<tr>
<td>Rumi et al. [80]</td>
<td>20</td>
<td>43</td>
<td>AD, decreased penetrance</td>
<td>Identified evidence of clonal hematopoiesis without JAK2 mutations in some familial MPN kindred members suggesting JAK2V617F as a secondary event in familial MPN</td>
</tr>
<tr>
<td>Kralovics et al. [44]</td>
<td>6</td>
<td>13</td>
<td>AD, decreased penetrance</td>
<td>Excluded linkage between familial PV and candidate loci including c-mpl, EPO-R, 20q, 13q, 5q, and 9p</td>
</tr>
<tr>
<td>Rumi et al. [79]</td>
<td>35</td>
<td>75</td>
<td>AD, decreased penetrance</td>
<td>Found evidence of anticipation of disease-onset with successive generations in familial MPN</td>
</tr>
<tr>
<td>Bellanne-Chantelot et al. [99]</td>
<td>72</td>
<td>174</td>
<td>AD, decreased penetrance</td>
<td>Found evidence of MPN kindreds with and without JAK2V617F mutation</td>
</tr>
<tr>
<td>Hussein et al. [58]</td>
<td>1</td>
<td>2</td>
<td>NE</td>
<td>Suggested JAK2V617F as a secondary event in familial MPN</td>
</tr>
<tr>
<td>Pardanani et al. [77]</td>
<td>2</td>
<td>6</td>
<td>NE</td>
<td>Noted 2 siblings with MPN: 1 with JAK2V617F mutant PV and the other with JAK2 wild-type ET</td>
</tr>
<tr>
<td>Pietra et al. [78]</td>
<td>2</td>
<td>4</td>
<td>NE</td>
<td>Identified somatic JAK2 exon 12 mutations in familial MPN cases suggesting possible genetic predisposition for both JAK2V617F and exon 12 mutants in familial MPN</td>
</tr>
<tr>
<td>Abdel-Wahab et al. [14]</td>
<td>48</td>
<td>28</td>
<td>NE</td>
<td>Examined TET2 mutations MPN kindreds and identified only somatic TET2 mutations</td>
</tr>
<tr>
<td>Saint-Martin et al. [100]</td>
<td>42</td>
<td>61</td>
<td>NE</td>
<td>As above. Also noted that TET2 mutations were associated with leukemic transformation in this small cohort</td>
</tr>
</tbody>
</table>

<sup>a</sup>Adapted from Rumi et al. [101]
The \( \textit{JAK2V617F} \) mutation and rs10974944 are contained in the same haplotype block, which is distinct from the promoter and 5' exons of \( \textit{JAK2} \). The mechanism by which a germline genetic variant in the 46/1 haplotype block could result in increased risk of developing the \( \textit{JAK2V617F} \) mutation and MPN development is not known. Potential mechanisms were investigated in the three aforementioned studies, in which negative results included genotype-specific differences in (1) \( \textit{JAK2} \) expression, (2) nonsynonymous alterations within the haplotype block, (3) alterations in the 3' UTR, and (4) differential splicing of \( \textit{JAK2} \) [11–13].

The current proposed hypotheses as to how the 46/1 haplotype confers risk of \( \textit{JAK2V617F} \) and MPN development includes two commonly cited hypotheses. First is the hypothesis that the haplotype confers an increased frequency of mutations at the \( \textit{JAK2} \) locus, and those mutations that result in a selective growth advantage manifest as a clonal disorder. This hypothesis is strongly supported by the examples described earlier of MPN patients that have acquired the \( \textit{JAK2} \) mutation multiple times in independent clones. However, this so-called “hyper-mutability” hypothesis is hindered by the fact that no mechanism has been proposed to explain the mechanism by which the haplotype would increase the frequency of mutations at a distant nucleotide. Moreover, the haplotype is also enriched in \( \textit{JAK2V617F} \)-negative MPN [82, 83].

The second hypothesis is the idea that the \( \textit{JAK2V617F} \) mutation is equally likely to occur in different haplotypes but cells in which the mutation occurs on the risk haplotype gain a stronger growth advantage. This so-called “fertile ground” hypothesis is hindered only by the fact that the mechanism by which the haplotype might confer a growth advantage has not been proposed or identified. The recent discovery of the haplotype also increasing the risk of \( \textit{JAK2} \) exon 12 and \( \textit{MPL} \) mutations has been cited to favor the fertile ground hypothesis over the hypermutability hypothesis [84, 85]. Nevertheless, the possibility still exists that the haplotype is in linkage with a yet unidentified functional variant, which influences disease pathogenesis.

In addition to the above evidence for a germline genetic cause for MPN pathogenesis, there also exists the possibility that clustering of MPNs may also occur because of yet unidentified environmental exposures, which may increase the risk of developing \( \textit{JAK2V617F} \). The most intriguing suggestion of a possible environmental link and MPNs comes from recent epidemiologic data from three counties in eastern Pennsylvania where the incidence of PV is almost triple that of other areas in the United States [86]. Most of these cases have clustered in specific regions of these counties with clear evidence of \( \textit{JAK2V617F} \)-positivity of many of the cases and no evidence for consanguinity in the region. So far, no clear etiologic environmental exposure has been identified, and the area is under investigation by several federal agencies.

**Novel Genetic Alterations in MPN: The Possibility of Epigenetic Alterations, Regulatory RNAs, and Post-translational Modifications**

Although the study of the genetic causes of MPNs has been extremely insightful and resulted in the identification of multiple novel alleles, less explored thus far has been the possibility of disease-modifying alterations outside of coding nucleotides. This includes the possibility of epigenetic modifications,
differential expression of microRNAs (miRNAs), and post-translational modifications, which might modify the phenotype of disease produced in patients with JAK2 mutations. Such “post-genetic” alterations could also possibly be responsible for establishing clonal hematopoiesis prior to acquisition of mutations in JAK2.

There has been increasing interest in the study of epigenetic regulation of the JAK-STAT pathway in MPNs, partly based on several important observations of the JAK-STAT pathway in Drosophilia. In Drosophilia, the JAK-STAT pathway is simpler than in mammals with only a single JAK member (termed Hopscotch) with a single downstream STAT (STAT92E). Studies of the JAK-STAT pathway in Drosophilia have proven that the Hopscotch ligand, Unpaired, is under direct epigenetic transcriptional regulation by PcG proteins. Thus, inactivation of PcG allows excessive expression of Unpaired with inappropriate activation of Hopscotch and resultant tumors in eye and wing imaginal discs of Drosophilia [87]. Similar epigenetic control of JAK-STAT pathway members in mammals has not been demonstrated so clearly. However, there are at least two reports, which suggest that SOCS3, a negative regulator of JAK2 signaling, is hypermethylated in MPNs [88, 89]. In one study, however, SOCS3 promoter hypermethylation was restricted to patients with MF and not to those with PV or ET, and it was not clear whether SOCS3 promoter hypermethylated cases had decreased SOCS3 expression by qRT-PCR. Although there is increasing evidence that epigenetic alterations exist in patients with MPN, specific gene targets of epigenetic alteration that might explain MPN pathogenesis have not been elucidated.

In addition to the search for epigenetic regulation of JAK-STAT pathway members in MPN, it has recently been discovered that JAK2 itself may potentially affect chromatin state. Here again, studies of JAK-STAT pathway in Drosophilia have been prescient as it has been known since 2006 that Hopscotch overactivation globally disrupts heterochromatic gene silencing and destabilizes heterochromatin in Drosophilia [90]. However, the exact mechanism by which Hopscotch affects chromatin structure in Drosophilia is still not clear. Equally exciting and unresolved is the recent discovery by Dawson et al. that JAK2 can be found in the nucleus and appears to phosphorylate histone H3 among all core histones [91]. This was confirmed through evidence of (a) decreased H3 phosphorylation in the presence of at least two different JAK2 inhibitors and (b) phosphorylation of H3Y41 only after JAK2 transfection in JAK2-null gamma-2A cells. The authors then demonstrate that phosphorylation of H3Y41 results in displacement of HP1-alpha. They then posit that HP1-alpha displacement results in overexpression of lmo2, an oncogene with a known role in leukemogenesis. There are many questions yet to be answered regarding the possible role of intranuclear JAK2, such as: (1) how is JAK2 entering the nucleus given that it has no classic nuclear localization sequence? (2) how is the kinase activity of JAK2 relevant to its nuclear function given that this activity has always been known to require expression of a cytoplasmic cytokine receptor? and (3) are there any nuclear targets of JAK2 kinase activity besides histones?

miRNAs are short (~22 nucleotides), phylogenetically-conserved, nonprotein coding RNAs, which regulate gene expression through sequence-specific base pairing with target mRNA. An increasing body of work has demonstrated that miRNAs have an important role in both normal and malignant hematopoiesis. As such, a number of groups have hypothesized that differential
expression of a single miRNA or a cluster of miRNAs could result in abnormal clonal hematopoiesis preceding acquisition of the JAK2V617F mutation in MPNs. To date, the study of miRNAs in MPNs has been nascent and has largely relied on the use of comparison of miRNA gene expression profiling of MPN hematopoietic subsets compared with that of normal subjects. Although this has resulted in a list of miRNAs, which are potentially differentially expressed in MPN relative to normal individuals, functional validation of any of these candidate miRNAs has not been performed. This is critical as the target genes of miRNAs range from 1 to >100 with many of miRNA target genes being only predicted putative targets.

One of the best examples of the possible involvement of a miRNA in the pathogenesis comes from O’Connell et al. who noted that lipopolysaccharide injection of mice resulted in transient induction of miR-155 followed by granulocyte/monocyte expansion [92]. They then validated this finding by overexpressing miR-155 in a murine BMT assay, which resulted in a clear MPN phenotype with neutrophilia, splenomegaly, and extramedullary hematopoiesis. To ascertain whether miR-155 had a role in human myeloid disease, the authors compared miR-155 expression by qRT-PCR in bone marrow cells of AML patients with that of controls. Although they noted increased miR-155 expression in AML, the expression of miR-155 in other myeloid malignancies, including MPNs, was not assessed.

There have been at least four studies of miRNAs expression profiling in MPN patients relative to controls published thus far with the goal of identifying miRNAs differentially regulated in the patients with MPN relative to normal individuals (Table 2.6) [93–96]. The list of miRNAs found to be differentially expressed in patient with MPN relative to control in these different studies to date only share one miRNA, namely miR-150. This is a potentially important finding as miR-150 has been recently recognized to regulate

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study design</th>
<th>GEP platform</th>
<th>Findings</th>
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<tbody>
<tr>
<td>Bruchova et al. [94]</td>
<td>Liquid culture of mononuclear cells from PV and normal patients. GEP at</td>
<td>CombiMatrix MicroRNA Custom Array</td>
<td>PV-specific difference in miR-150 expression (decreased in PV)</td>
</tr>
<tr>
<td>Guglielmelli et al. [95]</td>
<td>miRNA gene expression in MF granulocytes compared with PV/ET and normal</td>
<td>Quantification of 156 mature miRNAs by qRT-PCR</td>
<td>miR-31, -150, -95 decreased in MF relative to PV/ET/controls; miR-34a, -342, -326, -105, -149, and -147 in all MPN compared with controls</td>
</tr>
<tr>
<td>Bruchova et al. [93]</td>
<td>Comparison of GEP from hematopoietic subsets from 5 PV and 5 controls</td>
<td>CombiMatrix MicroRNA Custom Array</td>
<td>The following miRNAs were decreased in PV subsets relative to controls: reticulocytes- miR-150, -30b, -30c; granulocytes: let7a. Multiple miRNAs found to be upregulated in PV relative to controls as well.</td>
</tr>
<tr>
<td>Slezak et al. [96]</td>
<td>Comparison of GEP from granulocytes from 6 MPN and 5 controls</td>
<td>827 custom microRNA array</td>
<td>miR-133a and -1 most significantly downregulated in MPN subjects relative to controls</td>
</tr>
</tbody>
</table>
lineage fate in MEPs [97]. In a series of in vitro and in vivo experiments by Lu et al. it was shown that overexpression of miR-150 in MEP cells promotes development of megakaryocytic cells over erythroid cells. Thus, it is possible that downregulation of miR-150 in MEPs of MPN patients with predominant erythrocytosis over thrombocytosis could be a result of differential expression of miR-150. Additional functional work to validate findings miRNA gene-expression studies along with improved understanding of miRNA network in normal hematopoiesis will hopefully improve our understanding of the possible role of miRNAs in MPNs.

In addition to regulation at the genetic, epigenetic, and post-transcriptional levels, there exists the possibility that post-translational modifications may be important in creating the MPN phenotype. Some precedent for this already exists with the discovery by Zhao et al. that the presence of JAK2V617F influences the deamidation state of the proapoptotic protein Bcl-XL [98]. It is currently thought that when a cell encounters DNA damage, the activity of the amiloride-sensitive sodium-hydrogen exchanger isoform 1 (NHE1) increases. This increases the intracellular pH, which leads to nonenzymatic deamidation of Bcl-XL. This post-translational modification results in a conversion of an asparagine residue into isoaspartic acid, which, in turn, reduces the ability of Bcl-XL to inhibit the Bcl-2 homology 3 (BH3)-only family of proapoptotic proteins, thereby promoting apoptosis. Evaluation of this pathway in primary MPN samples revealed that DNA damage-induced Bcl-XL deamidation was inhibited in JAK2V617F bearing granulocytes but not in granulocytes from normal individuals or in lymphoid cells from diseased individuals with lower levels of JAK2V617F allele burden. Further cell line experiments suggest that the V617F mutation in JAK2 somehow affects the DNA damage response and ultimately affects deamidation of Bcl-XL. Thus far no further clear examples of post-translational regulation of the MPNs have been suggested.

**Conclusion: A Multi-Step Model of MPN Pathogenesis**

The discovery of gain-of-function mutations in JAK2 and MPL in MPNs led to initial enthusiasm that JAK2/MPL mutations were the disease-initiating events in MPN, and were responsible for the onset of disease as well as clonal hematopoiesis. The fact that JAK2/MPL mutations were transforming in in vitro as well as in vivo assays supported this “single-hit” model of MPN pathogenesis. However, careful genetic analysis of isolated clones from JAK2/MPL mutant MPN patients as well as familial MPN kindreds provided clear evidence that alterations other than JAK2 mutation must be present.

The fact that clonal hematopoiesis can exist in members of MPN kindreds before JAK2 development as well as the fact that JAK2 wild-type leukemic blasts were found derived from JAK2V617F mutant MPN samples suggest that genetic alteration(s) can precede acquisition of JAK2/MPL mutations. The discovery of mutations in TET2, ASXL1, and the strong, yet unidentified germline genetic risk haplotype for MPNs, has led to the hypothesis that these genetic alterations can in fact establish clonal hematopoiesis without causing overt disease (Fig. 2.7). In this multi-step model of MPN pathogenesis, it is proposed that a genetic alteration, somatic or inherited, occurs in a hematopoietic stem/progenitor cell. This state of clonal hematopoiesis may
not yield an overt clinical phenotype but likely increases the likelihood of JAK2/MPL mutation acquisition; clones that acquire activating mutations in the JAK-STAT mitogenic pathway gain further clonal advantage. The finding that mutations in \textit{TET2} and \textit{ASXL1} can occur years before the clinical onset of MPN phenotype has led to strong interest that mutations in either of these genes could establish clonal hematopoiesis. However, definitive evidence that alterations in \textit{TET2} and \textit{ASXL1} lead to clonal hematopoiesis has not been shown, and mutations in \textit{TET2} have now been found to occur at all stages in MPN pathogenesis (before or after \textit{JAK2V617F} acquisition) making it clear that \textit{TET2} mutations are not a canonical “pre-JAK2” event [64, 65]. This has led some authors to speculate that disease pathogenesis in MPNs is heterogeneous with some patients acquiring \textit{JAK2/MPL} mutations as the initiating and sole event whereas others develop nonphenotype defining “pre-JAK2” mutations first [7]. The realization that the JAK2 haplotype leads to increased acquisition of both \textit{JAK2} and \textit{MPL} mutations suggests germline genetic variation is relevant to MPN pathogenesis. At the same time, functional data to

Fig. 2.7 Hypothesized multi-step model of MPN pathogenesis. Prior to the acquisition of mutations that activate the JAK-STAT pathway, it is proposed that genetic abnormalities may occur, which establishes a state of clonal hematopoiesis. The events that establish clonal hematopoiesis could occur as a poorly understood effect from the presence of the MPN risk haplotype, acquisition of mutations in \textit{TET2} or \textit{ASXL1}, and/or loss of an unidentified gene located on 20q. Establishment of clonal hematopoiesis is thought to increase the likelihood of acquisition of phenotype-defining mutations (such as mutations in \textit{JAK2} and \textit{MPL}). Later, stepwise acquisition of disease-transforming genetic events promotes transformation from a chronic MPN state to a state of acute myeloid leukemia. No canonical genetic events have been identified at leukemic transformation of MPNs, but genes that are enriched in MPN state compared with leukemic state in paired samples include mutations in p53, TET2, and N/K-Ras. It has also recently been observed that Ikaros deletions may occur at leukemic transformation.
support the role of the JAK2 haplotype and other germline MPN alleles in MPN pathogenesis does not yet exist.

Despite knowledge of specific genetic alterations in MPNs over the last 5 years, it is clear that yet more unidentified genetic and “post-genetic” events must occur in MPN patients. A significant proportion of ET and MF patients present without any known MPN disease alleles. Moreover, a full explanation for the intriguing MPN risk haplotype has not been identified, and the identity of which genes within recurrent cytogenetic alterations identified in MPN patients contribute to transformation is not known. Moreover, systematic evaluation of the epigenome, regulatory non-coding RNAs, and post-translational regulatory events in MPNs are just now being performed.

Improvements in next-generation sequencing technologies as well as increased access to these technologies will allow groups to better characterize the MPN genome. This will, in turn, hopefully provide a full catalogue of the somatic mutations, chromosomal rearrangements, and copy-number alterations, which exist in MPNs. In parallel, increasing use of transcriptome sequencing will likely promote efforts to identify alterations present at the transcriptional level, which may or may not be identified at the level of DNA. At the same time, improvements in the understanding and throughput of technologies to catalogue epigenetic alterations will hopefully allow for a better understanding of the methylation and chromatin states of MPN genomes. Although much progress has been made in improving our understanding of the pathogenesis of the different MPNs, there is much more work to be done!

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