CLONAL EVOLUTION OF MUTATION EVENTS IN MYELOPROLIFERATIVE NEOPLASMS

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1. SUMMARY

1. Summary

Myeloproliferative disorders (MPD) are a heterogeneous group of diseases characterized by aberrant proliferation of the myeloid lineages. They represent clonal stem cell disorders with an inherent tendency towards leukemic transformation. Currently, MPD are subdivided into three disease entities: polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). A mutation in *JAK2* (*JAK2*-V617F) is frequently found in all three entities. The discovery of this mutation had a major impact on the diagnostic workup of patients with suspected MPD, increased our understanding of the pathogenesis and has led to first clinical trials testing the effects of *JAK2* inhibitors on late stages of MPD.

We now have convincing evidence that there are additional mutational events preceding *JAK2*-V617F. They are likely to be involved in initiating the disease or are acting as collaborating mutations to promote progression to myelofibrosis and acute leukemia. In this thesis, the clonal evolution of deletions on chromosome 20q (del20q) and *TET2*, a member of the *Ten-Eleven-Translocation* (*TET*) family of genes, were studied. In addition, the functional consequence of del20q was also analyzed by shRNA-mediated knockdown.

We developed a real time copy number PCR assay for deletions on chromosome 20q (del20q), screened peripheral blood granulocytes from 664 patients with myeloproliferative disorders (MPD) and identified 19 patients with del20q (2.9%), of which 14 (74%) were also positive for *JAK2*-V617F. We analyzed 8 patients with del20q using array comparative genomic hybridization (aCGH) and defined a 9 Mb common deleted region with 93 genes. To examine the temporal relationship between the occurrence of del20q and *JAK2*-V617F we performed colony assays in methylcellulose, picked individual BFU-E and CFU-G colonies and genotyped each colony individually for del20q and *JAK2*-V617F. In 2/9 patients we found del20q colonies with and without *JAK2*-V617F, suggesting that del20q preceded *JAK2*-V617F. 3/9 patients showed the inverse order of events, suggesting that del20q is not a general predisposing event for *JAK2*-V617F. Interestingly, 2 patients showed a complex pattern of mutation acquisition including several events of del20q

affecting alleles of different parental origin and uniparental disomy on chromosome 9p (9p-UPD). The fact that rare somatic events, such as del20q or 9p-UPD, occurred more than once suggests that these patients carry a predisposition to acquire such genetic alterations.

In serial samples from patients with del20q, we observed an increase in the percentage of del20q positive cells, suggesting that del20q as the sole genetic alteration may provide a competitive advantage. To find genes involved in the del20q induced proliferation advantage, we analyzed changes in the gene expression pattern induced by del20q. Screening for such differences in expression patterns is most powerful if inter-individual differences in genetic background and differences in the composition of the tissues to be analyzed can be eliminated. Therefore we used pools of individually *JAK2*-V617F and del20q genotyped colonies grown in methylcellulose, which provided a pure enough cell population to detect small differences in expression and compared the expression pattern of colonies with and without del20q. We discovered 680 genes, which had significantly changes ($p<0.05$) in expression. Inside the CDR we found 28 genes downregulated, of which we selected three genes for further functional analysis: *serin threonin kinase 4 (STK4)*, *topoisomerase 1 (TOP1)* and *protein tyrosine phosphatase receptor T (PTPRT)*. A pool of shRNA targeting these genes was used to retrovirally transfect murin bone marrow (BM) cells before they were transplanted to lethally irradiated reciepient mice. Because del20q was preferentially found together with *JAK2*-V617F, we decided to use BM cells derived from transgenic *MxCre;FF1* mice expressing *JAK2*-V617F. In addition, we also included two additional candiate genes, *V-MYB myeloblastosis viral oncogene homolog (avian)-like 2 (MYBL2)* and *L3MBTL*, a human homolog of the *Drosophila lethal (3) malignant brain tumor (D-l(3)mbt) polycomb protein*, in the bone marrow transplantation experiments. This is an ongoing project and we are monitoring the GFP expression in peripheral blood and the distribution of the integrated shRNAs. So far we did not see any proliferative advantage of the cells transfected with the shRNA pool targeting the del20q candidate genes.

Recently, a new gene was found to be mutated in MPN patients: *TET2*, a member of the *Ten-Eleven-Translocation* (*TET*) family of genes. Initial studies suggested, that *TET2* mutations may represent a tumor suppressor gene and may precede the acquisition of *JAK2*-V617F. We therefore studied the clonal evolution of *TET2* mutations by analyzing individually genotyped colonies of 8 patients. In 4/8 MPN patients we found that some colonies with mutated *TET2* carried wild type *JAK2*, whereas others were *JAK2*-V617F positive, indicating that *TET2* occurred before *JAK2*-V617F. One of these patients carried a 4 bp germline deletion, located in the C-terminal conserved region, leading to a frameshift and premature stop. This is the first report of a inherited mutation in *TET2*. In two other patients we observed the opposite order of events, with *JAK2* exon 12 mutation preceding *TET2* mutation in one case. These findings suggest, that mutations in *TET2* do not represent a general predisposing event for acquiring mutations

in *JAK2*. Two additional patients showed two separated clones for *TET2* and *JAK2*-V617F, which further supports this theory

The present work provided additional insights for understanding the complex clonal pattern in patients with MPN and the role of del20q and *TET2* mutations in the clonal evolution of the disease.

2. INTRODUCTION

2.1 Hematopoiesis

Hematopoiesis summarizes all aspects involved in the formation of blood cells. This dynamic process is highly regulated in order to fulfill the requirements of the body for the transport of oxygen, blood coagulation and immune defense. An adult produces approximately 200 billion erythrocytes, 100 billion leukocytes and 100 billion platelets each day. Mutations affecting control mechanisms of this highly orchestrated process can lead to cancer.

The initial hematopoiesis starts in the first few weeks of gestation in the yolk sac. For the definitive hematopoiesis, stem cells, so called hemangioblasts, seed liver, spleen and bone marrow and produce most of the hematopoietic cells. Liver and spleen continue to produce blood cells for the first two months after birth. Starting from the last two month of gestation, the bone marrow is the most important site of hematopoiesis.

The main components of the blood are erythrocytes, platelets and leukocytes separated into myeloid and lymphoid cells. Myeloid cells include phagocytes such as macrophages and granulocytes, whereas the lymphoid lineage summarizes T, B and natural killer (NK) cells.[1] All lineages can be reconstituted from a single bone marrow derived cell, the hematopoietic stem cell (HSC). This cell is very rare in the bone marrow and although the exact phenotype is yet unknown, it is phenotypically described as $CD34^+$ $CD38^-$. In a specialized cell division, HSC are able to replicate while keeping a constant number of stem cells. The division can occur in two different ways: symmetrical, producing two new daughter cells with same properties like the parental HSC; asymmetrical, resulting in a new HSC and a progenitor. The HSC stays in a quiescent state (G_0 phase) under normal conditions, as soon as the HSC is stimulated by stress such as bleedings or infections, proliferation and differentiation is induced.[2]

We distinguish between long-term and short-term repopulating HSCs. This subdivision is based on the observations made in mouse experiments: only the long-term repopulating cells are able to reconstitute a mouse in secondary transplants, because these cells are still able to self-renew,[3,4] whereas the short-term repopulating cells (multipotent progenitors) are still able to

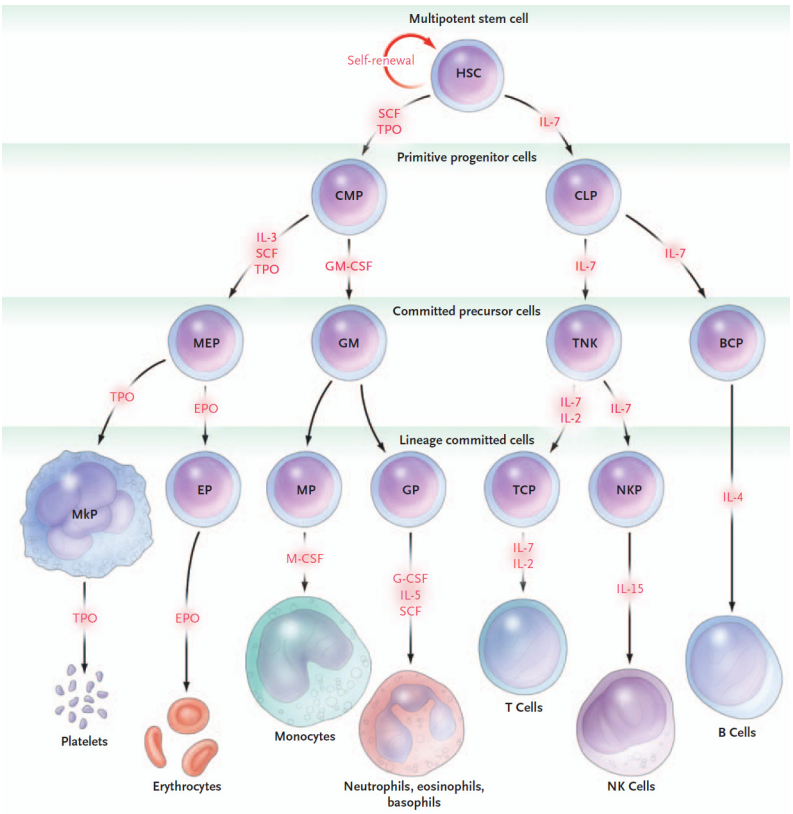


Figure 1:
Blood cell development starts at the level of the HSC, which can either self renew or generate primitive progenitor cells. We distinguish between a common myeloid progenitor (CMP) and a common lymphoid progenitor (CLP). These cells can then further differentiate to cells committed to NK and T cells (NKT), to B cell precursors (BCP), to cells committed to granulocytes and monocytes (GM) and to megakaryocyte/erythrocyte progenitors (MEP). Cytokines and growth factors supporting the survival, proliferation and differentiation of the different lineages are shown in red. [7]

differentiate into all lineages, but loose their ability to self renew.

The multipotent progenitors become more restricted in their developmental potential and are summarized as oligo-potent progenitors or primitive progenitor cells. We distinguish between common myeloid progenitor (CMP)[5] giving rise to all myeloerythroid lineages and common lymphoid progenitor (CLP)[6] giving rise to mature lymphoid effector cells (B, T, dendritic and NK cells). On the level of the committed precursor cells we distinguish between megakaryocyte/erythrocyte progenitors (MEP's), granulocyte-macrophage progenitor (GMP), T and NK cell progenitor (TNK) and B cell progenitor (BCP). These cells give then rise to the lineage committed cells.

Some questions about the exact lineage restriction of the different progenitor still remain, but there is no doubt about the sequential differentiation of HSCs via progenitors to fully differentiated blood cells. This process is irreversible under physiological steady-state conditions. By combining different steps of differentiation with increasing proliferative potential, this system allows an enormous amplification in the numbers of differentiated cells starting from a single stem cell.[8,9]

Hematopoiesis is regulated by growth factors, which are needed for survival, proliferation, differentiation and maturation of hematopoietic cells at all stages of development. These glycoprotein hormones act either locally at the site where they are produced by cell-cell contact or they circulate in plasma. Growth factors also bind to the extracellular matrix to form niches to which stem and progenitor cells adhere. Depend-

ing on the growth factor, the action can be restricted to a single lineage at a late differentiation stage, or can affect multiple lineages by acting on earlier progenitors. Erythropoietin (Epo), for example, promotes the proliferation of erythroid progenitors by reducing the level of cell-cycle inhibitors and increasing transcription of cyclins and supports their survival by augmenting anti-apoptosis protein $Bclx_L$.[10] Mice lacking the Epo or its receptor (EpoR) suffer from severe anemia and die. Administration of Epo in humans or mice, on the other hand, increases the number of erythroid progenitor cells, which differentiate into normoblasts, enucleate, and leave the bone marrow. The juxtatubular interstial cells of the renal cortex producing 90% of the erythropoietin in the blood are able to sense oxygen levels through an oxygen-dependant prolyl hydroxylase, which regulates the stability of the primary transcription factor of *EPO*, *hypoxia-inducible factor 1 α (HIF-1α)*.[11] *Thrombopoietin (TPO),* the main regulator of platelet production, is promoting proliferation of megakaryocytes by similar mechanism like *EPO* (cyclins, $Bclx_L$).[12] At the same time, Tpo is also involved in platelet aggregation.[13] Tpo is produced mostly in liver, but also in kidney and skeletal muscles and is regulated inversely to the number of platelets in the blood and megakaryocytes in the bone marrow.[12,14] Mature platelets and megakaryocytes express also the Tpo receptor and are therefore able to remove Tpo from the circulation.[15] Beside the role in stimulating megakaryocytopoiesis, Tpo also has an non-redundant function in the survival and proliferation of hematopoietic stem cells.[16] Mice lacking *TPO* or its receptor have 7 to 8 times less stem cells with the capacity to repopulate the bone marrow and the expansion of HSC after transplantation is reduced 17 times.[17,18]

The signals from the cytokines are processed and transmitted mostly through the JAK/STAT signaling pathway, which will be described in the following chapter.

2.2 JAK/STAT Pathway

The JAK/STAT pathway is one of the key pathways in the regulation of hematopoiesis and therefore also implicated in many different hematopoietic malignancies. Today, many genetic alterations, activating mutations and translocations affecting the signaling pathway are known. Recent insights into structure and mechanisms of the JAK/STAT pathway highlighted JAKs as therapeutic targets.

2.2.1 Janus kinase family

The JAK family of kinases includes 4 different members: *JAK1*, *JAK2*, *JAK3* and *TYK2*. They have seven defined regions of homology, called JAK homology domains (JH1-7)[19] The JH1 domain contains all feature needed for a catalytically active tyrosine kinase including the tyrosine residues in the activation loop, the canonical GXGXXG motif in the nucleotide-binding loop and a conserved aspartic acid residue involved in the phosphotransfer reaction in the catalytic loop.[20] Even though JH2 is highly homologous to the kinase domain JH1, there is no catalytic activity.[21] We know today that this domain, also called the pseudokinase domain, has an important regulatory role in the activity of JAK. Deletion of the JH2 domain in *JAK3* and *JAK2* lead to increased phosphorylation of the mutants and Stat5 suggesting that the pseudokinase domain is inhibiting the basal activity of the kinase domain.[22] JH3 and JH4 form together the Src homology-2 (SH2) domain based on their sequence homology, but do not bind to any phosphotyrosine residue.[23,24] JH6 and JH7 domains comprise the Four-point-one, Erzin, Radixin, Moesin (FERM) domain, which regulates the activity and is needed cytokine receptor binding (Figure 2).[25-27]

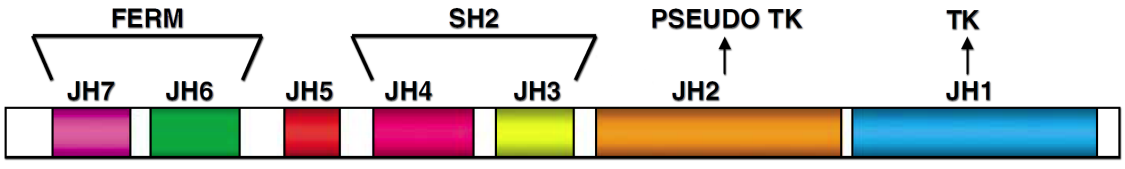


Figure 2:
Structure of Janus kinases (JAKs). The protein has 4 functional domains: the FERM domain, the SH2 domain, the pseudo tyrosine kinase domain (pseudo TK) and the catalytically active tyrosine kinase domain (TK).[28]

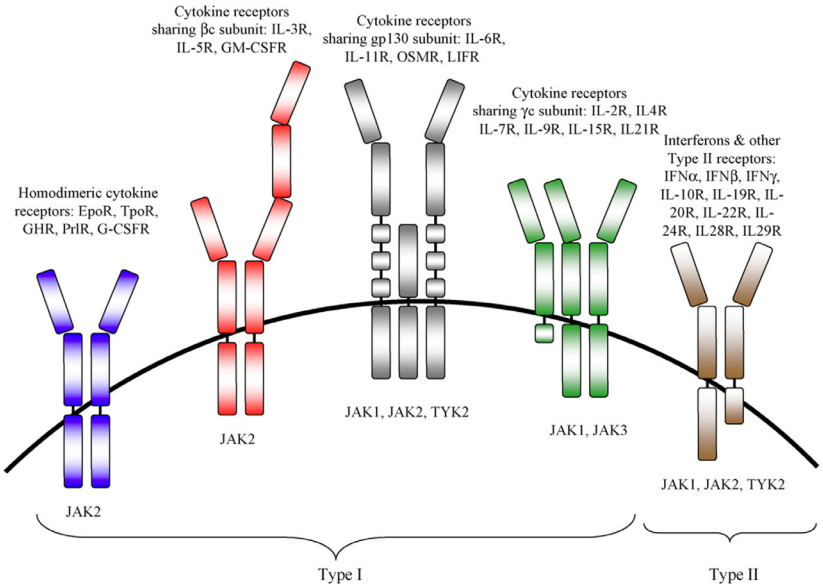


Figure 3:
Different cytokine receptors interact with different family members of JAKs. Jak2 interacts with all cytokine receptors, except with receptors sharing the γ_c subunit and is the only one to interact with homodimeric and β_c subunit sharing cytokine receptors.[29]

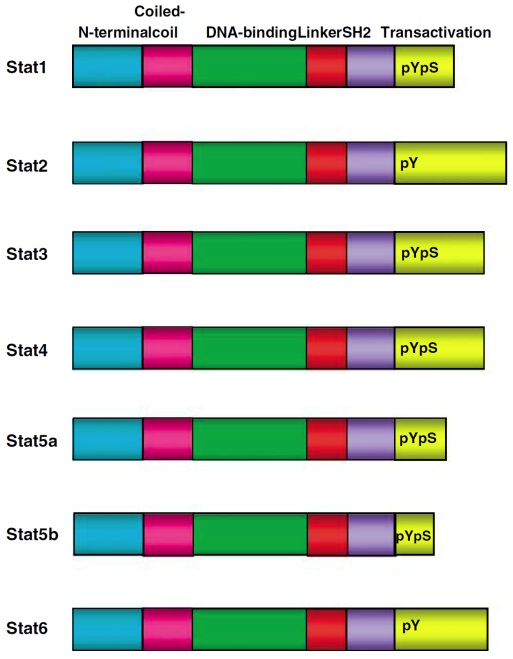


Figure 4:
Structure of STAT proteins. STATs harbor 6 different domains: N-terminal, coiled-coiled, DNA-binding, linker, SH2 and transactivator domain. The N-terminal and the DNA binding domain are binding to the promoter region of target genes. pY and pS mark regulatory phosphotyrosine and phosphoserine residues.[27]

STAT	Gene	STAT	Gene
STAT1	Bcl-xL	STAT3	Bcl-xL
	Bcl-2		Bcl-2
	bFGF		cdc25a
	CIITA		c-myc
	Caspase-1		cyclin A
	Caspase-2		cyclin D1
	Caspase-3		cyclin D2
	Caspase-7		cyclin D3
	c-myc		Fas
	mcp-1		HIF-1a
	MMP-9		IL-6
	MMP-2		Mcl-1
	p21		survivin
STAT5	Bcl-xL		MMP-2
	Bcl-2		MMP-9
	c-myc		Pim-1
	Cyclin D1		Pim-2
	Cyclin D2		p21
	Cyclin D3		Survivin
	Cyclin E		VEGF
	Pim-1		

Table 1:
Summary of Stat1, Stat3 and Stat5 target genes. Abbreviations: HIF, hypoxia inducible factor; IL, interleukin; MMP, matrix metalloproteinase; STAT, signal transducers and activators of transcription; VEGF, vascular endothelial growth factor.[34]

2.2.2 Cytokine receptors

The cytokine superfamily includes a set of single span membrane proteins without any enzymatic activity in their cytosolic domain. They instead bind members of the Janus kinase family (JAKs) via their cytosolic domain. Cytokine receptors can be divided into type I and type II receptors based on conserved motifs in the extracellular and intracellular part.[29] The receptors can be further subdivided based on their subunit composition. For type I we further distinguish between homodimeric cytokine receptor (e.g. Epo, Tpo receptor), receptors sharing the β_c subunit (e.g. IL-3, GM-CSF receptor), cytokine receptors having the gp130 subunit in common (e.g. IL-6, IL-11 receptors) and receptors sharing the γ_c subunit (e.g. IL-2, IL-7 receptors). Each subgroup is associated with one more members of the JAK family (Figure 3).

2.2.3 Signal transducers and activators of transcription

The signal from the cytokine receptor is transferred via JAKs to the nucleus by signal transducers and activators of transcription (STAT).[31,32] There are seven different mammalian STATs described: *STAT1* to *STAT6*, with *STAT5a* and *STAT5b*. All STATS have a modular structure with 6 different domains, including N-terminal conserved region, a coiled-coiled domain, a DNA binding domain, a linker region, a SH2 domain and a C-terminal transactivation domain. The N-terminal domain is very critical for the STAT function. If small deletions occur in this domain, STATs cannot phosphorylate anymore. The N-terminal domain also functions as nuclear import signal and it is involved in the receptor binding in cooperating with the DNA-binding domain. The coiled-coiled domain has an α-helical structure, is involved in the binding to the receptor and associates with regulatory proteins.[33] The DNA-binding and the SH2 domain are highly conserved among the different STATs. The SH2 domain is crucial for the recruitment of STATs to the activated receptor complex and the interaction with JAK and Src kinases. The SH2 domain is necessary for the homo- and heterodimerization of STATs, which enables them to enter the nucleus and bind the DNA. The transactivation domain is variable among the different STAT members and is implicated in the transcriptional activation.

2.2.4 The JAK/STAT pathway

Binding of ligand to the receptor induces either homo- or dimerizations of the receptor and induces juxtapositioning of the JAKs. The recruitment of JAKs to the receptor leads to JAK phosporylation through autophosphorylation and/or cross phosphorylation by other JAKs or other member of the tyrosine kinase family. The activated JAKs are then able to phosphorylate the receptor on target tyrosine sites. This phosphotyrosine sites at the receptor serve as on docking station for STATs via their SH2 domain. By binding to the receptor, the STATs will become also phosphorylated at specific

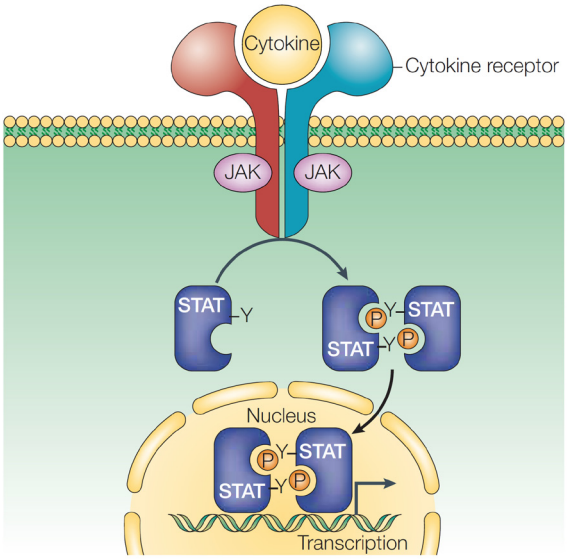


Figure 5:
A schematic illustration of the JAK/STAT pathway. The binding of cytokines to the receptor leads to the activation of JAKs, which are then able to activate STATs. The activated STATs dimerize and translocate to the nucleus to induce transcription of target genes.[34]

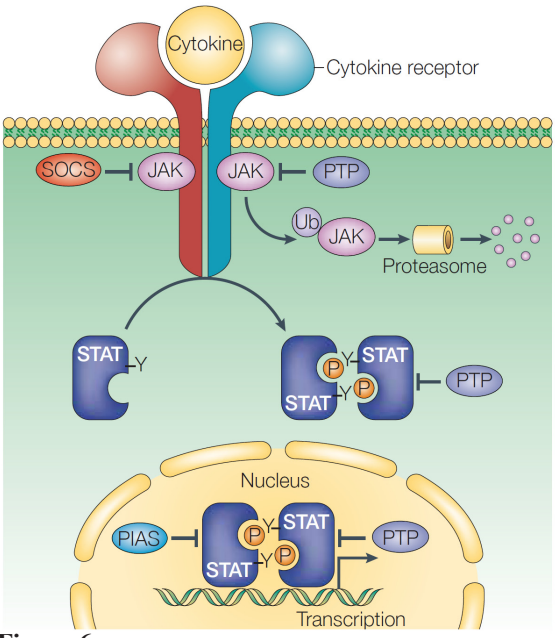


Figure 6:
Negative regulation of JAK/STAT signaling pathway. JAKs are inhibited by suppressor of cytokine signaling family proteins (SOCS/CIS), protein tyrosine phosphatases (PTPs) and ubiquitylation. STATs are blocked by PTPs and protein inhibitors of activated STAT proteins (PIAS).[34]

sites at a conserved C-terminal tyrosine. This phosphorylation allows then homo- or heterodimerization of STATs via the interaction of the SH2 domain of one STAT with the phosphotyrosine of the other STAT. Following dimerization STATs are able to translocate to the nucleus to activate specific genes.[32,34]

2.2.5 Target genes of STATs

There is a wide spectrum of genes with STAT responsive elements and they can be activated or repressed by STATs. Some of them induce a pattern of induced genes which is common among different cell types, like for example Stat1, which induces an “interferon signature”. Other STATs regulate genes in a more cell-type restrictive manner, meaning that the set of genes activated or inactivated by a specific STAT can differ between different cell-types.[36] A summary of selected STATs with their target genes can be found in table 1.

2.2.6 Negative regulators

The activity of the JAK/STAT pathway is controlled by several negative regulators, including protein tyrosine phosphatases (PTPs), suppressor of cytokine signaling family proteins (SOCS/CIS), protein inhibitors of activated STAT proteins (PIAS) and ubiquitylation.

Three different types of PTPs are known to regulate the JAK/STAT pathway. Firstly, SH2-containing phosphatases (Shp) associate with SH2 domains with phosphorylated tyrosine residues and are able to

dephosphorylate activated cytokine receptors and JAKs. *SHP1* primarily expressed in hematopoietic tissues, whereas *SHP2* is ubiquitously expressed. The second phosphatase, which negatively regulates JAK/STAT signaling is the transmembrane PTPase (CD45) expressed at high levels in all hematopoietic lineages at all stages. In vitro, CD45 directly dephosphorylates and binds to JAKs and negatively regulates IL3 mediated cellular proliferation.[37] The third group includes *phosphotyrosine phosphatase 1B (PTP1B)* and *T-cell protein tyrosine phosphatase (TC-PTP)*. Ptp1B is localized in the endoplasmatic reticulum and is expressed in many different tissues. *TC-PTP* is expressed in hematopoietic cells and exists in two isoforms, a long cytoplasmatic isoform (p48) and a short isoform (p45) located in the nucleus. Jak2 and Tyk2 are substrates for Ptp1B,[38] whereas Jak1 and Jak3 are substrates for Tc-PTP.[39]

Socs are small proteins with a central SH2 domain and conserved C-terminal SOCS/CIS boxes. The 8 members of this family (*SOCS1-7* and *CIS*) regulate the JAK/STAT pathway in several different ways. Cis binds to the Stat5 binding sites on the receptor, prevents Stat5 binding and therefore interrupts Stat5 signaling.[40] Socs proteins can also directly bind to the activation loop of JAKs.[41] Finally, Socs1 has been shown to target Jak2 for ubiquitination and subsequent proteasom-mediated degradation of JAKs.[42]

The PIAS family was initially described in STAT signaling,[43] but they show also activity towards a variety of other transcription factors. Five different PIAS proteins are described and all five bind to activated STAT dimers and inhibit STAT-mediated transcription by different mechanisms.

It was also shown that Jak2 can be ubiquitinated in vitro and in vivo,[42] which leads to rapid degradation of Jak2. The ubiquitination is increased in response to stimulation by IL-3 and IFN-γ.

2.2.7 Independent functions of JAKs

Even though, JAKs are mainly active in the JAK/STAT pathway, they have also STAT independent functions. JAKs are also involved in the activation the kinase Pyk2 in IL-2 signaling.[44] They are able to stimulate the RAS-MAPK pathway[45-47] and to induce the *C-FOS* and *C-MYC* genes.[48] Recently it was also shown that human Jak2 is present in the nucleus and directly phosphorylates histone H3. Heterochromatin protein 1alpha (HP1alpha) specifically binds to the phosphorylation site of H3 through its chromoshadow domain. Phosphorylation of H3Y41 by Jak2 prevents this binding. The haematopoietic oncogene *LMO2* was shown to be one target of the phosphorylated H3.[49]

2.3 Myeloproliferative Neoplasms

Myeloproliferative Neoplasms (MPN) summarizes a group of diseases with clinical and biologic similarities. They were described for the first time by Wiliam Damashek in 1951.[50] At this time point, the disease group included four entities: polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF) and chronic myeloid leukemia (CML). In 1960, CML became the first cancer to be connected with a specific cytogenic marker, the Philadelphia chromosome (PH). This reciprocal chromosomal translocation, t(9;22)(q(34;q11), generates a fusion between *Abelson murine leukemia viral oncogene homolog 1 (ABL)* and *Breakpoint cluster region protein (BCR)*. This fusion leads to constitutive activation of the *ABL* kinase, which represents the disease-causing mutation.[51-53] For this reason, CML is considered as a disease group for itself, whereas the other three entities (PV, ET, PMF) are summarized as Philadelphia chromosome negative (PH^-) MPN's.[54] They are characterized by overproduction of blood cells, mostly of the myeloid lineage, and variable predisposition for transformation into another disease entity or to leukemia (Figure 7).

2.3.1 Clinical characteristics

Polycythemia Vera patients have mild to overt excess of normochromic, normocytic red blood cells (RBCs) in peripheral blood with neutrophilia and rarely, basophilia. The bone marrow is hypercelluar due to this trilineage proliferation. Most of the clinical findings in PV are related to hypertension or vascular abnormalities caused by the excess of RBCs. In 20% of the cases a history of venous or arterial thrombosis is present, which is in many cases the first indication for PV. In a full-blown polycythemic stage, splenomegaly can be found in 70% of the

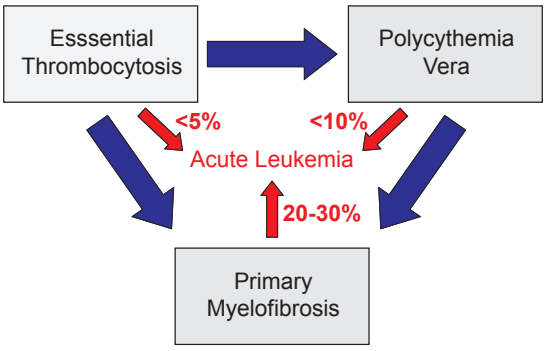


Figure 7:
Philadelphia chromosome negative myeloproliferative neoplasms. Arrows mark possible transitions. The percentage indicates the fraction of patients transforming to AML.

patients and hepatomegaly in 40% of cases. The median survival time is longer than 10 years and most patients die of thrombosis or hemorrhage, whereas 20% die due to MDS or AML.

Essential Thrombocythemia is marked by persistent thrombocytosis with abnormally shaped platelets. In most patients the thrombocytosis is discovered in a routine blood check in asymptomatic patients, however some of the patients are also diagnosed due to manifestation of vascular occlusion or hemorrhage. Splenomegaly is only seen in a small subset of ET patients. Both ET and PV may transform to PMF.

In early phase primary myelofibrosis patients show hypercellular bone marrow, characterized by prominent granulocytic and megakaryocytic proliferation and a reduction and maturation block in nucleated erythroid precursors. Reticulin fibrosis is absent in the bone marrow. After this prefibrotic or early stage of PMF, the more advanced fibro-osteosclerotic phase of the disease starts. This stage is characterized by significant amount of reticulin deposition and the appearance of collagen fibers in the bone marrow. About 30% of the patients are asymptomatic at the time of diagnosis and the disease is discovered by routine examinations which detects the presence of splenomegaly, anemia, leukocytosis and/or thrombocytosis. Median survival time depends on the disease stage in which PMF is diagnosed. For early stage patients it is about 10 to 15 years and for patients in a fibrotic stage, it is about 3 to 7 years. [55-58]

2.3.2 Clonal stem cell disorder

Fialkow and colleagues analyzed polymorphisms in the X-linked *glucose-6-phosphate dehydrogenase (G6PD)* locus in female patients. They were able to show that the same isoform was present in all granulocytes, platelets and erythrocytes, consistent with a myeloid clonal expansion.[59] This initial finding was also confirmed in ET and PMF.[60,61] The stem cell origin of the disease was further proven by demonstrating the involvement of B, T, and natural killer (NK) lymphocytes.[62-65]

2.3.3 Chromosomal aberrations

Based on the successful discovery of the *BCR/ABL* fusion, the cytogentic approach was also used in Philadelphia chromosome negative myeloproliferative neoplasms. Even though karyotype abnormalities are frequent at the diagnosis of MPN, no specific aberration for PV, ET or PMF was identified. The most frequent are 9p uniparental disomy (UPD), numerical aberrations of chromosomes 1, 8, and 9, deletions on chromosomes 5q, 13q, 20q and frequent gains of 9p.[66-77] Except for 9pUPD, all cytogentic aberrations can also be found in other clonal myeloid disorders (meylodisplastic syndrome, CML and acute myeloid leukemia). Therefore, it is likely that these chromosomal abnormalities have a more general role in the pathogenesis of hematological malignancies.

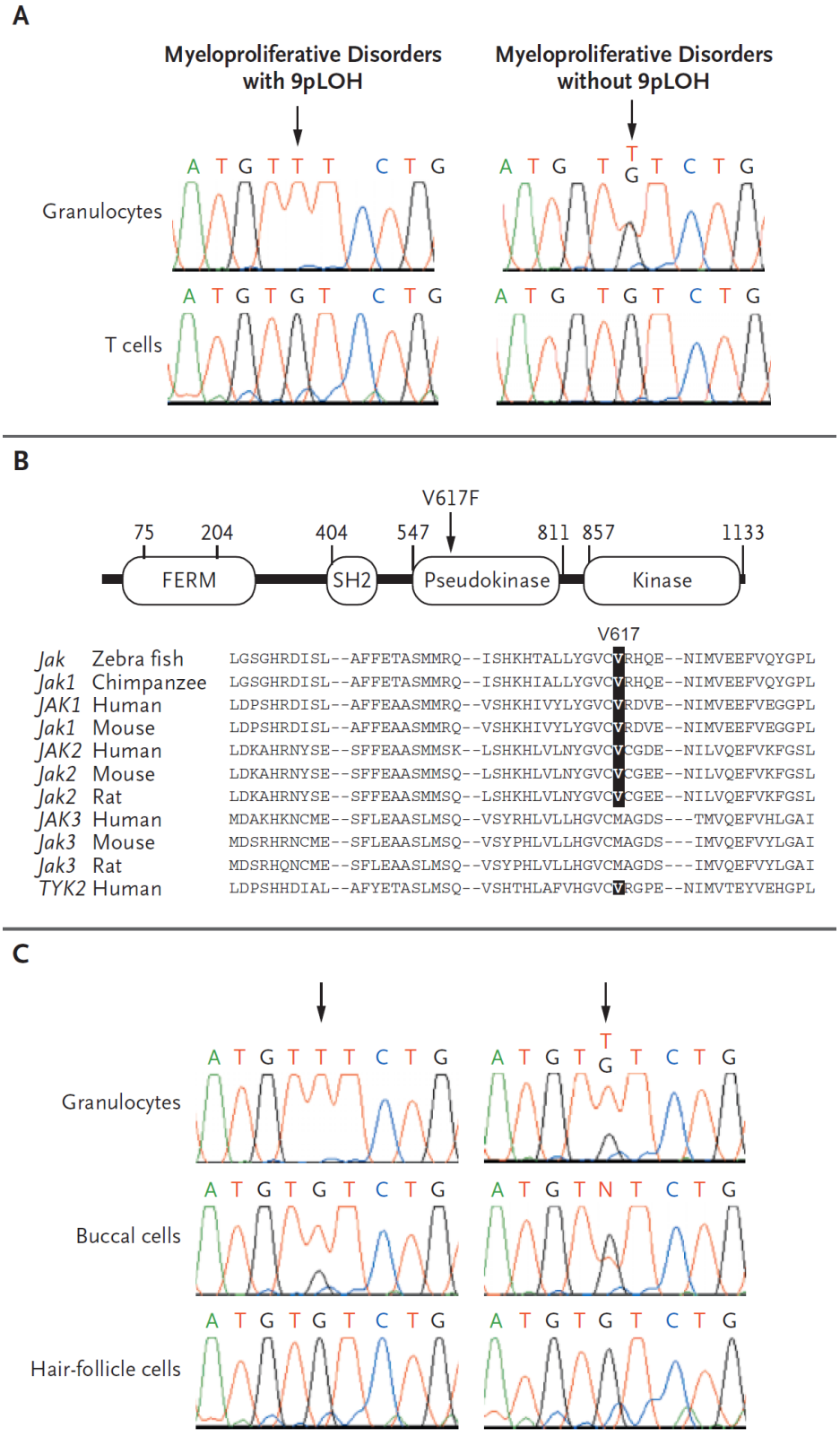


Figure 8:
JAK2-V617F mutations in patients with myeloproliferative neoplasms. Panel A shows the heterozygous mutation in a patient without 9pUPD and the homozygous mutation in a patient with 9pUPD. Panel B shows the domain structure of *JAK2*. The mutation is located in highly conserved residue. Panel C shows the somatic origin of the mutation. *Jak2*-V617F was present in buccal swab and granulocyte DNA, but was absent in hair follicle DNA.[66]

2.3.4 Epo independant growth

The next important observation was made by Jaroslav Prchal and Arthur Axelrad: They were able to show that bone marrow cells from PV patients, but not from healthy controls, were able to form erythroid colonies in the absence of exogenous Epo. These cytokine independent colonies are called endogenous erythroid colonies (EEC).[78] The formation of EECs is found in most of the PV patients and in a subset of ET and PMF patients without cytoreductive treatment.[79] The increased response to the limiting concentration of Epo proves the cell-autonomous characteristics of the observed bone marrow proliferation, as had it been suggested by Dameshek.

2.3.5 The discovery of *JAK2*-V617F

More than 50 years after Dameshek's editorial in Blood, a new era in MPN research started with the discovery of the *JAK2*-V617F mutation. The mutation was found simultaneously in four different labs using different genetic, functional and genomic approaches. The group around William Vainchenker discovered that the growth of EEC was abrogated by siRNA mediated knock-down of *JAK2*. This finding led them to search for mutations in *JAK2*.[80] Anthony Green and colleagues used candidate gene resequencing to find the mutation.[81] The lab of Radek Skoda was building their research strategy on the observation of Josef Prchal, that a acquired uniparental disomy (UPD) of chromosome 9p24 is common in PV[67]. The *JAK2*-V617F mutation was found by sequencing genes located in the minimal region of the UPD.[66] Gilliland and colleagues used high throughput sequencing to screen the tyrosine kinome, because activating mutations were previously described in other MPDs.[82]

The guanine to tyrosine substitution results in a valine to phenylalanine substitution at amino acid 617 of Jak2. This mutation was never found in germline and is therefore acquired as a somatic disease allele in the hematopoietic system. The valine 617 to phenylaline substitution occurs inside the JH2 domain (also called pseudo kinase domain). However, the complete 3D structure of Jak2 is not yet solved and therefore it is not completely clear how the *JAK2*-V617F mutation can activate the kinase. However, deletion of the JH2 domain leads to increased activation of Jak2, which is consistent with an autoinhibitory role of the pseudo-kinase domain.[22] Interestingly, there are no reports for alternative mutations at this codon, even though a recent report showed, that any of the 4 alternative residues (tryptophan, methionine, isoleucine and leucine) for valine 617 leads to constitutive activation of the kinase.[83] This finding potentially supports the idea of a pre-*JAK2* event inducing mutation at a specific sequence background. Depending on the detection method, the mutation can be found in approximately 95% of PV patients, 50% of ET patients and in 50% of PMF patients. The discovery of the *JAK2*-V617F mutation was a big breakthrough and modified the classification and diagnosis of myeloproliferative neoplasms.

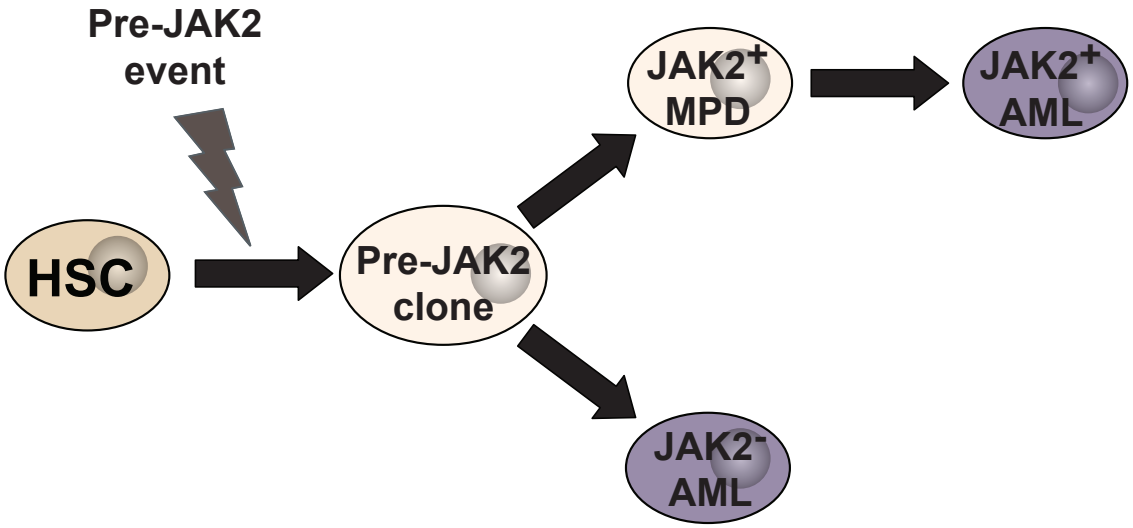


Figure 9:
Model of leukemic transformation in MPN. Some patients develop a *JAK2*-V617F positive leukemia consistent with a *JAK2*-V617F positive progenitor undergoing leukemic transformation. However, some patient also develop a *JAK2*-V617F negative leukemia, consistent with a pre-*JAK2* clone susceptible to leukemic transformation.

2.3.6 *JAK2* exon 12 Mutations

After the discovery of *JAK2*-V617F, the JAK2 gene was screened for additional mutation. Scott at al. described in *JAK2*-V617F negative PV a subset of patients with a somatic gain of function mutation in exon 12 of *JAK2*. In contrast to *JAK2*-V617F, exon 12 mutations include a variety of amino acid changes, deletions and insertion. *JAK2* exon 12 mutations can only be found in patients with PV and are associated with a nearly pure erythrocytosis.[84] However, it was also shown, that the two *JAK2* mutation can coexist in two different clones in the same patient.[65]

2.3.7 *MPL* mutations

The JAK/STAT pathway can also be activated by mutations in *myeloproliferative leukaemia virus oncogene homologue gene (MPL)*, which is the receptor for *TPO*. Germline gain of function mutations in *MPL* were already described in familial thrombocytosis,[85] when in 2006 a somatic acquired *MPL* mutation (*MPL*-W515L) was described in *JAK2*-V617F negative PMF.[86] Subsequently, an additional mutation at the same codon was discovered: *MPL*-W515K. Taken together, this two mutations can be found in 5% of PMF patients and 1% of ET patients.[87]

2.3.8 Mouse Models for *JAK2*-V617F

The oncogenic activity and disease causing potential of the *JAK2*-V617F mutation was proven by in vitro and in vivo experiments. BAF3 cells grow faster at low levels of IL-3 and become cytokine independent upon expression of *JAK2*-V617F.[66] Bone marrow transplant models were able to show, that *JAK2*-V617 can induce a myeloproliferative

phenotype in mice resembling MPN in humans.[80,88-90] Depending on the genetic background of the mice, the phenotype differed between the various experiments: erythrocytosis, dramatically increased leukocytoosis and bone marrow fibrosis was observed in BALB/c, whereas C57BL/6 showed increased erythrocytosis with mild leukocytosis. Beside the transplantation models, several transgenic mouse lines were generated [91-93].

Ralph Tiedt and colleagues developed a transgenic moue model expressing human *JAK2*-V617F which can be induced.[91] These mice were crossed with transgenic mice expressing Cre recombinase through various promoters. The *MxCre* strain expresses *Cre* recombinase under the control of the *Mx* promoter, which can be activated by interferon. The *Cre* recombinase activates the *JAK2*-V617F transcript. *MxCre;FF1* mice showed a phenotype resembling PV with increased hemoglobin levels, thrombocytosis and neutrophilia. Depending on the number of pIpC injection, the copy number changes. If the *Mx* promoter is activated over a longer time, more copies of the transgene get excised. In *VavCre* mice, the expression of *Cre* recombinase is driven by the *Vav* promoter, which is shown to direct constitutive *Cre* expression to all hematopoietic cells.[94] The *VavCre;FF1* mice only have elevated platelet counts and therefore resemble more the ET phenotype in patients. In the third transgenic mouse line, *SclCre*, the expression of a tamoxifen inducible *Cre* recombinase is driven by 5' endothelial enhancer of *stem cell leukemia (SCL)*. The *SclCre;FF1* mice have high numbers of erythrocytes, platelets and white blood cells. The phenotype is stronger in *SclCre;FF1* mice than in *MxCre;FF1* mice.

The comparison of these transgenic mouse models indicates, that the disease phenotype is depending on the expression level of *JAK2*-V617F. High levels of expression, comparable to the transplantation model, induce erythrocytosis, whereas low levels of *JAK2*-V617F cause thrombocytosis. This observation can be translated into patients with MPN: ET patients stay in a heterozygous *JAK2*-V617F state, whereas PV patients often acquire UPD in 9p24 and therefore become homozygous for *JAK2*-V617F.[91]

2.3.9 One mutation, three diseases

Although the dosage effect provides some evidence how one mutation can cause three different disease entities, many question are still open. Additional mutation events are also likely to modify the disease phenotype.

Since the discovery of *JAK2*-V617F, the question is raised whether this mutation represents the disease-initiating event. It was shown in mouse experiment that *JAK2*-V617F alone is able to reproduce the disease phenotype in animals, similar to *BCR/ABL*. However, these models do not completely reflect the situation in humans, where the mutation initially occurs in one stem cell, which has to out-compete the normal hematopoiesis. There is also evidence, that the clonal hematopoiesis can exceed the *JAK2*-V617F harboring clone, thus hypothesizing that an initial mutation event induces clonal

hematopoiesis.[95-98] In addition, the presence of *JAK2* wild type EECs together with *JAK2*-V617F EECs was shown in PV.[99]

Another indication that *JAK2*-V617F might not be the "first-hit", originates from the analysis of MPD patients transformed to AML. It was shown that many of the patients progress from a *JAK2*-V617F positive MPN to a *JAK2*-V617F negative AML (Figure 9).[100,101]

2.3.10 Predisposition

One hypothesis would be, that MPN occurs in patients carrying a predisposition to genomic instability, which makes the more susceptible to acquire mutations causing the disease phenotype. The same observation was also made in familial MPN's. In this families the risk to acquire *JAK2*-V617F and to get MPN is increased several hundred times. Consistent with the genetic predisposition, a specific constitutional haplotype in *JAK2*, called 46/1 or "GGCC", was recently described. Several groups showed, that *JAK2*-V617F and *JAK2* exon 12 mutations are associated with a certain haplotype.[102-105] In addition it was also shown that this haplotype is in general associated with MPN, regardless the mutational status of *JAK2*.[106,107] This haplotype might be necessary to propagate the MPN phenotype or might favor mutagenesis, but the exact function is not yet known.

2.3.11 Mutations in *TET2*

We know today, that additional mutation events contribute to the disease phenotype and that they could be shared between different hematological malignancies. Based on this hypothesis, Delhommeau and colleagues analyzed samples from myelodisplastic syndrome (MDS), acute myeloid leukemia (AML) and MPN using various genetic approaches. They discovered a recurrent deletion on 4q24, which in some AML samples included just one gene: *TET2*, a member of the *Ten-Eleven-Translocation* (*TET*) family of genes. They also analyzed a group of MPD, which had an early expansion of the *JAK2*-V617F clone, meaning that the proportion of CD34+ cells with *JAK2*-V617F was very high. They found a region of UPD at 4q24 and a deletion including only *TET2*. Sequencing of patient samples discovered a wide range of missense, nonsense mutations and deletions.[108] In MPN, *TET2* mutations can be found in approximately 12% of the patients.[108]

TET2 consists of 11 exons spread over 150kb and is expressed in a wide range of tissues. The family of Tet protein consists of 3 members: *TET1*, *TET2* and *TET3*, which share two highly conserved regions. Not much is known about TET2 yet. However, *TET1* is involved in a rare translocation in AML where it is fused to the *mixed-lineages leukemia (MLL)*.[109,110]

In addition to the initial publication by Delhommeau and colleagues, various groups showed the occurrence of *TET2* mutation in different hematopoietic malignancies, as in systemic mastocytosis (SM)[111], MPN[112], chronic myelomonocytic leukemia (CMML)[113], MDS[113,114] and AML[113].

The functional effect of the *TET2* mutations is not yet known because only limited information about the wt *TET2* function is

known. It has been shown, that $CD34^+$ derived from patients with *TET2* mutations are able to expand in a NOD/SCID transplantation mouse model.[108] It was also shown that a subset of patients looses the second copy of *TET2* either through a second mutation, or UPD, consistent with the standard model of a tumor suppressor mutation.

2.3.12 Outlook

In the last few years, there was a tremendous progress with the discovery of *JAK2*-V617F mutation and other genetic alterations in the field of MPN research. However, the molecular as well as the phonotypical heterogeneity of MPN patients is not well understood. Future studies will undoubtedly discover additional mutations associated with both phenotype as well as disease progression. Therefore it will be important to know more about the complex molecular heterogeneity of the patients to provide a successful, individualized therapy.

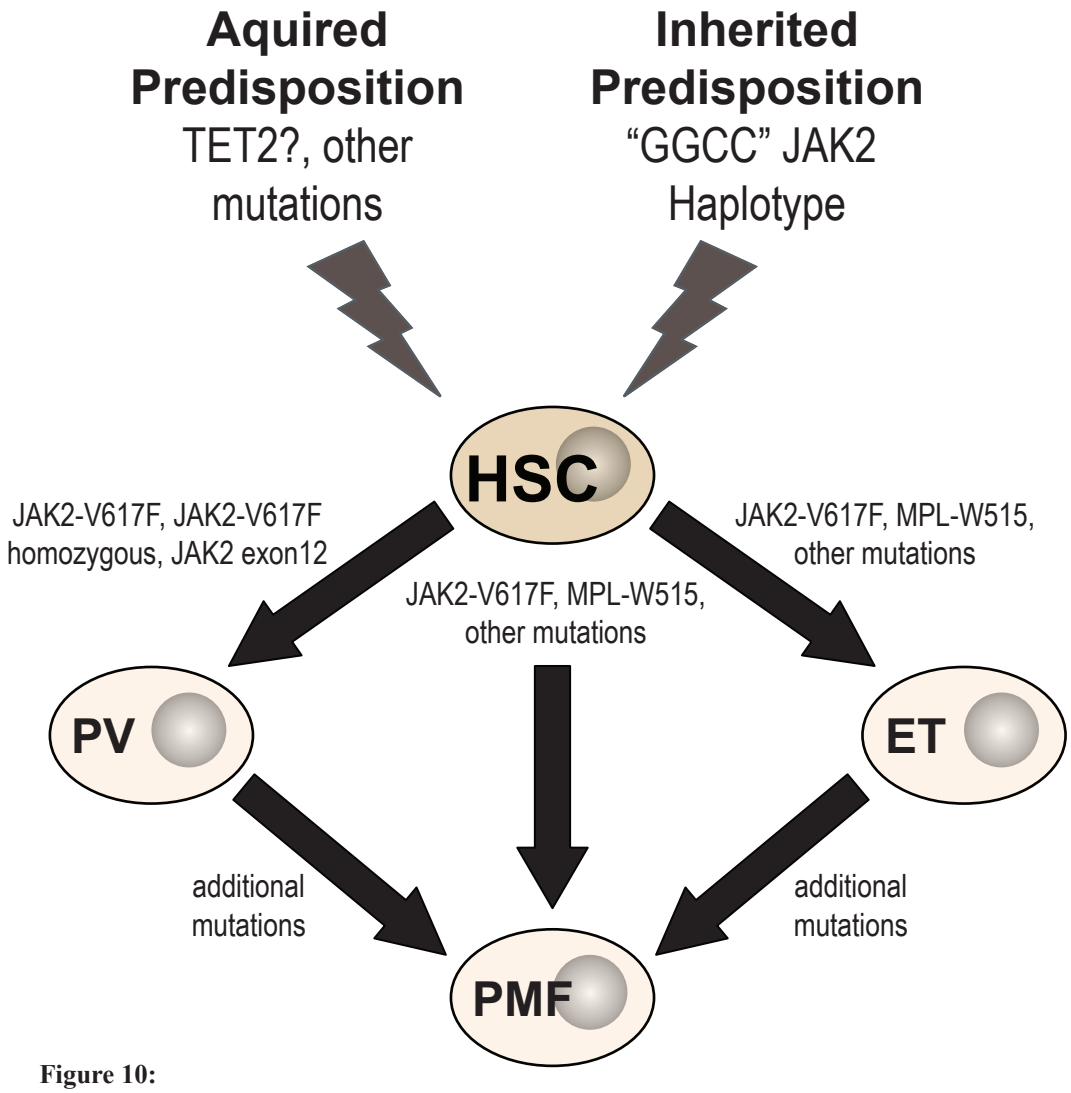


Figure 10:
Proposed model for MPN heterogeneity.

3. RESULTS

3.1 Clonal analysis of deletions on chromosome 20q and *JAK2*-V617F in MPD suggests that del20q acts independently and is not one of the pre-disposing mutations for *JAK2*-V617F

3.1.1 Abstract

<<We developed a real time copy number PCR assay for deletions on chromosome 20q (del20q), screened peripheral blood granulocytes from 664 patients with myeloproliferative disorders (MPD) and identified 19 patients with del20q (2.9%), of which 14 (74%) were also positive for *JAK2*-V617F. To examine the temporal relationship between the occurrence of del20q and *JAK2*-V617F we performed colony assays in methylcellulose, picked individual BFU-E and CFU-G colonies and genotyped each colony individually for del20q and *JAK2*-V617F. In 2/9 patients we found that some colonies with del20q carried only wild type *JAK2*, whereas other del20q colonies were *JAK2*-V617F positive, indicating that del20q occurred before the acquisition of *JAK2*-V617F. However, in colonies from 3/9 patients we observed the opposite order of events. The lack of a strict temporal order of occurrence makes it unlikely that del20q represents a predisposing event for *JAK2*-V617F. In two patients with *JAK2*-V617F and one patient with *MPL*-W515L, microsatellite analysis revealed that del20q affecting chromosomes of different parental origin and/or 9pLOH occurred at least twice. The fact that rare somatic events, such as del20q or 9pLOH occurred more than once in subclones from the same patients suggests that the MPD clone carries a predisposition to acquiring such genetic alterations.>>

2.1.2 Introduction

Deletions of the long arm of chromosome 20 (del20q) have been observed frequently in patients with myeloproliferative disorders (MPD), myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML).[68-70,115] A common deleted region (CDR) of 2.7 megabases (Mb) was mapped for patients with MPD and a 2.6 Mb CDR was defined for MDS/AML with an overlap of 1.7 Mb between the two CDRs.[75,116-120] However, to date it was not possible to identify a gene mutation within the CDR that is functionally linked to the expansion of the del20q clone.[121] In MPD patients, del20q was found in the bone marrow from about 1% of patients with essential thrombocythemia (ET), up to 9% of polycythemia vera (PV) and up to 12% of primary myelofibrosis (PMF).[76,120] The del20q occurred preferentially together with the *JAK2*-V617F mutation in 28/29 MPD patients studied.[100] We previously analyzed two such patients with del20q and *JAK2*-V617F and found that the size of the del20q clone by far exceeded the size of the clone carrying *JAK2*-V617F,[97] suggesting that del20q preceded the acquisition of the *JAK2*-V617F mutation. Here we expanded these studies by screening for del20q in 664 patients with MPD and examined the temporal order of the acquisition of *JAK2*-V617F and del20q in patients carrying both mutations by analyzing single colonies grown in methylcellulose.

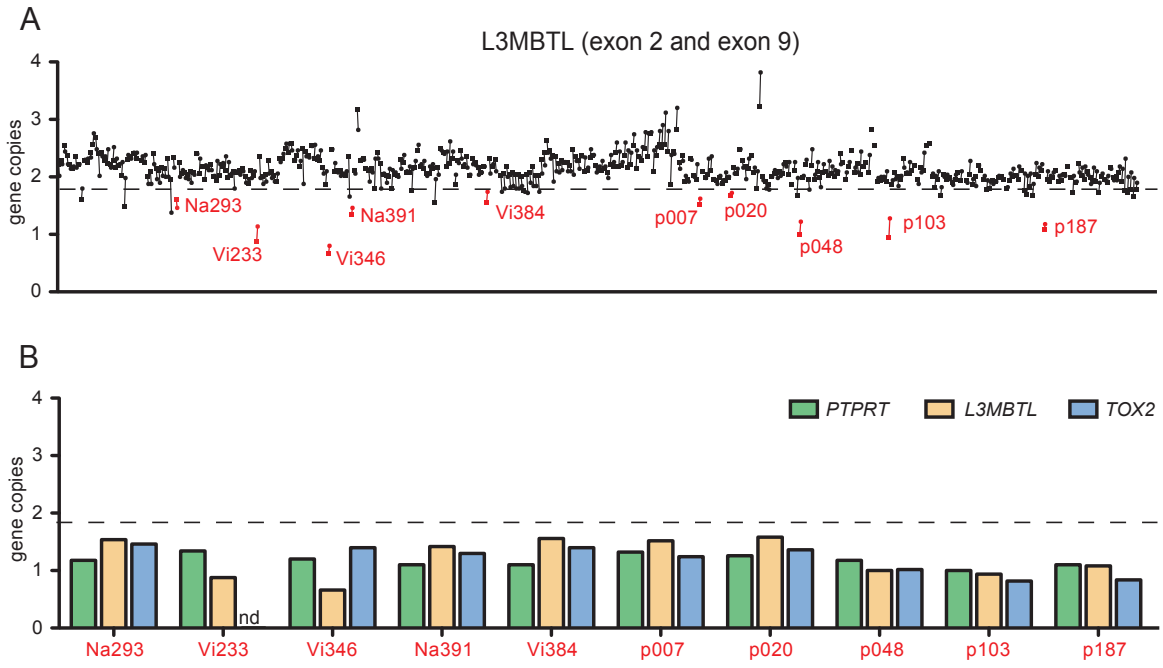


Figure 11. Screening for del20q by real time PCR.
A) The copy number of the L3MBTL gene was determined in DNA from purified peripheral blood granulocytes using two SYBR real time assays with primer pairs located in L3MBTL exon 2 (quadrangles) and exon 9 (circles). Samples with gene copy values below a threshold of 1.8 in both assays are marked in red and identified by unique patient numbers. B) Patients with decreased L3MBTL gene copy number were further examined with real time assays for the neighboring genes PTPRT and TOX2. The decreased copy number was confirmed in all cases. Not determined, nd.

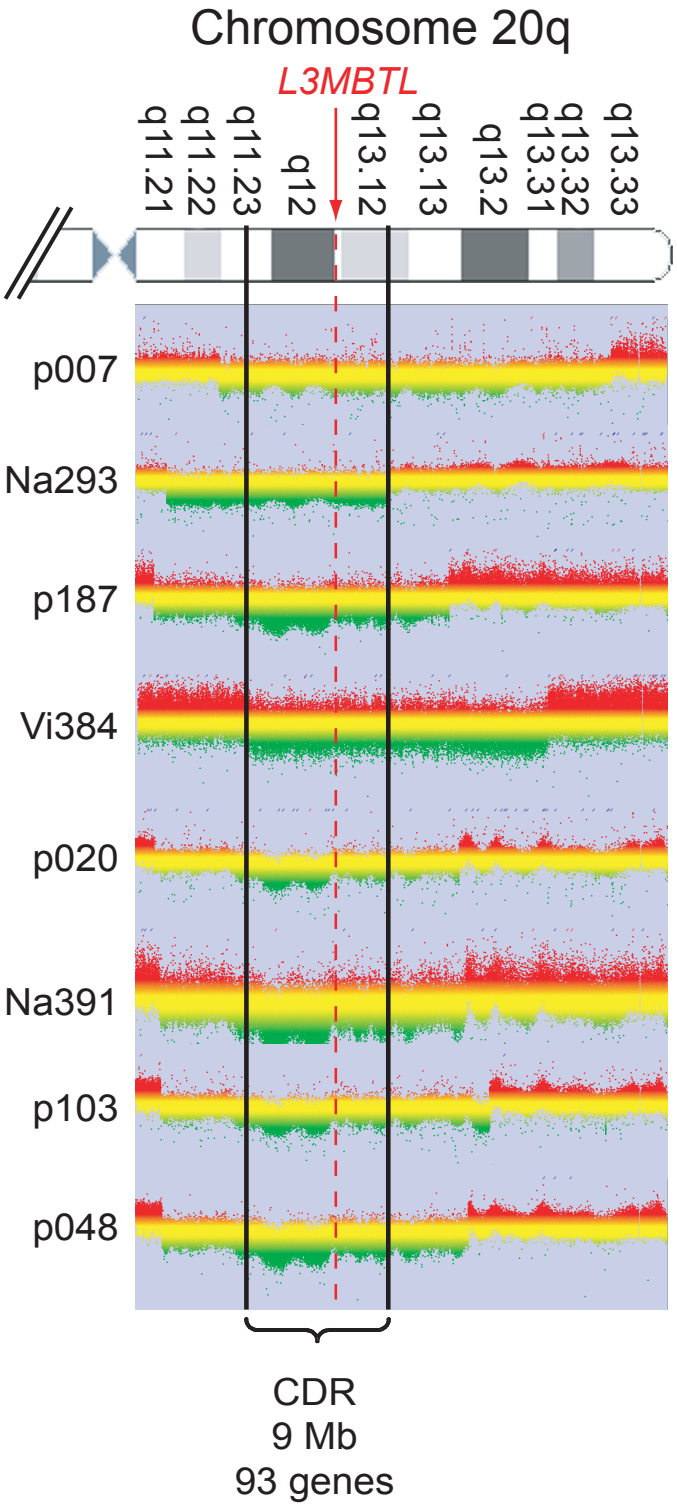


Figure 12. Comparative genomic hybridization
Granulocyte DNA from patients and a reference DNA were used to perform custom high-density oligonucleotide CGH arrays. The graphs show the log ratios for all probes located on chromosome 20q. Green color marks regions deleted in the patients DNA. The boundaries are marked by a clear drop in the ratio between the patient's DNA and the reference. Patient Vi384 delineates the centromeric and patient Na293 the telomeric border of the common deleted region (CDR), which spans 9 megabases (Mb) and includes 93 genes.

2.1.3 Results

Purified peripheral blood granulocyte DNAs of 664 MPD patients (320 ET, 262 PV, 82 PMF) were screened for del20q by monitoring the copy number of *L3MBTL*, a gene located in the common deleted region on chromosome 20q. Two real time PCR assays with primers placed in exons 2 and 9 of *L3MBTL* were used in parallel (Figure 11A). Patients with decreased gene copy numbers in both assays were further examined with real time PCR assays for *PTPRT* and *TOX2*, two genes flanking *L3MBTL* (Figure 11B). The del20q was confirmed in 19/664 (2.9%) of MPD patients, or specifically in 4/320 (1.3%) ET, 7/262 (2.7%) PV, and 8/82 (9.8%) PMF. The *JAK2*-V617F mutation was present in 14/19 (74%) of patients with del20q, whereas the *MPL*-W515L was present in one patient without *JAK2*-V617F. No mutations in *JAK2* exon 12 were found among the patients with del20q (Table 2). Cytogenetic analysis was available for 8 of the 19 patients with del20q and confirmed the results obtained by real time PCR in all 8 cases. To map the breakpoints of the individual deletions, we

UPN	Diagnosis	Gender	Age at sampling	Disease duration (months)	*JAK2*-V617F (%T)	Complications	Treatment
p007	PV	M	76	264	92	sMF, thrombosis	phlebotomy, IFN-alpha, hydroxyurea
p020	PMF	F	75	132	0	thrombosis	hydroxyurea
p048	PMF	M	77	168	95	splenomegaly	n.a.
p103	PV	F	82	600	95	none	phlebotomy, hydroxyurea, ^{32}P
p187	PMF	M	74	186	59	splenomegaly	no treatment
Di433	PV	F	79	22	94	none	hydroxyurea
Na293	ET	M	68	28	16	anemia, splenomegaly	no treatment
Na391	ET	F	41	240	68	SVT and other thromboses, sMF, anemia	hydroxyurea
Vi062	ET	F	54	257	10	abortion (3x)	anagrelide
Vi102	ET	M	51	46	23	MI, melanoma	anagrelide, hydroxyurea, IFN
Vi108	PMF	F	59	39	72	PAD, insult, splenomegaly	IFN
Vi117	PMF	M	64	43	0	anemia, splenomegaly	IFN, lenalidomide
Vi139	PV	F	50	52	0	none	IFN, anagrelide, phlebotomy
Vi141	PV	F	79	211	11	bleeding, TIA	anagrelide, hydroxyurea, IFN, phlebotomy
Vi162	PMF	M	67	142	0	anemia, splenomegaly	IFN
Vi233	PMF	M	80	53	9	lung cancer, DVT, CAD	hydroxyurea
Vi318	PV	F	65	30	12	PAD	phlebotomy , hydroxyurea
Vi346	PMF	M	56	72	0	lung cancer	IFN
Vi384	PV	M	64	197	98	none	phlebotomy

Table 2 Characteristics of patients with del20q
UPN, unique patient number; sMF, secondary myelofibrosis; n.a. , not available; IFN, interferon; SVT, splanchnic vein thrombosis; TIA, transient ischemic attack; MI, myocardial infarction; PAD, Peripheral artery disease; DVT, deep vein thrombosis; CAD, Coronary artery disease.

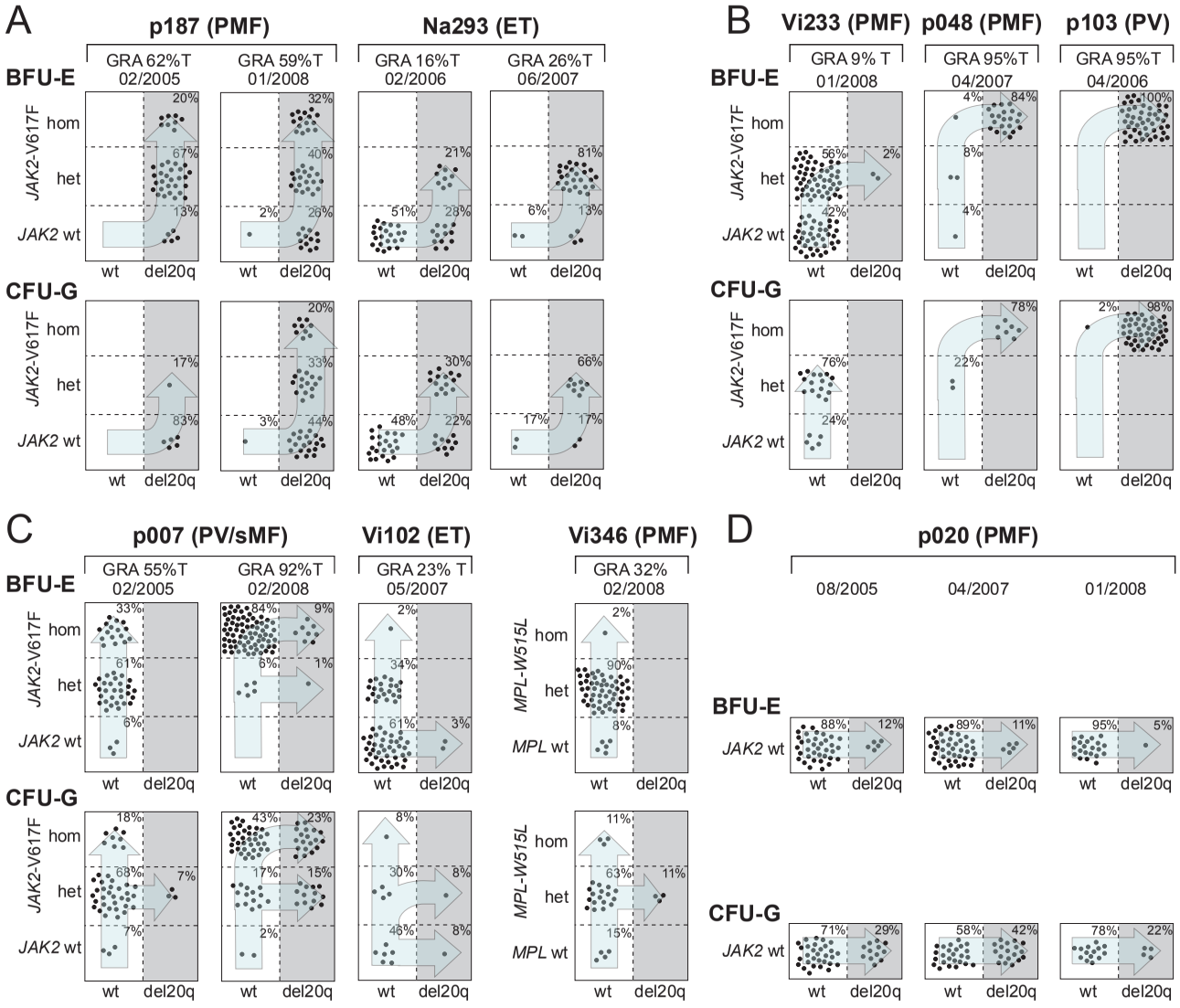


Figure 13. Single colony assays for del20q and JAK2-V617F.
Mononuclear cells from peripheral blood were grown in methylcellulose in the presence of erythropoietin. Single erythroid colonies (BFU-E) and granulocytic colonies (CFU-G) were picked and analyzed individually for the presence of del20q and JAK2-V617F by microsatellite PCR and allele-specific PCR, respectively. Each colony is represented by a dot that is placed into one of six quadrangles representing the 6 possible genotypes: wild type (wt), heterozygous (het) and homozygous (hom) for JAK2-V617F or MPL-W515L on the vertical axis, and absence (open quadrangles) or presence of del20q (gray quadrangles) on the horizontal axis. Results for BFU-Es are shown in the upper part and CFU-Gs in the lower part of the panels. The unique patient numbers, the diagnoses (PMF, primary myelofibrosis; ET, essential thrombocythemia; PV, polycythemia vera; sMF secondary myelofibrosis), the allelic ratio of JAK2-V617F in purified granulocytes (%T) and the date of the sample drawing are shown above the corresponding boxes. Light blues arrows indicate the suggested order of mutation events. A) This group of patients has acquired the del20q before JAK2-V617F, as demonstrated by the presence of JAK2 wt colonies carrying del20q. For both patients shown, two sequential samples were analyzed. B) In this group of patients, del20q was acquired in a cell already carrying JAK2-V617F, as indicated by the presence of JAK2-V617F positive colonies with or without del20q. C) Patients with a complex pattern of del20q acquisition that does not comply with a linear temporal order of events. Note that in patient Vi346 the del20q co-exists with MPL-W515L. D) A patient with del20q that is negative for the JAK2-V617F mutation.

performed custom high-density oligonucleotide CGH arrays on 8 del20q patients from whom DNA of sufficient quality was available. A common deleted region (CDR) of 8.98 Mb (35.95-44.93 Mb) containing 93 genes was defined (Figure 12).

To determine the temporal relationship between the occurrence of del20q and *JAK2*-V617F, we performed colony assays in methylcellulose, picked single BFU-E and CFU-G colonies grown in the presence of erythropoietin and genotyped each colony individually for del20q, *JAK2*-V617F, or in patient Vi346 for *MPL*-W515L (Figure 13). We observed three different patterns: First, in patients p187 and Na293 we found that some del20q positive colonies carried the wild type *JAK2*, whereas other del20q positive colonies were positive for *JAK2*-V617F, indicating that the del20q clone is larger than the *JAK2*-V617F clone (Figure 13A). Since all colonies positive for *JAK2*-V617F also displayed del20q, we infer that del20q occurred before the *JAK2* mutation. Second, in 3 patients we observed the reverse order, with *JAK2*-V617F preceding del20q, as illustrated by the presence of *JAK2*-V617F-positive colonies with and without del20q (Figure 13B). Patient Vi233 appears to have acquired del20q in a cell heterozygous for *JAK2*-V617F, whereas in patients p048 and p103 the transition to del20q occurred in a cell homozygous for *JAK2*-V617F. Third, in patients p007, Vi102 and Vi346 we found a more complex pattern (Figure 13C). In the first sample of p007 from 2/2005 only a few CFU-G heterozygous for *JAK2*-V617F carried del20q, while colonies homozygous for *JAK2*-V617F already existed. Two years later, del20q was also present in a subset of colonies homozygous for *JAK2*-V617F. This pattern could be explained ei-

	del20q		
	absent	**present**	**present Campbell et al**
MPD (total 664)	645	19	28
***JAK2*-V617F positive**	449 (70%)	14 (74%)	27 (96%)
***JAK2*-V617F negative**	196 (30%)	5 (26%)	1 (4%)
	p=0.805		
ET (total 320)	316	4	5
***JAK2*-V617F positive**	200 (63%)	4 (100%)	5 (100%)
***JAK2*-V617F negative**	116 (37%)	0 (0%)	0 (0%)
	p=0.3		
PV (total 262)	255	7	12
***JAK2*-V617F positive**	213 (84%)	6 (86%)	12 (100%)
***JAK2* exon 12**	5 (2%)	0	
***JAK2*-V617F/ exon 12 negative**	37 (14%)	1 (14%)	0 (0%)
	p=1		
PMF (total 82)	74	8	10
***JAK2*-V617F positive**	37 (50%)	4 (50%)	9 (90%)
***JAK2*-V617F negative**	37 (50%)	4 (50%)	1 (10%)
	p=1		

Table 3 Relationship between del20q and JAK2 mutations. P values represent Fisher's exact test.

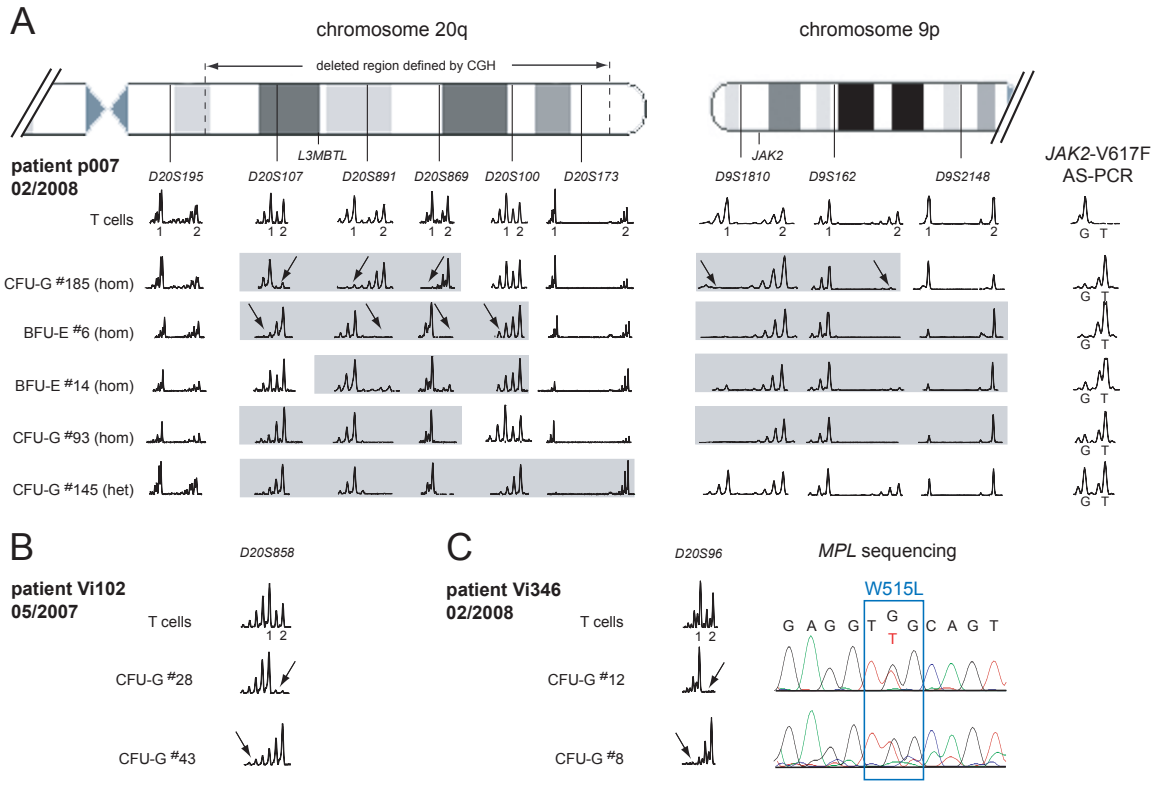


Figure 14. Multiple del20q and 9pLOH events in colonies from three MPD patients.
A) The deleted region on chromosome 20q was mapped in individual colonies from patient p007 using 6 microsatellites distributed along chromosome 20q. T cell DNA was used to define the two alleles for each informative microsatellite. The gray boxes mark the deleted region for each individual colony. Note that del20q in colony #185 created a different haplotype than the deletion in colony #6 (marked by arrows), indicating that two del20q events occurred independently and affected the chromosome 20q of different parental origin. Furthermore, 4 different sizes of the del20q haplotype were detected when additional colonies were analyzed. The results from the mapping of the 9pLOH region are shown on the right side. Note, that the 9pLOH region is smaller in colony #185, as shown by the heterozygosity of D9S2148 in this colony. B) Two separate del20q events affecting the chromosome 20q of different parental origin occurred in patient Vi102. C) Two separate del20q events affecting the chromosome 20q of different parental origin occurred in patient Vi346. The chromatograms for the MPL-W515L mutation are shown for the two colonies analyzed.

ther by postulating two independent del20q events or two independent 9pLOH events. The del20q positive BFU-E colonies in patient Vi102 were wild type for *JAK2,* and the *JAK2*-V617F positive colonies were all negative for del20q, which is compatible with originating from two independent clones. However, the finding of a CFU-G colony positive for both *JAK2*-V617F and del20q suggests that either del20q or JAK2-V617F occurred twice independently. Patient Vi346 was negative for *JAK2*-V617F, but carried the *MPL*-W515L mutation. The del20q was only detectable in CFU-G colonies and all of these colonies were also heterozygous for *MPL*-W515L. Some colonies were homozygous for *MPL*-W515L, but negative for del20q.

In one patient (Na391) with 50% of erythroblasts in the peripheral blood, all colonies were positive for both del20q and *JAK2*-V617F, which precluded us from determining the order of events (not shown) and one patient (p020) had del20q without a mutation in the *JAK2* or *MPL* gene (Figure 13D). We analyzed p020 at three different time points and we were able to detect an increase in del20q positive colonies in CFU-Gs in

2007. However, one year later, the percentage of del20q positive colonies was again comparable with the first time point. An increase in the percentage of del20q positive colonies was observed in serial samples from Na293 and p007, whereas in p187 the colonies were all del20q positive and remained so during follow up, except for one BFU-E and one CFU-G (Figure 13A and C).

To examine the complex pattern in patients p007, Vi102 and Vi346, we performed a more detailed microsatellite analysis for regions on chromosomes 9 and 20 (Figure 14). In p007, the deletion in colony #185 affected the chromosome 20q of a different parental origin than in colony #6, indicating that two independent deletion events must have occurred (Figure 14A, left panel). Furthermore, 4 different sizes of the latter del20q haplotype were detected when additional colonies were analyzed. This pattern could arise when a small del20q event was followed by sequential events that increased the size of the deleted region in the same cell, or alternatively, each of the del20q regions could represent a separate *de novo* deletion event. At present, we cannot distinguish between these possibilities. In addition, colony #185 also differed in the size of the 9pLOH region, suggesting the presence of two different 9pLOH events (Figure 14A, right panel). In contrast, analysis of the deleted regions in colonies from other patients showed a unique size for each of del20q regions (not shown), making it unlikely that the pattern observed in p007 is due to artifacts that occurred during the methylcellulose culture. Evidence for two independent del20q events was also found in patients Vi102 (Figure 14B) and Vi346 (Figure 14C). In both cases, colonies were found that showed loss of heterozygosity affecting two different parental chromosomes 20.

2.1.4 Discussion

In the present study we assessed the potential connection between *JAK2*-V617F and del20q by analyzing the temporal order of acquisition of the two events. Our copy number assay allowed us to screen for del20q by real time PCR in peripheral blood from a large number of MPD patients. The *L3MBTL* gene, which we have chosen for the copy number assay, is located within the CDR shared between patients with MPD and MDS/AML.[119] This assay requires that the majority of granulocytes carry del20q and therefore selects for del20q events that dominate in granulocytes in the peripheral blood. Nevertheless, our observed frequencies in patients with ET, PV and PMF of del20q are comparable with results obtained with cytogenetic studies of bone marrow.[76,120] Furthermore, real time PCR and cytogenetic analysis were in agreement in all 8 patients in whom both analyses have been performed. We found no difference in the frequencies of *JAK2*-V617F in PV and PMF patients with and without del20q. Our results do not confirm a previously reported preferential association of del20q with *JAK2*-V617F positive cases of PMF (Table 3).[100] The reason for this discrepancy is currently unclear. In ET, 4/4 patients with del20q were *JAK2*-V617F positive, similar to 5/5 del20q positive ET cases reported

previously (Table 3).[100] Thus, by combining the studies, 9/9 ET patients with del20q were also *JAK2*-V617F positive. However, this association should be examined in a larger series of patients with del20q. Mapping of the del20q region by CGH revealed that in most patients large deletions have occurred (Figure 12) and that the CDR derived from analyzing granulocytes overlaps with the published region obtained by mapping bone marrow samples.[119]

Disparity between the size of the JAK2-V617F positive clone and the clone determined by analysis of the X-chromosome inactivation pattern,[96,97] presence of endogenous erythroid colonies that are negative for JAK2-V617F,[65,99,122] and co-existence of JAK2-V617F and MPL-W515L/K in the same patients,[123] suggested that clonal events preceding the acquisition of JAK2-V617F exist in patients with MPD. We previously found two patients in whom the clone carrying del20q was larger than the clone positive for *JAK2*-V617F and we hypothesized that *JAK2*-V617F preferentially occurs on the background of clonal hematopoiesis, which in some cases may be caused by del20q.[97] Our single clone analysis confirmed the previously observed temporal order of events in 2 patients (Figure 13A), but in 3 additional patients we detected the inverse order, i.e. *JAK2*-V617F preceding del20q (Figure 13B). Thus, there appears to be no strict temporal order of acquisition making it unlikely that del20q represents a predisposing event for *JAK2*-V617F. At present, we cannot formally exclude the possibility that del20q may represent a "passenger mutation", i.e. a genetic alteration without functional consequence.[124,125] The fact that in serial samples of patients Na293 and p007 we observed an expansion of the del20q subclone (Figure 13) could be interpreted in favor of a growth advantage provided by del20q and thus for a role as a "driver mutation". Similarly, in patients p048 and p103 nearly all colonies were del20q positive, suggesting a growth advantage of the cells harboring del20q.

Unexpectedly, in 3 patients (p007, Vi102, and Vi346) we found evidence for several independent del20q events and in p007 for two independent 9pLOH events (Figure 14). Patient p007 was diagnosed with PV 22 years ago and since 11 years showed signs of a progression to secondary myelofibrosis. The long disease duration and/or progression to spent phase may favor accumulation of de novo mutation events. However, the disease duration in ET patient Vi102 (4 years) and PMF patient Vi346 (6 years) was shorter. Genomic instability as a consequence of JAK2-V617F was recently reported.[126] This could explain the findings in p007. However, in patient p102 one of the del20q events occurred in a *JAK2*-V617F negative cell (Figure 13C) and patient Vi346 was negative for *JAK2*-V617F, suggesting that genomic instability in these patients is independent of *JAK2*.

3.2 Whole genome expression analysis in single colonies and shRNA mediated knock-down in murine bone marrow cells allows to discover del20q candidate genes

3.2.1 Abstract

<<Even though del20q is the most frequent chromosomal aberration in patients with MPN, no candidate gene inside of the common deleted region was so far linked to the disease phenotype. We analyzed differences in gene expression of hematopoietic colonies pooled according to their genotype. With this technique, we were able to analyze highly pure cell populations and to eliminate inter-individual differences in genetic background and differences in the analyzed cell fraction. The expression of 680 genes was significantly ($p<0.05$) changed by the presence of del20q. Out of this 680 genes, we selected three genes from the CDR, which were significantly downregulated for further analysis: *serin threonin kinase 4 (STK4)*, *topoisomerase 1 (TOP1)* and *protein tyrosine phosphatase receptor T (PTPRT)*. We generated a pool of shRNAs targeting these three genes and two additional candidate genes. This mixture of shRNA was then used to transfect NIH3T3 cells for in-vitro assays and murine bone marrow cells for bone marrow transplantations in mice. The first experiment showed that downregulation of these genes did not have any functional consequences for the cells. However, we were able to show that the simultaneous transfection using several shRNAs works and we will investigate the functional consequence of the knockdown more in detail.>>

3.2.2 Introduction

Abnormal karyotypes are often detected at diagnosis of ET, PV and PMF, however no recurrent cytogenetic aberration was so far discovered. The most frequent are 9p uniparental disomy (UPD), numerical aberrations of chromosomes 1, 8, and 9, deletions on chromosomes 5q, 13q, 20q (del20q) and frequent gains of 9p.[66-77] The fact that all of them, except 9pUPD, are also found in other clonal myeloid disorders, suggests that their role in hematological malignancies could be universal. Deletions of the long arm of chromosome 20 (del20q) have been observed frequently in patients with myeloproliferative disorders (MPD), myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML).[68-70,115] A common deleted region (CDR) of 2.7 megabases (Mb) was described for patients with MPD and a 2.6 Mb CDR was defined for MDS/AML. The MPD and MDS/AML regions overlap with 1.7 Mb.[75,116-120] Deletions often mark sites of mutations in tumor suppressor genes, because the remaining allele can be inactivated by a mutation. However, no mutation was so far found in a gene inside the deleted region on chromosome 20q. Alternatively, imprinting may also inactivate the second allele. The methylation pattern of *lethal(3) malignant brain tumor (L3MBTL)*, a gene located in the CDR was analyzed in detail, however both the methylated and the unmethylated copy was lost in patients with del 20q and the expression level did not correlate with the methylation status.[121,127]

In this study, we analyzed del20q-induced changes in gene expression by examining pools of individually genotyped hematopoietic colonies grown in methylcellulose. This approach reduces inter-individual differences in genetic background and differences in the composition of the tissues to a minimum and allows detecting small changes in expression levels.

3.2.3 Results

To detect changes in gene expression which are specifically induced by del20q, we analyzed CFU-G colonies grown in methylcellulose from two patients (p020 and Na293). Individual colonies were picked for subsequent DNA and RNA isolation and analyzed for del20q and *JAK2*-V617F (Figure 15). One patient, p020, was negative for *JAK2*-V617F and therefore we only distinguished between wt and del20q colonies. The colonies of Na293 were grouped into wt/wt, del20q/wt and del20q/*JAK2*-V617F heterozygous. The RNA from these colony groups were pooled into two samples and used for Affymerix Human Gene 1.0 ST array analysis.

The mean ratio of expression levels between wt colonies and del20q positive colonies shows a clear drop on chromosome 20q. This region correlates with the deleted region defined by aCGH in granulocyte DNA of the respective patient (Figure 15). Next, we combined the data from both patients and performed ANOVA analysis to detect differentially expressed genes between colonies with and without del20q. A total of 680 significant expression differences ($p<0.05$) were observed (data not shown). From all genes downregulated, 28 were lo-

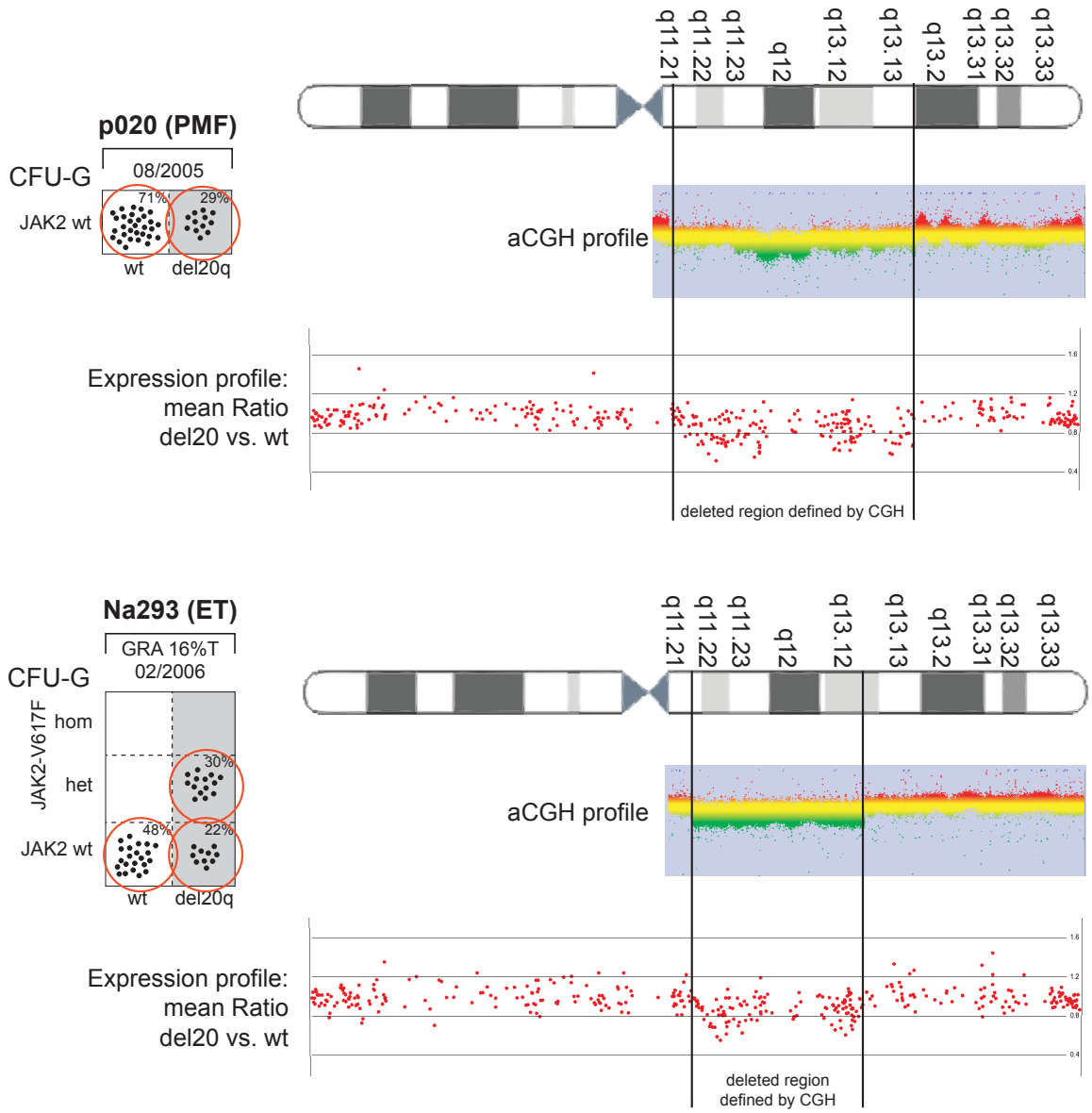


Figure 15: Expression analysis of pools of individually genotyped colonies.
Mononuclear cells from peripheral blood were grown in methylcellulose in the presence of erythropoietin. Single granulocyte colonies were picked and analyzed individually for the presence of del20q and JAK2-V617F by microsatellites and allele-specific PCR, respectively. The colony pattern with the different genotypes is shown on the left site. CGH array data combined with expression array data is shown for two patients, p020 and NA293, on the right site. A region of decreased copy number was observed in granulocyte DNA as compared to a normal control. Expression analysis of del20q CFU-G colonies compared to CFU-G colonies without del20q from the same patient was able to show that the copy number change also affected the gene expression of a subset of genes.

cated inside the CDR described in chapter 3.1, 10 genes were located inside the MPD CDR and 3 genes inside the MDS/MPD CDR[75,116-120] (Figure 16A). Three genes (*STK4*, *TOP1* and *PTPRT*) were selected for further analysis and the reduced expression was confirmed by qPCR in cDNA islolated from colony pools. The downregulation of *STK4* and *TOP1* was also found in total granulocyte RNA of two patients which had a high percentage of del20q positive cells. *PTPRT* expression was not detected in total granulocytes (Figure 16B). Two additional genes, *MYBL2* and *L3MBTL*, which were

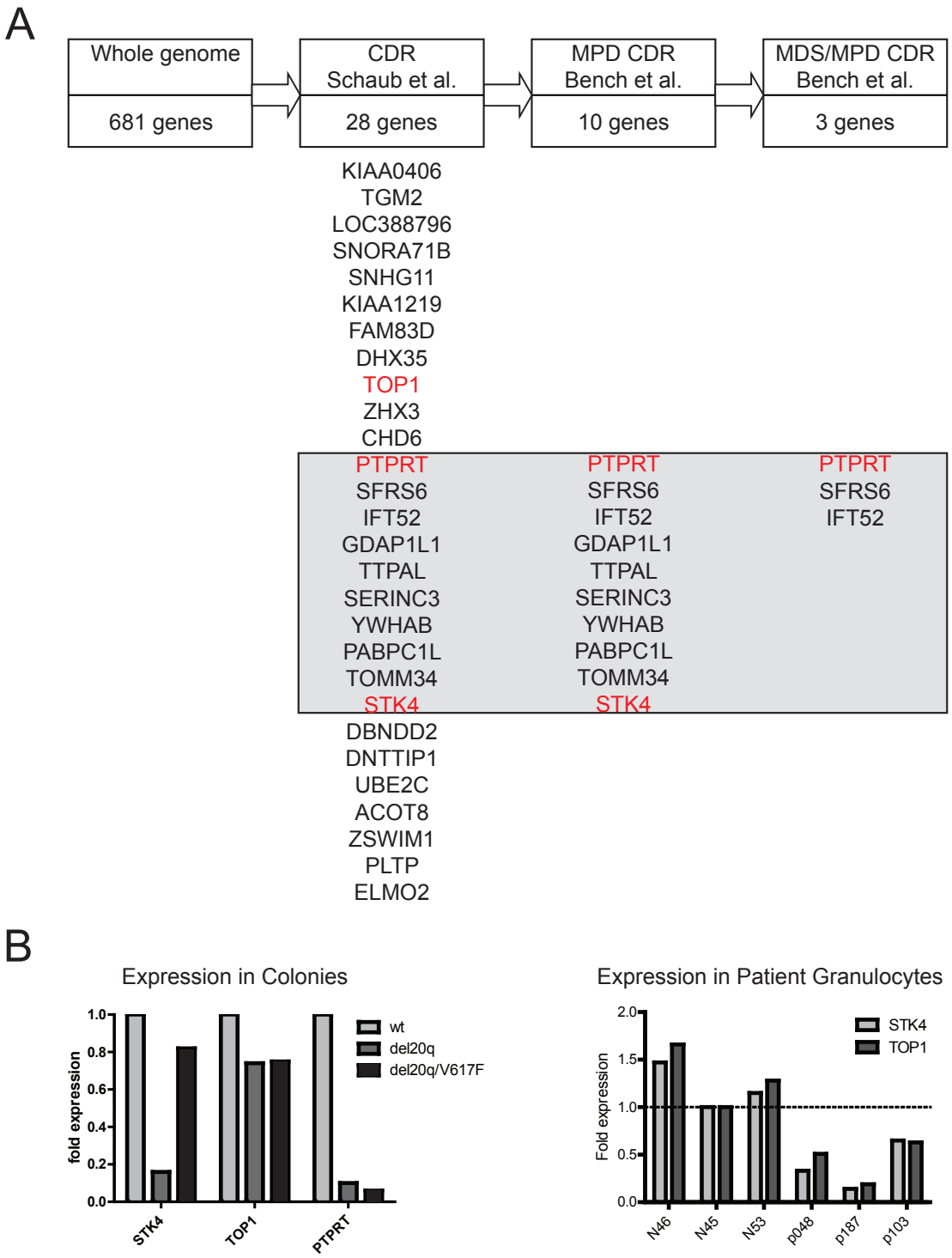
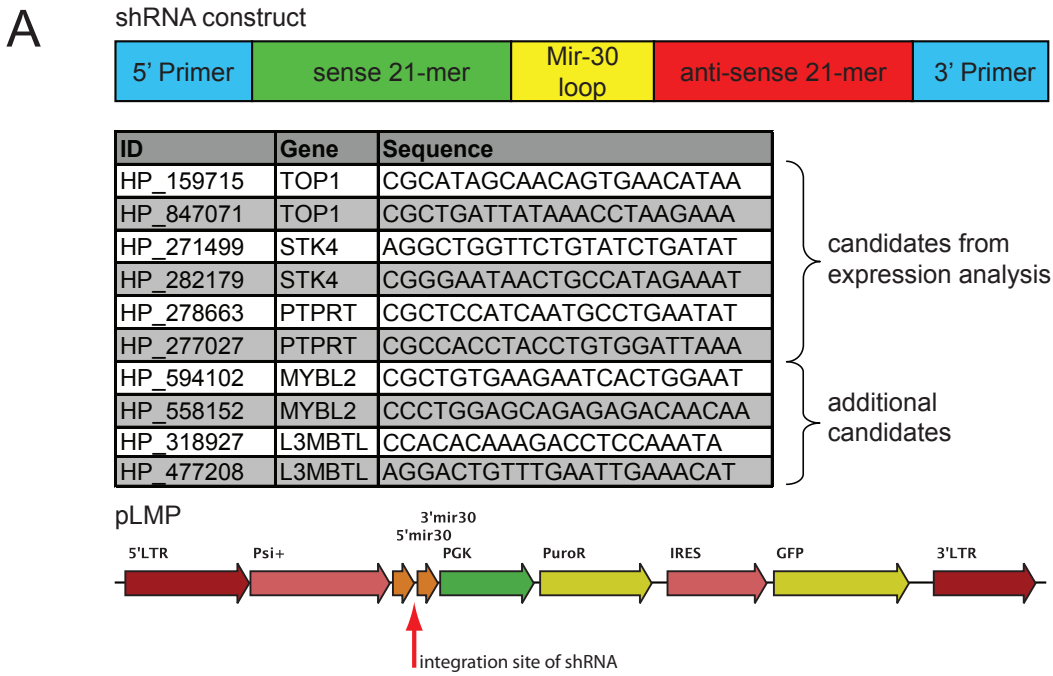
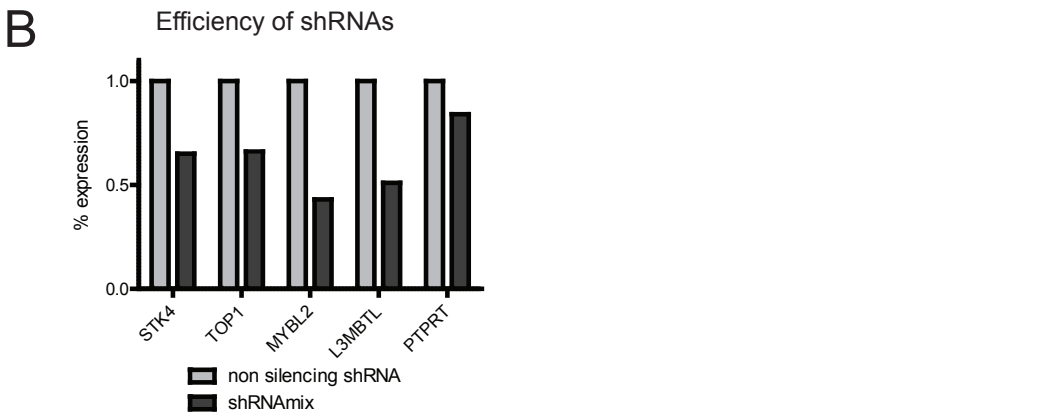


Figure 16: Gene expression analysis results.
A) We have found 680 genes, which were significantly ($p<0.05$) changed in expression between colonies with the deletion on chromosome 20q and colonies without the deletions. The CDR described in the first chapter of this thesis includes 28 downregulated genes, the MPD CDR includes 10 genes and the MDS/MPD region includes 3 genes. B) The expression changes of the candidate genes were confirmed by qPCR in cDNA from colonies with and without del20q. The downregulation of TOP1 and STK4 was also observed in patients with a percentage of del20q positive cells.



ID	Gene	Sequence
HP_159715	TOP1	CGCATAGCAACAGTGAACATAA
HP_847071	TOP1	CGCTGATTATAAACCTAAGAAA
HP_271499	STK4	AGGCTGGTTCTGTATCTGATAT
HP_282179	STK4	CGGGAATAACTGCCATAGAAAT
HP_278663	PTPRT	CGCTCCATCAATGCCTGAATAT
HP_277027	PTPRT	CGCCACCTACCTGTGGATTAAA
HP_594102	MYBL2	CGCTGTGAAGAATCACTGGAAT
HP_558152	MYBL2	CCCTGGAGCAGAGAGACAACAA
HP_318927	L3MBTL	CCACACAAAGACCTCCAAATA
HP_477208	L3MBTL	AGGACTGTTTGAATTGAAACAT



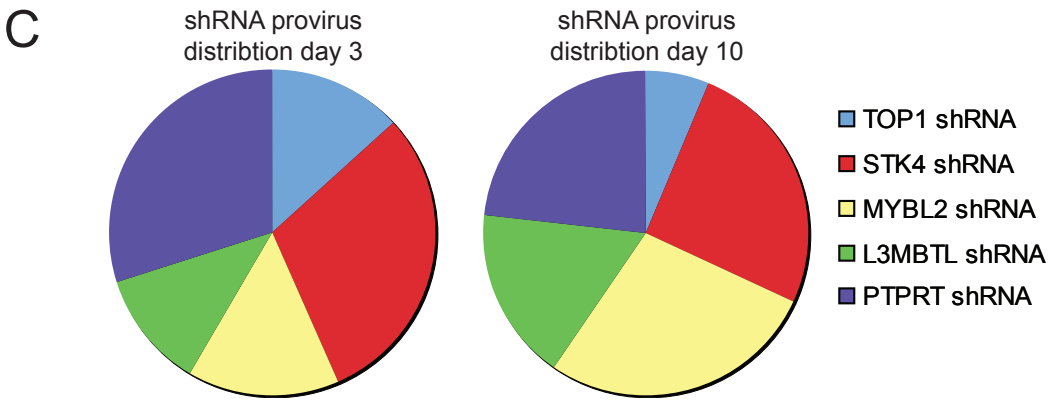


Figure 17: shRNA constructs and transfection of NIH3T3 cells
A) The basic structure of mir30 adapted shRNA construct is shown. The table below lists all shRNA sequences used for the shRNAmix together with the RNAi codex database accession number. The shRNAs were integrated into pLMP, which is a retroviral construct expressing GFP and puromycin resistance as selective marker. B) The efficiency of the shRNAs was tested in NIH3T3 cells. Briefly, retrovirus containing a mixture of all shRNAs was produced and used to transfect NIH3T3 cells. The cells were then sorted for GFP marker and used for RNA and DNA extraction. The qPCR results are shown in the graph. C) This panel sows the assessed distribution of the provirus with the different shRNA by cloning at day 3 and day 10 of the NIH3T3 culture.

also interesting candidate genes, were also included for the following analysis.

We selected for each candidate gene two shRNAmir sequences from the RNAi codex database[128] targeting the murine homologs and cloned them into a pLMP vector[129] (Figure 17A). NIH3T3 cells were transfected with retrovirus containing a non-silencing control shRNA or a pool of all shRNAs targeting the candidate genes (shRNAmix) to test the efficacy of the knockdown. The expression level in GFP+ sorted cells of the candidate genes was reduced between 14% and 57% compared to the non silencing control (Figure 17B). Cloning of the integrated provirus DNA showed, that at least one shRNA sequence for each gene was present in about 12%-30% of the cells (Figure 17B). The NIH3T3 cells containing the shRNAmix were then kept in culture for 10 days and underwent several passages. After this period, the distribution of the integrated

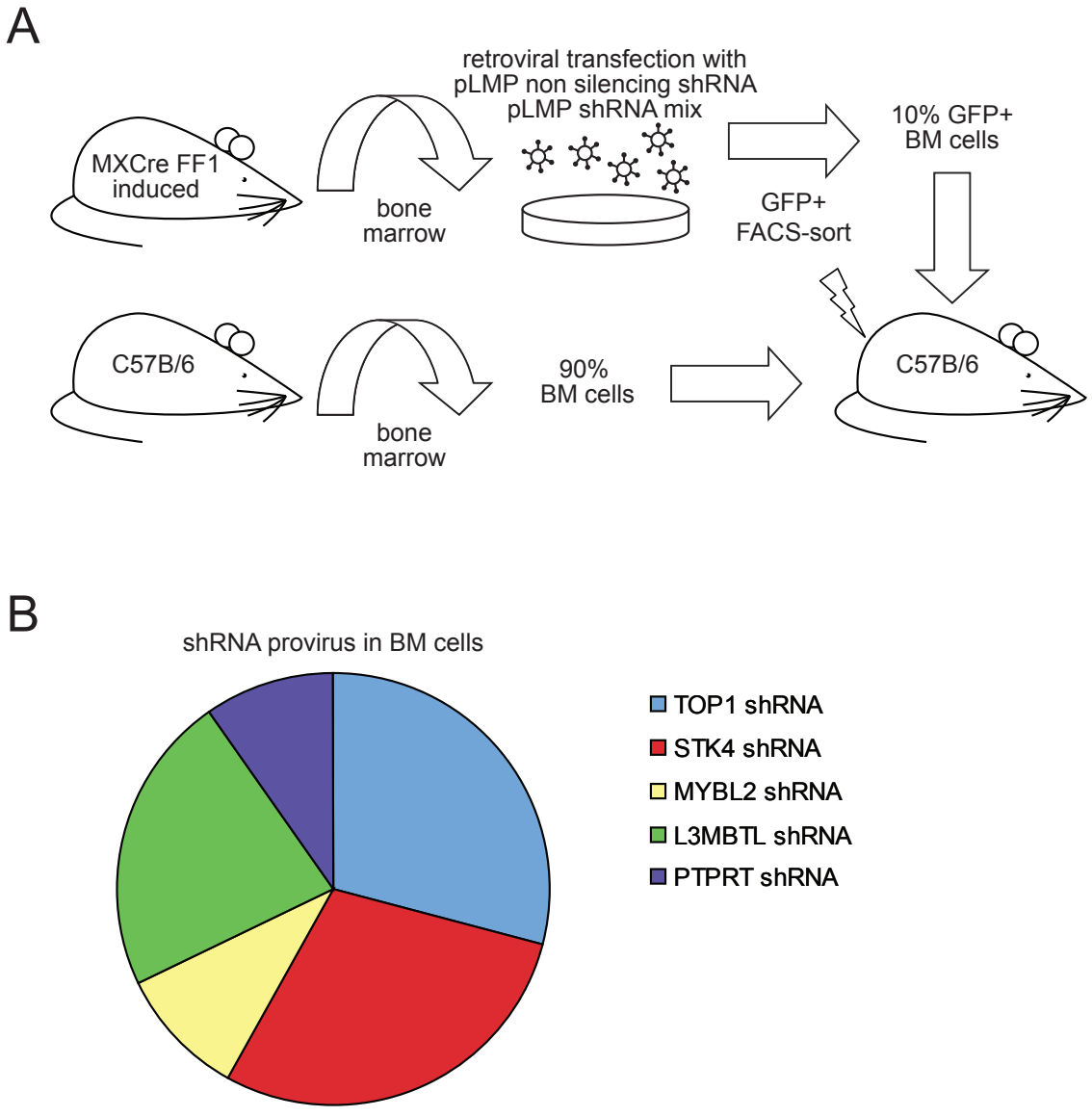


Figure 18: Experimental Set-up of BM transplantation
A) Bone marrow cells from MxCre;FF1 mice were harvested and retrovirally transfected with mixture shRNAs targeting all candidate genes. After 2 days, GFP+ cells were sorted and transplanted together with wt C57B/6 bone marrow in ratio of 1/10 to lethally irradiated C57B/6 mice. B) Distribution of integrated provirus in BM transfected with shRNAmix. All candidate genes are targeted by at least 10% of the proviruses.

shRNAs was analyzed again by cloning. However, no change in the distribution was observed (Figure 17C).

Next, we used this shRNAs to transfect bone marrow (BM) from induced *MxCre;FF1* mice expressing *JAK2*-V617F.[91] The BM cells were either transfected with retrovirus containing a non silencing control shRNA or with a pool of all shRNAs targeting the candidate genes (shRNAmix). The cells were then sorted for GFP marker and together with C57B/6 wt BM cells transplanted into wt C57B/6 mice (Figure18A). Each mouse received 9% of GFP positive BM cells containing the shRNA. Both experimental groups included five animals. The distribution of the integrated shRNAs was again analyzed by cloning and showed that all candidate genes were targeted with at least 10% of the shRNAs (Figure 19B).

At the time point of the first bleeding, 4 weeks after transplantation, we were able to detect around 5% GFP^+ erythrocytes, granulocytes, monocytes and about 2% GFP^+ platelets in both groups of recipient mice. The shRNAmix group showed a slightly higher percentage of GFP^+ cells. However, 8 weeks after transplantation, the amount of GFP^+ cells was reduced and 12 weeks after transplantation all GFP+ cells disappeared, except for erythrocytes in one mouse transfected with shRNA mix where a small portion still remained (Figure 19A).

There were no differences observed in the blood counts between the control group and the animals transfected with shRNAmix and all values were within the physiological range (Figure 19B).

3.2.4 Discussion

The aim of this study is to discover potential candidate genes located inside the MPD CDR on chromosome 20q by analyzing the gene expression profile from individually genotyped colonies. By analyzing CFU-G colonies, which were pooled according to their genotype, we were able to compare an exactly defined cell population and to reduce individual differences in genetic background and composition of the tissues to a minimum. The sensitivity of this assay is shown by the clear drop in the ratio between wt and del20q expression, covering exactly the region which was previously shown to be deleted by aCGH (Figure 15). Because of the close similarity of the samples, only a relatively small number of genes (680) were differentially expressed ($p<0.05$). Out of the 93 genes of our CDR, only 28 genes were significantly downregulated and this number is even smaller if we only consider genes inside the MPD CDR.

Based on this expression data, we decided to select in a first experiment 3 downregulated genes from the CDR to analyze them in more detail. We performed a pathway analysis using the STRING database with all genes which were significantly changed in expression. Two genes were involved in the same pathways like genes outside of the CDR: *serin threonine kinas 4 (STK4)* and *topoisomerase 1 (TOP1)*. *STK4* is a serine/threonine protein kinase that is activated in response to a variety of apoptotic stimuli and overexpression of *STK4* induces apoptosis. *TOP1* is a key enzyme at the interface between DNA replication, transcription and

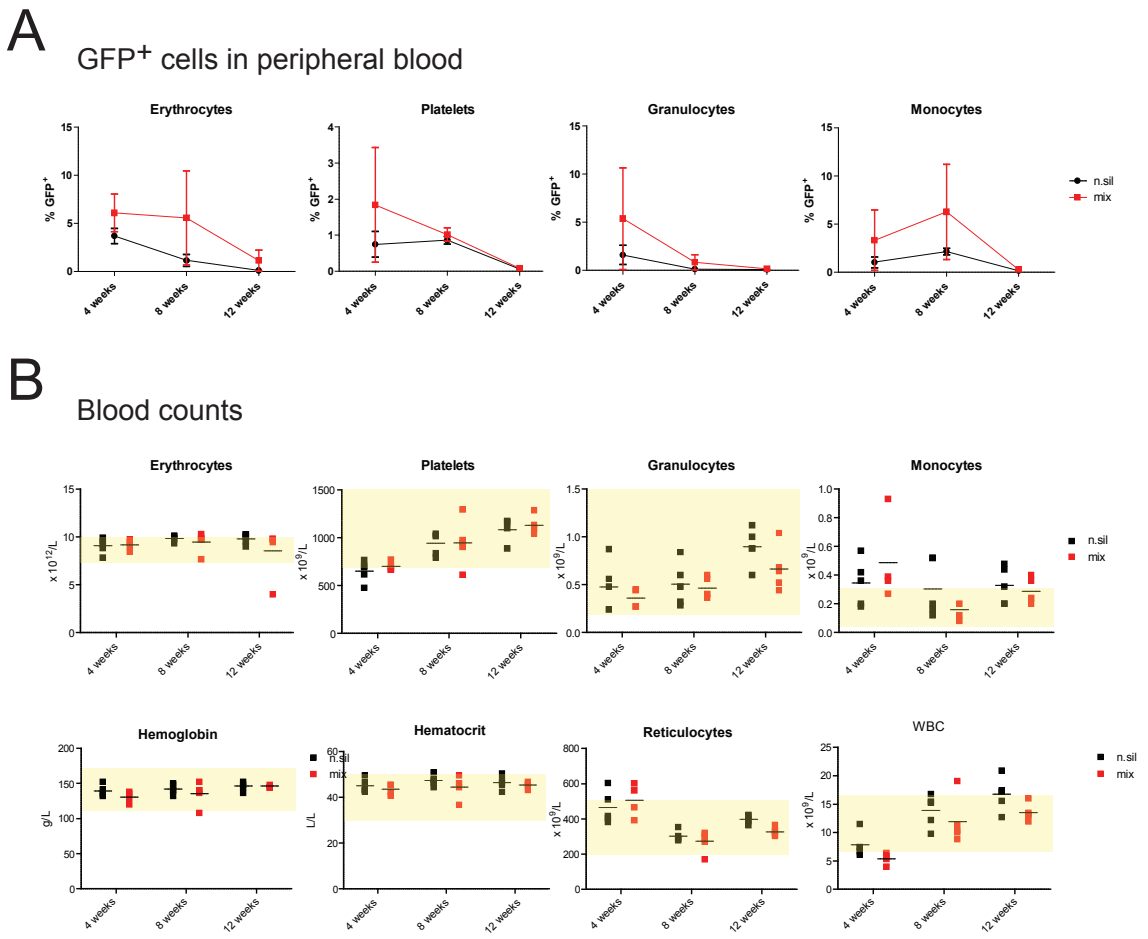


Figure 19: BM transplantation results
A) This panel shows the percentage of GFP+ cells in different blood cell lineages over time in mice transfected with non silencing shRNA (black) or shRNAmix (red). The results from 5 mice are shown per group. B) Blood counts of mice transplanted with non silencing shRNA (black) and shRNAmix (red). Each dot represents a single mouse. The yellow bar marks the physiological range of the respective blood parameter.

mRNA maturation. A recent study showed, that *TOP1* is suppressing genomic instability in mammalian cells by preventing a conflict between DNA transcription and replication.[130] The third candidate, *protein tyrosine phosphatase receptor T (PTPRT)*, was also downregulated but not connected to the same genes like *STK4* and *TOP1*. Recently it was shown, that *signal transducer and activator of transcription 3 (STAT3)* is a substrate of *PTPRT*.[131] For our analysis we also included two genes which were not significantly changed in expression, but represent potential candidate genes*: V-MYB myeloblastosis viral oncogene homolog (avian)-like 2 (MYBL2),* which encodes a transcription factor involved in cell cycle progression and *L3MBTL*, a human homolog of the Drosophila lethal (3) malignant brain tumor (D-l(3)mbt) polycomb protein, which functions as transcriptional repressor and is known as tumor suppressor in the larval brain of drosophila.

NIH3T3 cells were used to check the efficiency in knock-down of the different shRNA's. The expression of all targeted genes was reduced by 14% to 57% compared to the non silencing control. This reduction is lower than expected since not all cells carry all shRNAs. Therefore, we analyze a mix-

ture of different cell populations expressing different levels of the candidate genes. NIH3T3 cells were also used to check the influence of the different shRNAs on cell growth. For this purpose, we analyzed the distribution of the different shRNAs at day 3 and day 10 in cell culture. If one shRNA would provide a growth advantage in NIH3T3 cells, the prevalence of this specific shRNA should be higher after a certain time. However, the shRNA distribution was stable, suggesting that none of the shRNA's provided a selective advantage for NIH3T3 cells.

In most of the patients del20q occurs together with *JAK2*-V617F. Even though the temporal order of these two events can vary, *JAK2*-V617F could potentially be needed for the effect of the del20q gene. Therefore we decided to test the candidate genes on the background of *JAK2*-V617F in *MxCre;FF1* mice[91]. These mice express *Cre* recombinase under the control of the *Mx* promoter, which can be activated by injection of polyinosine-polycytosine (pIpC). The *Cre* recombinase activates the *JAK2*-V617F transcript. *MxCre;FF1* mice showed a phenotype resembling PV with increased hemoglobin levels, thrombocytosis and neutrophilia. This phenotype can be also transplanted in a competitive repopulation assay with normal C57B/6 BM cells down to a 1/10 ratio. We transplanted 9% of *MxCre;FF1* BM cells transfected with non silencing control or shRNAmix together with 91% of normal C57B/6 BM cells. By monitoring the GFP expression we saw, that GFP^+ cells quickly disappeared in the peripheral blood. After 12 weeks, basically no GFP^+ cells were detected in the peripheral blood. This goes inline with the observation, that the expected phenotype of *MxCre;FF1* BM cells transplanted in a 1/10 ratio did not develop. This data suggest that during the process of viral transduction, stem cells cells were lost and normal C57B/6 cells mostly accomplish the hematopoiesis. Moreover, the normal BM cells, which were used for the remaining 91% of the transplant, did not undergo the transplantational procedure and therefore may have stronger repopulating capacity. In addition, the *Mx* promoter driving the expression of *Cre* recombinase may also be activated by the virus infection due to the interferon response. This would lead to the excision of additional transgene copies and reduce the expression level of mutated *JAK2*-V617F.[91]

The present study shows the feasibility and the advantages of using a pool of different shRNA's. Studying the distribution of integrated shRNAs provides an marker for increased proliferation induced by the respective shRNA. This experimental set-up also allows to decrease the number of mice used in an experiment and may also help to discover synergistic effect of the candidate genes. This work is still in progress and the future direction and optimization of this project will be discussed in the "Perspectives".

3.3 Clonal analysis of *TET2* and *JAK2* Mutations suggests that *TET2* can be a late event in the progression of myeloproliferative neoplasms

3.3.1 Abstract

<<Somatic mutations in *TET2* occur in patients with myeloproliferative neoplasms (MPN) and other hematological malignancies. It has been suggested that *TET2* is a tumor suppressor gene and mutations in *TET2* precede the acquisition of *JAK2*-V617F. To examine the order of events we performed colony assays and genotyped *TET2* and *JAK2* in individual colonies. In 4/8 MPN patients we found that some colonies with mutated *TET2* carried wild type *JAK2*, whereas others were *JAK2*-V617F positive, indicating that *TET2* occurred before *JAK2*-V617F. One of these patients carried a germline *TET2* mutation. However, in two other patients we observed the opposite order of events, with *JAK2* exon 12 mutation preceding *TET2* mutation in one case. Finally, in 2/8 patients the *TET2* and *JAK2*-V617F mutations defined two separate clones. The lack of a strict temporal order of occurrence makes it unlikely that mutations in *TET2* represent a predisposing event for acquiring mutations in *JAK2*.>>

3.3.2 Introduction

TET2, a member of the *Ten-Eleven-Translocation* (*TET*) family of genes,[109,110] can be mutated in various hematopoietic disorders including myeloprolieferative neoplasms (MPN), myelodysplastic syndromes (MDS), acute myeloid leukemia (AML) and chronic myelomonocytic leukemia (CMML).[108,112,113,132-137] *TET2* is mutated in 13% of the MPN cases with the highest frequency occurring in patients with primary myelofibrosis (PMF) and polycythemia vera (PV) (17% and 16%), and lowest in patients with essential thrombocythemia (ET) (5%).[108] The mutations in *TET2* do not cluster in a particular region and show a very diverse pattern of frame shift, nonsense and missense mutations. The *TET2* mutations are generally present in a heterozygous state and only a minority of patients displays mutations in both *TET2* alleles. A recent study showed that loss of heterozygosity (LOH) in *TET2* might occur through mitotic recombination or gene copy number changes.[137] The loss of both gene copies through mutations and chromosomal aberrations is compatible with a potential tumor suppressor activity of *TET2*. However, the mechanism of how *TET2* is involved in disease initiation and/or progression is unknown.

UPN	gender	diagnosis	age at diagnosis [year]	disease duration [month]	*TET2* mutation
p021	M	PV	55	107	p.Thr1372Ile
p101	M	ET	61	38	p.S1285del
p136	M	PV	75	38	p.Gln891X
p191	M	PMF	66	59	p.Leu1210Pro
p209	M	PV	64	30	p.Arg544X
p225	F	PV	75	1	splice donor c.3980+2G>T
p226	F	PV	49	28	p.Asp1858fs
p234	M	PV	71	1	p.Gly1275Glu

UPN	*JAK2*-V617F %T	*JAK2*-exon 12 % mutation	complications	treatment
p021	3%	21%	none	aspirin, phlebotomies
p101	19%		bleeding	aspirin, hydroxyurea
p136	40%		deceased, cause of death unknown	n.a.
p191	100%		intracranial bleeding, pulmonary embolism	oral anticoagulants
p209	88%		post-PV myelofibrosis	aspirin, hydroxyurea
p225	82%		cerebro-vascular insult, arterial occlusion	aspirin, hydroxyurea
p226	82%		none	aspirin, hydroxyurea
p234	20%		transient ischemic attack	clopidogrel, hydroxyurea

Table 4 Characteristics of MPN patients with mutations in *TET2*

3.3.3 Results and Discussion

To study the order of events in the clonal evolution of TET2 and JAK2 mutations, we examined individual colonies derived from peripheral blood of 8 MPN patients with TET2 mutations (6 PV, 1 ET and 1 PMF) that were identified by sequencing of DNA from 57 MPN patients. All patients with TET2 mutation were also positive for JAK2-V617F (Table 1).

Mutational analysis of single colonies allowed us to distinguish 3 different patterns of mutation accumulation (Figure 1): In 4/8 MPN patients we found that some colonies with mutated TET2 carried wild type JAK2, whereas others were JAK2-V617F positive, indicating that TET2 occurred before the acquisition of JAK2-V617F (Figure 1A). These 4 patients confirm previous data by Delhommeau, and colleagues, who showed that TET2 occurs before JAK2-V617F.3 A second group showed the inverse order of events, i.e. JAK2 mutations occurred before the acquisition of TET2 mutations (Figure 1B): patient p191 displayed colonies positive for JAK2-V617F without the TET2 mutation and all colonies with mutated TET2 were also positive for JAK2-V617F. The transition from heterozygous to homozygous JAK2-V617F appears to have independently occurred twice. Indeed, the analysis of individual colonies homozygous for JAK2-V617F revealed the presence of two subclones with different sizes of the 9p uniparental disomy (UPD) region (Supplementary Figure 1A). By analyzing an informative single nucleotide polymorphism (SNP) in exon 11 of TET2 (Figure 1B), we can exclude the possibility that the JAK2-V617F positive and TET2-L1210P-negative colonies arose through mitotic recombination, in which the TET2-L1210P was lost. In patient p021 two independent clones were present, a smaller clone positive for JAK2-V617F and a larger clone positive for a JAK2 exon 12 mutation (JAK2-N542-E543del).21,22 Interestingly, the TET2 mutation in this patient occurs only in combination with the JAK2 exon 12 mutation and the TET2 mutation in this patient was acquired after the JAK2 exon 12 mutation. A third pattern consisted of bi-clonal disease, as illustrated in patient p101 with colonies positive either for JAK2-V617F or TET2, but absence of double-positive colonies (Figure 1C). A similar biclonal pattern was previously described in one patient with del20q and JAK2-V617F, consistent with the presence of a predisposition to independently acquire two otherwise rare somatic events.23 Patient p234 also shows a pattern compatible with bi-clonal disease. However, to progress to the double positive stage either JAK2 or TET2 must have mutated twice independently. Again, SNP analysis excluded the possibility that the JAK2-V617F positive and TET2-G1275E negative colonies arose through mitotic recombination (Figure 1C). Since it is unlikely that TET2 independently mutated twice at the exactly same position (TET2-G1275E), we conclude that the majority of colonies first acquired TET2-G1275E followed by JAK2-V617F. A second independent event produced the subclone that is positive for JAK2-V617F only.

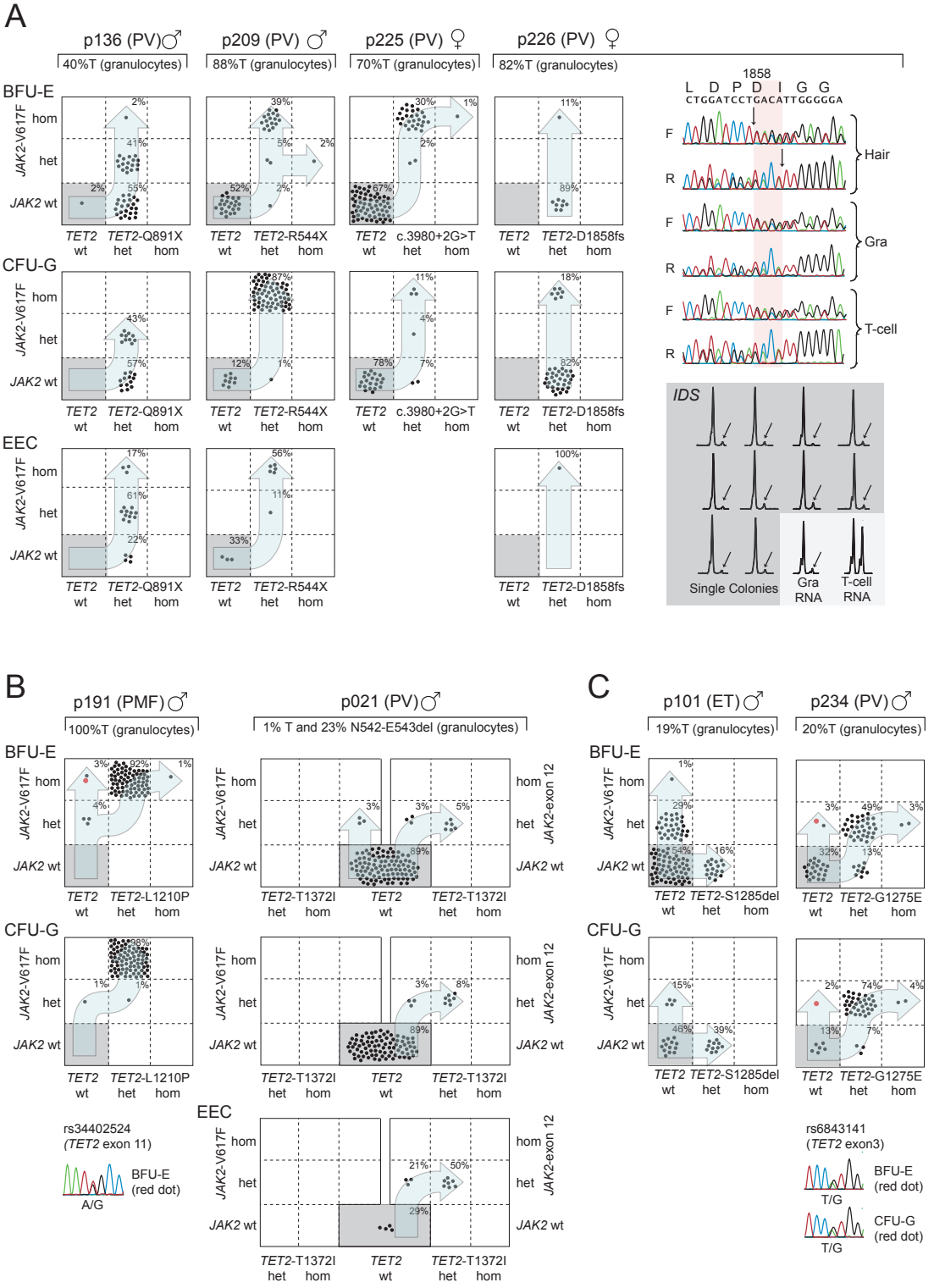

A
p136 (PV)♂
40%T (granulocytes)
p209 (PV) ♂
88%T (granulocytes)
p225 (PV) ♀
70%T (granulocytes)
p226 (PV) ♀
82%T (granulocytes)
BFU-E
CFU-G
EEC
JAK2-V617F
hom
het
JAK2 wt
TET2 wt
TET2-Q891X het hom
TET2-R544X het hom
TET2 c.3980+2G>T het hom
TET2-D1858fs het hom
1858
L D P D I G G
CTGGATCCTGACATTGGGGGA
F
R
Hair
Gra
T-cell
IDS
Single Colonies
Gra RNA
T-cell RNA
B
p191 (PMF)♂
100%T (granulocytes)
p021 (PV)♂
1% T and 23% N542-E543del (granulocytes)
JAK2-exon 12
TET2-L1210P het hom
TET2 wt
TET2-T1372I het hom
rs34402524
(TET2 exon 11)
A/G
BFU-E
(red dot)
C
p101 (ET)♂
19%T (granulocytes)
p234 (PV)♂
20%T (granulocytes)
TET2-S1285del het hom
TET2-G1275E het hom
rs6843141
(TET2 exon3)
T/G
D
L1210P
G1275Q
S1285del
R544X
Q891X
R1167fs
T1372I
D1858fs
conserved region
del
S354X
Q557X
R550X
P1237-S1239del
P1540fs

Figure 20 (page 46). Analysis of single colonies for mutations in TET2 and JAK2.
Mononuclear cells from peripheral blood were grown in methylcellulose in the presence or absence of erythropoietin. Single burst forming units erythroid (BFU-E), endogenous erythroid colonies (EEC) and colonies forming units granulocytes (CFU-G) were picked and analyzed individually for the presence of TET2 and JAK2-V617F mutations by DNA sequencing and allele-specific PCR, respectively. Each colony is represented by a dot that is placed into one of six quadrangles representing the 6 possible genotypes: wild type (wt), heterozygous (het) and homozygous (hom) for JAK2-V617F on the vertical axis, and for TET2 mutations on the horizontal axis. The unique patient numbers, the diagnoses (PMF, primary myelofibrosis; ET, essential thrombocythemia; PV, polycythemia vera) and the allelic ratio of JAK2-V617F in purified granulocytes (%T) are shown above the corresponding boxes. Light blues arrows indicate the suggested order of mutation events. A) Patterns compatible with TET2 mutations occurring before JAK2-V617F. The sequencing chromatograms for patient p226 show the presence of TET2 mutation in DNA from hair follicles, T cells and granulocytes, demonstrating the germline origin of the mutation. Allele-specific PCR assay for the X-chromosomal gene IDS is shown for p226. The genomic DNA from patient p226 was heterozygous for a C/T single nucleotide polymorphism (not shown). The relative expression of the two IDS alleles was determined by comparing the C and T peak intensities obtained by the allele-specific RT-PCR assay in T cells and granulocytes. The skewing of expression towards the C-allele is shown for 10 individual colonies (gray area). The inactivated IDS allele is marked with an arrow. B) Patterns compatible with JAK2 mutations occurring before TET2 was mutated. Patient p021 carries two JAK2 mutations, JAK2-V617F and JAK2 N542-E543del, but the TET2 mutation can be only found together with the deletion in exon 12 of JAK2. In patient p191, the sequencing chromatogram for the single nucleotide polymorphism (SNP) rs34402524 located in TET2 is shown for one BFU-E colony marked in red. The presence of a heterozygous SNP excludes the possibility, that this colony is the product of a mitotic recombination event. C) Patterns compatible with a bi-clonal state of the disease. In patient p234, the sequencing chromatogram for the SNP rs6843141 located in TET2 is shown for one BFU-E and one CFU-G colony marked in red. Again, the presence of a heterozygous SNP excludes the possibility, that these colonies are the product of a mitotic recombination event. D) Location of mutations in the Tet2 protein in patients from whom data on single colonies is available. Mutations from this study are shown above the protein strand and mutations analyzed in previous publications (refs.3,26) are shown below. The grey boxes represent regions conserved between the different TET family members. Blue boxes, TET2 mutations that occur before JAK2 mutations; yellow boxes, TET2 mutations that occurred after JAK2; white boxes, TET2 and JAK2 mutations compatible with bi-clonal disease; red box, germ line TET2 mutation.

The SNPs in close proximity (rs10974944 and rs12343867) were non-informative and were not part of the recently described GGCC or 46/1 JAK2 haplotype (data not shown).24,25

In 4/8 patients we observed a small number of colonies that were homozygous for the TET2 mutations. Gene copy number analysis revealed that p209 and p191 retained two copies of the TET2 gene, while in p225 loss of one copy of the TET2 gene was found indicating that the normal TET2 allele was lost through a deletion (Supplementary Figure 1B). In patient p234, homozygosity was achieved through UPD in some colonies and deletion in other colonies. Colonies homozygous for the TET2 mutations from all four patients displayed LOH of SNPs or microsatellite markers in the TET2 locus, as expected for a deletion or UPD at chromosome 4q (Supplementary Figure 1B).

The heterozygous TET2-D1858fs mutation in patient p226 was also present in DNA from hair roots, indicating that the mutation was germline. This 4 base pair deletion, located in the C-terminal conserved domain of TET2, results in a frame shift and premature stop and is likely to be functionally relevant. The same mutation was also found in buccal DNA from an asymptomatic sister of p226 (data not shown). This appears to be the first report of a germline mutation in TET2. Accordingly, all colonies in this patient were positive for the TET2 mutation. The X-

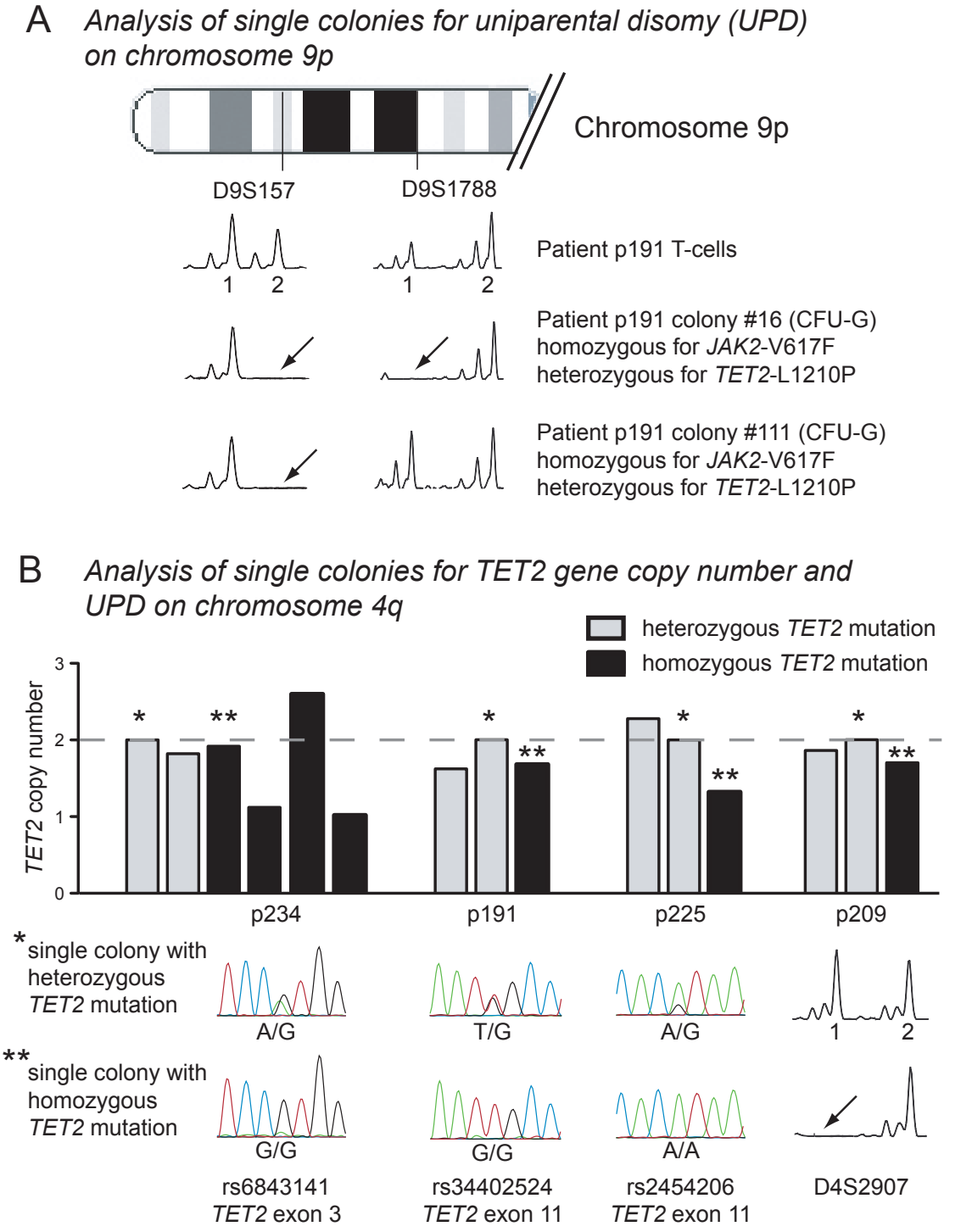


Figure 21
A) Mapping of the 9p UPD region in patient p191. The top row shows heterozygosity in T cells for the two microsatellite markers and below two single colonies are shown. Note, that the 9p UPD region of the two colonies is different, as shown by the loss of allele number 1 with marker D9S1788 in colony #16. B) TET2 gene copy number analysis and detection of 4q UPD in individual colonies heterozygous or homozygous for the TET2 mutation. Heterozygous and homozygous colonies for which chromatograms are shown are marked by asterisk or a double asterisk, respectively.

chromosome inactivation pattern (XCIP) in individual colonies from p226 with wild type JAK2, as determined by scoring a C/T polymorphism in the 3'-untranslated region of the IDS mRNA,18-20 revealed a strong skewing (10/10 expressed the C allele of IDS), indicating that these progenitors were of clonal origin (Figure 1A). The finding of clonality suggests that this patient has a second significant disease clone which does not carry a mutation in JAK2.

We show that JAK2-V617F or JAK2 exon 12 mutations can precede mutations in TET2. A similar conclusion was reached in one patient with familial MPN positive for TET2 and JAK2-V617F mutations.26 In 4/8 patients, some colonies carried a homozygous TET2 mutation that was due to the loss of the wild type allele through deletion or UPD. The percentage of such homozygous colonies in all 4 patients was very low (<5%), opening the possibility that the loss of the wild type TET2 may not provide an additional competitive advantage. The location of the TET2 mutation that have been analyzed using single colony assays is summarized in Figure 1D. Most of the TET2 frame shift and non-sense mutations occurred in patients in whom TET2 preceded the acquisition of JAK2. The lack of a strict temporal order of occurrence resembles the findings obtained for del20q and makes it unlikely that mutations in TET2 represent a predisposing event for acquiring JAK2.

4. MATERIAL AND METHODS

Patient and Patient Material

The collection of patient blood samples was performed at the study centers in Basel, Switzerland, Vienna, Austria, and Dijon and Nantes, France and was approved by the local Ethics Committees ("Ethik Kommission Beider Basel", the "Ethik Kommission der Universität Wien und des Allgemeinen Krankenhauses der Stadt Wien-AK", and the institutional boards of Dijon and Nantes). Written consent was obtained from all patients in accordance with the Declaration of Helsinki. The diagnosis of MPD was established according to the criteria of the World Health Organization. [55-57]

Peripheral-blood mononuclear cells (PBMCs) were prepared with the use of Ficoll gradient centrifugation. Granulocytes were isolated,[141] and analysis of cytospin preparations verified that the purity exceeded 90 percent. CD4+ T cells from patient were obtained either by isolation using fluoresence activated cell sorting using BD Influx (BD Biosience, San Jose, CA , USA) or T cells cultures from PBMCS. For this purpose, PBMC's from patients were co-cultured with irradiated feeders in IL2-media (with 200U/ml IL2, non-essential amino acids, AB^+ serum, kanmycin, Na-pyruvat and L-glutamin) with 2µg/ml phytohemagglutinin (Murex, Dartford, England). After two weeks of culture, the purity of T cells was checked by FACS and if necessary, the cells were purified using MACS columns to deplete NK-T cells. Buccal mucosal cells were obtained with cytologic brushes, and hair-follicle DNA was prepared from plucked hair. Genomic DNA was isolated with the use of the NucleoSpin Tissue (Macherey-Nagel, Düren, Germany).

Copy Number Analysis

The granulocyte DNA was assayed using a real time PCR based copy number assay as previously published.[66] The following primers were used to detect the copy number of two different exon of *L3MBTL*: 1736-GATCCCAATCAGGACCCCC, 1737-CGCCTGGCACTGACAGGT for exon 2 and 1995-CATGAAGCTGGAGGCTGTTG, 1996-GCCACGCAGACAAGGGAC for exon 9. Primers for *LOC221154* (1283-CCATGGACGACGGGTTTCT,

ID	Sequence	Purpose
TET2_ex2F	ATATCCAATTATAGGTATCCAAAACC	*TET2* genomic seqeuncing
TET2_ex2R	TTGAAGTTTGGGAGAGAGATTACC	*TET2* genomic seqeuncing
TET2_ex3.1F	CAGTTTGCTATGTCTAGGTATTCCG	*TET2* genomic seqeuncing
TET2_ex3.1R	TCACCATGTGTGTGTTCCAC	*TET2* genomic seqeuncing
TET2_ex3.2F	AATTGTGATGGTGGTGGTG	*TET2* genomic seqeuncing
TET2_ex3.2R	TAAGCCAAGAAAGAAATCCAG	*TET2* genomic seqeuncing
TET2_ex3.3F	CCTGGTGGCAGCTCTGAAC	*TET2* genomic seqeuncing
TET2_ex3.3R	TTGATTGGAGAGATTGGGTTG	*TET2* genomic seqeuncing
TET2_ex3.4F	CCCCAACACAGCACTATCTG	*TET2* genomic seqeuncing
TET2_ex3.4R	CTCGAACTCGCTTGATTTTG	*TET2* genomic seqeuncing
TET2_ex3.5F	CTCCAGACTTTTCCTCACCC	*TET2* genomic seqeuncing
TET2_ex3.5R	CAGGTTCCACCTTAATTGGC	*TET2* genomic seqeuncing
TET2_ex3.6F	AGCATGCTGCTCTAAGGTGG	*TET2* genomic seqeuncing
TET2_ex3.6R	TCACAAGACACAAGCATCGG	*TET2* genomic seqeuncing
TET2_ex4F	GGGGTTAAGCTTTGTGGATG	*TET2* genomic seqeuncing
TET2_ex4R	TGCTTTGTGTGTGAAGGCTG	*TET2* genomic seqeuncing
TET2_ex5F	TGCCTCTTGAATTCATTTGC	*TET2* genomic seqeuncing
TET2_ex5R	GGGTAACCCAATTCTCAGGG	*TET2* genomic seqeuncing
TET2_ex6F	TGCAAGTGACCCTTGTTTTG	*TET2* genomic seqeuncing
TET2_ex6R	AACCAAAGATTGGGCTTTCC	*TET2* genomic seqeuncing
TET2_ex7F	CAGCTGCACAGCCTATATAATG	*TET2* genomic seqeuncing
TET2_ex7R	TCACTTCATCTAAGCTAATGAATTCTC	*TET2* genomic seqeuncing
TET2_ex8F	GGGATTCAAAATGTAAGGGG	*TET2* genomic seqeuncing
TET2_ex8R	TGCAGTGGTTTCAACAATTAAG	*TET2* genomic seqeuncing
TET2_ex9F	TGTCATTCCATTTTGTTTCTGG	*TET2* genomic seqeuncing
TET2_ex9R	TCCTCATTTGCCTTCAGC	*TET2* genomic seqeuncing
TET2_ex10F	ACACACACGTTTTCTTTGGG	*TET2* genomic seqeuncing
TET2_ex10R	CAGAACTTACAAGTTGATGGGG	*TET2* genomic seqeuncing
TET2_ex11.1F	CCTACATTTAAGTATCCTCACTAGCC	*TET2* genomic seqeuncing
TET2_ex11.1R	TGAAGTGGCCATCCATCTC	*TET2* genomic seqeuncing
TET2_ex11.2F	TTGGAAATAGCCAGAGTTTTACATC	*TET2* genomic seqeuncing
TET2_ex11.2R	AGCCATGTTTTGGCTCATTC	*TET2* genomic seqeuncing
TET2_ex11.3F	GTGAGCTGCATGCCACAAC	*TET2* genomic seqeuncing
TET2_ex11.3R	TGTGTAGAGTTGTAAGCGGGG	*TET2* genomic seqeuncing
TET2_ex11.4F	AATTTTCACTGGCTCCCAAG	*TET2* genomic seqeuncing
TET2_ex11.4R	AAAGCTTAGGTAGACAAAGTGCTTC	*TET2* genomic seqeuncing
TET2_ex11.5F	GCTCATCCAGTGAAGTCCTTG	*TET2* genomic seqeuncing
TET2_ex11.5R	TGGAACTAACCCTGTTTATTCATC	*TET2* genomic seqeuncing
TET2_ex11.6F	CTTCAGAGAACTGAATGGCAG	*TET2* genomic seqeuncing
TET2_ex11.6R	ACAGTACTAAAATACTAAAGCATTGC	*TET2* genomic seqeuncing
TET2_ex11.7F	GAGTTTGAAGCAGAATTCACATC	*TET2* genomic seqeuncing
TET2_ex11.7R	AAAGGGTTAAATCCCTGACAAAG	*TET2* genomic seqeuncing
TET2_ex11.8F	ATGCTCTCCCTATGCCAA	*TET2* genomic seqeuncing
TET2_ex11.8R	TGGGGCTAGATATTATGGT	*TET2* genomic seqeuncing

ID	Sequence	Purpose
TET2_ex11.9F	CTTGAATTTGTGGTTGTGTC	*TET2* genomic seqeuncing
TET2_ex11.9R	TTCATTGAGGTGGCAGTC	*TET2* genomic seqeuncing
JAK2 ex13F	AACCGAGTAGAGCCAAAATACACT	*JAK2* happlotype
2665	AGCACAATAAAGCAAGGTGC	*JAK2* happlotype
IDS-F-C (HEX)	GATATCTTCTAACCATACC	XCIP
IDS-F-T (HEX)	tttGATATCTTCTAACCATACT	XCIP
IDS-R-gDNA	GTTTCTTCGTGGTATATAACCAG	XCIP
IDS-R-RNA	GTTTCTTGAACATCCAGCACATCCAC	XCIP

Table 5: Primers for TET2 sequencing, JAK2 haplotype and XCIP. ttt=non-annealing tail

1284-TGTACAGGACGTAGGAGGGTGA) were used as a diploid reference. Samples that showed a decrease in copy number in both exons were validated using primers in the neighboring genes *PTPRT* (1740-TGCCCCGGAACCATGATA, 1741-GGTCCAGAGGCAGCACGT) and *TOX2* (1949-CCTGCCTACTCCTATCAGGCC, 1950- GTTGGACACCATGATGGCTG).

The following primers were used to assess the copy number of TET2: TET2_copy_F-AGTGTGGAAGCTCAGGAGGA and TET2_copy_R- TGTACGTGATGGGGCTGAC.

Colony Assays

The colony assays were performed using PBMC from patients as published.[65] Methylcellulose based media (#4431 and #4434) containing erythropoietin (Epo) and methylcellulose based media without EPO (#4531) from Stem Cell Technologies (Vancouver, Canada) were used. To ensure that plates with an optimal density were obtained so that colonies can be picked without contamination by cells from neighboring colonies, PBMCs were plated at three different concentrations (50'000, 100'000 and 200'000 cells per ml). Single colonies were picked either into 5% Chelex (BioRad, Hercules, USA) and 0.1% Triton-X (Sigma-Aldrich, Buchs, Switzerland) for DNA or into Trifast (PeqLab, Erlangen, Germany) to extract RNA and DNA following the manufacturer guidelines.

Comparative genomic hybridization (CGH)

The custom oligo CGH array was designed using the eArray platform from Agilent (Agilent, Santa Clara, USA). The chips were processed following the instructions of the supplier. Either a pool of male whole blood DNAs (Promega, Madison, USA) or T-cell DNAs from the respective patients were used as reference DNA. The data was analyzed using Capweb software (http://bioinfo-out.curie.fr/CAPweb/).[142]

Affymetrix expression Arrays

We picked single colonies and isolated DNA and RNA using Trifast (PeqLab, Erlangen, Germany). The DNA was used to genotype each colony for the presence or absence of del20q. RNA from colonies with the same genotype was subsequently pooled

Target genes	Assay ID (ABI)	Sequence
mSTK4	Mm00451755_m1	n.a.
mTOP1	Mm00493749_m1	n.a.
mL3MBTL	Mm01239974_m1	n.a.
mMYBL2	Mm00485340_m1	n.a.
mTbP	mM00446973_m1	n.a.
mGAPDH	n.a.	CTCAGCTCCCCTGTTTCTTG CCTTCCACAATGCCAAAGTT
hSTK4	Hs00178979_m1	n.a.
hTOP1	Hs00243257_m1	n.a.
hTbP	HS99999910_m1	n.a.

Table 6: Gene expression assays.

and analyzed by Affymetrix Human Gene 1.0 ST arrays. Prior to the analysis, the RNA was amplified using the WT-Ovation™ Pico RNA Amplification System (Nugen Inc., San Carlos, USA). The data was analyzed using Partek Genomic Suite (Partek, St. Louis, USA) and STRING database[142] for protein interactions.

TET2 Sequencing

The exons of *TET2* were amplified with primers specified in table 5. The PCR fragments were purified using shrimp slkaline phosphatase (Promega, Madison, Wi, USA) and exonuclease I (Fermentas, Ontario, Canada) und sequenced with BigDye Terminator v3.1 Cycle Sequencing kit. After purification with BigDye XTerminator Purification kit, the samples were analyzes with a 3130xl GeneticAnalyzer (Amplied Biosystems, Foster City, CA, USA).

JAK2 haplotype and IDS clonality

The *JAK2* haplotype was assessed by determining the alleles of SNPs in proximity of *JAK2*-V617F. The primers used for this purpose are listed in table 4. The clonality in patients and single colonies was determined by analyzing the genotype of a C/T polymorphism in the 3'-untranslated region of the *IDS* mRNA. The primers, which are labeled with fam, are listed in table 5. The PCR product was analyzed with a 3130xl GeneticAnalyzer (Amplied Biosystems, Foster City, CA, USA).

Retrovirus production

293T cells were transfected with eaqual amounts of pLMP (containing the shRNA's) and packaging plasmid (Ecopack) using Turbofect (Fermentas, Ontario, Canada). The first supernatant was harvested 48 hours after transfection and the second 72 hours after transfection. The supernatant was filtered through a 4µm filter and used directly for the infection of murine bone marrow cells.

Transfection and transplantation of murine bone marrow cells

The donor mice (*MxCre;FF1*) were treated with 150mg 5-fluoroucil per kg bodyweight 8 days before harvesting the bone marrow. After harvesting the bone marrow, it was

kept in transplant media (RPMI-1640, 10% fetal calf serum, 6ng/ml mIL3, 10ng/ml hIL6 and 6ng/ml mSCF) for 24 hours, before the first spin infection (2500rpm, 90min) was performed. 1ml of viral supernatant was used together with 4 x 10^6 bone marrow cells and 2µl of polybrene transfection reagent (Millipore, Billerica, MA, USA). A second spin infection was performed one day later. The recipient mice were lethally irradiated (11 Gy) at the day of transplantation. Each mouse got 10^6 bone marrow cells. Dead cells were removed before transplantation using Ficoll gradient centrifugation.

pLMP integration cloning

DNA was extracted from sorted retrovirally transfected NIH3T3 or mouse bone marrow cells using NucleoSpin Blood Kit (MachereyNagel, Düren, Germany). The integrated provirus was amplified using the following primers: 2834- ACTTGCTGGGATTACTTCT and 2835- GTGGATGTGGAATGTGTG. The PCR product was purified on an agarose gel and extracted with NucleoSpin Extract II (MachereyNagel, Düren, Germany). The fragment was cloned into pSC-A-amp/kan using the StrataClone Kit (Agilent, Santa Clara, USA) and plated on S-Gal/LB Agar plates (Sigma-Aldrich, Buchs, Switzerland). Single white colonies were picked into 5% Chelex (BioRad, Hercules, USA) and 0.1% Triton-X (Sigma-Aldrich, Buchs, Switzerland). The cloned PCR fragment was amplified using M13 and T3 primers and sequenced with M13 using BigDye Terminator v3.1 Cycle Sequencing kit. After purification with BigDye XTerminator Purification kit, the samples were analyzed with a 3130xl GeneticAnalyzer (Amplied Biosystems, Foster City, CA, USA). The sequencing data was analyzed using CLC DNA Workbench 5 (Aarhus, Denmark)

Gene expression analysis

Gene expression analysis in cDNA was performed either with commercial Taq-Man assays from Appliedbiosystems or self-designed primer pairs for SYBR-Green qPCR. The different assays are listed in table 6. Either qPCR Mastermix for Taq-Man (Eurogentec, Seraing, Belgium) or PowerSYBR Green (Amplied Biosystems, Foster City, CA, USA) were used and the samples were analyzed on a ABI Fast 7500 (Biosystems, Foster City, CA, USA). *Tata-binding protein (TbP)* and GAPDH were used as internal controls.

5. PERSPECTIVES

The discovery of *JAK2*-V617F has substantially changed the understanding of MPN, but this finding opened a lot of new questions. It is widely accepted, that *JAK2*-V617F does not represent the disease initiating mutation and additional hits may occur in earlier stage of the disease and may influence the disease phenotype, severity and complications. The pre-*JAK2* phase of the disease may even be without an obvious disease phenotype. For this reason, it is important to know the temporal relationship of *JAK2*-V617F and other mutations to understand the evolution of different disease clones in patients.

Several Jak2 inhibitors based on small molecules to inhibit the kinase activity are in development and first clinical trials have been started. The present work showed, that some patients carry significant disease clone negative for *JAK2*-V617F with marked proliferation potential. It would be therefore interesting to monitor the clonal distribution in these patients treated with Jak2 inhibitors. The treatment may indeed reduce the *JAK2*-V617F positive clone, but also provide new "space" for a different clone. This experiment would help to discover the efficacy of Jak2 inhibitors at the molecular level of the clonal distribution.

The analysis of del20q induced changes in expression provided an interesting basis for further investigation of candidate genes. We developed an experimental set-up, which allows us to screen large numbers of target genes in BM transplantation experiments by using pools of shRNAs. In future, we plan to optimize the experimental set-up and include additional target genes from the expression analysis. In a first experiment, GFP expressing cells disappeared in the peripheral blood, probably due to a lack of transfected stem cells. For this reason, We will optimize the production of retrovirus to achieve higher titers and also use lentiviral vectors instead of retroviral, which makes it easier to transfect resting cells. Because the interferon response induced by the retroviral infection may reactivate the *Mx* promoter, we will use *SCLCre;FF1* transgenic *JAK2*-V617F mouse model for our experiments. In this case, the expression of a tamoxifen inducible *Cre* recombinase is driven by 5' endothelial enhancer of *stem cell leukemia (SCL)*. We will also perform shRNA mediated knockdown in BM from wt C57B/6

mice and *VavCre;FF1* transgenic mice. The readout for all experiments will be complete blood counts (CBC's), percentage of GFP^+ cells in peripheral blood and distribution of integrated shRNA. The latter readout is based on the assumption that the shRNA providing a proliferative advantage to the cell will expand. Positive candidates will be further analyzed by transfecting them separately. The results received in the mouse model can be further confirmed by transfecting human $CD34^+$ from *JAK2*-V617F positive patients without del20q with the respective shRNA and to monitor colony numbers in methylcellulose cultures.

While studying the clonal evolution of *TET2* mutations in MPN patients, we discovered one patient carrying a 4 bp deletion in exon 11 of *TET2*, which was present in germ line. We will sequence additional family members (parents, siblings and children) of this initial patient and compare the mutational status with blood counts of the respective family members. To prove the functional relevance of the *TET2* mutation, we will test the effect of the mutation in a functional assay in collaboration with Prof. P. Schär (DBM, Basel).

Similar to the experiments in del20q we will also follow up patients with *TET2* mutations and monitor the clonal distribution at different time points. We have found several patients carrying a small clone homozygous for the *TET2* mutation. It would be of special interest to investigate whether homozygous *TET2* mutations provide any advantage, which would allow this clone to expand. The function of *TET2* protein is still unknown and has not been shown to be implicated in human cancers. Similar to the analysis of del20q, we will also extract RNA and DNA from single colonies grown in methylcellulose and analyze them for the presence of *TET2* and *JAK2*-V617F. Colonies with the same genotype will then be pooled and analyzed on expression arrays. This obtained data should provide us information about the pathways involved in the *TET2* induced pathogenesis.

During our studies of the clonal evolution of *TET2* in MPN patients we discovered two patients with an interesting colony picture. Both patients were nearly 100% clonal in total granulocytes, but kept a substantial amount of colonies without a mutation in *JAK2* or *TET2*. This data suggests that the expansion of this "wt" clone is driven by a yet unknown mutation. We will screen additional patients to find more informative cases and use DNA from these colonies to analyze with SNP arrays. Regions of LOH and copy number alteration would then provide information about region with potential candidate genes.

It's widely accepted, that we are not dealing with a single mutation (*JAK2*-V617F) causing the disease. A complex interplay between different disease clones defines the course of the disease and may be responsible for complication and transformation into acute myeloid leukemia. The present thesis and the perspective will help to understand interaction between different clones, functional consequences of additional mutations and may facilitate the discovery of new mutations responsible for the disease phenotype of MPN.

6. REFERENCES

1. Hoffbrand AV, Pettit JE, Moss P. Essential haematology (ed 4th). Oxford ; Malden, MA: Blackwell Science; 2001.

2. Bradford G, Williams B, Rossi R, Bertoncello I. Quiescence, cycling, and turnover in the primitive hematopoietic stem cell compartment. Exp Hematol. 1997;25:445-453.

3. Zijlmans J, Visser J, Kleiverda K, Kluin P, Willemze R, Fibbe W. Modification of rhodamine staining allows identification of hematopoietic stem cells with preferential short-term or long-term bone marrow-repopulating ability. Proc Natl Acad Sci U S A. 1995;92:8901-8905.

4. Uchida N, Aguila H, Fleming W, Jerabek L, Weissman I. Rapid and sustained hematopoietic recovery in lethally irradiated mice transplanted with purified Thy-1.1lo Lin-Sca-1+ hematopoietic stem cells. Blood. 1994;83:3758-3779.

5. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature. 2000;404:193-197.

6. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell. 1997;91:661-672.

7. Kaushansky K. Lineage-specific hematopoietic growth factors. N Engl J Med. 2006;354:2034-2045.

8. Hodgson G, Bradley T. In vivo kinetic status of hematopoietic stem and progenitor cells as inferred from labeling with bromodeoxyuridine. Exp Hematol. 1984;12:683-687.

9. Passegué E, Wagers A, Giuriato S, Anderson W, Weissman I. Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. J Exp Med. 2005;202:1599-1611.

10. Krantz S. Erythropoietin. Blood. 1991;77:419-434.

11. Ratcliffe P, O'Rourke J, Maxwell P, Pugh C. Oxygen sensing, hypoxia-inducible factor-1 and the regulation of mammalian gene expression. J Exp Biol. 1998;201:1153-1162.

12. Kaushansky K. Thrombopoietin. N Engl J Med. 1998;339:746-754.

13. Kojima H, Shinagawa A, Shimizu S, et al. Role of phosphatidylinositol-3 kinase and its association with Gab1 in thrombopoietin-mediated up-regulation of platelet

function. Exp Hematol. 2001;29:616-622.

14. Qian S, Fu F, Li W, Chen Q, de Sauvage FJ. Primary role of the liver in thrombopoietin production shown by tissue- specific knockout [letter]. Blood. 1998;92:2189-2191.

15. Yang C, Li YC, Kuter DJ. The physiological response of thrombopoietin (c-Mpl ligand) to thrombocytopenia in the rat. Br J Haematol. 1999;105:478-485.

16. Sitnicka E, Lin N, Priestley GV, et al. The effect of thrombopoietin on the proliferation and differentiation of murine hematopoietic stem cells. Blood. 1996;87:4998-5005.

17. Solar GP, Kerr WG, Zeigler FC, et al. Role of c-mpl in early hematopoiesis. Blood. 1998;92:4-10.

18. Fox N, Priestley G, Papayannopoulou T, Kaushansky K. Thrombopoietin expands hematopoietic stem cells after transplantation. J Clin Invest. 2002;110:389-394.

19. Ziemiecki A, Harpur A, Wilks A. JAK protein tyrosine kinases: their role in cytokine signalling. Trends Cell Biol. 1994;4:207-212.

20. Leonard W, O'Shea J. Jaks and STATs: biological implications. Annu Rev Immunol. 1998;16:293-322.

21. Wilks AF, Harpur AG, Kurban RR, Ralph SJ, Zurcher G, Ziemiecki A. Two novel protein-tyrosine kinases, each with a second phosphotransferase-related catalytic domain, define a new class of protein kinase. Mol Cell Biol. 1991;11:2057-2065.

22. Saharinen P, Silvennoinen O. The pseudokinase domain is required for suppression of basal activity of Jak2 and Jak3 tyrosine kinases and for cytokine-inducible activation of signal transduction. J Biol Chem. 2002;277:47954-47963.

23. Higgins D, Thompson J, Gibson T. Using CLUSTAL for multiple sequence alignments. Methods Enzymol. 1996;266:383-402.

24. Kampa D, Burnside J. Computational and functional analysis of the putative SH2 domain in Janus Kinases. Biochem Biophys Res Commun. 2000;278:175-182.

25. Pellegrini S, Dusanter-Fourt I. The structure, regulation and function of the Janus kinases (JAKs) and the signal transducers and activators of transcription (STATs). Eur J Biochem. 1997;248:615-633.

26. Richter M, Duménil G, Uzé G, Fellous M, Pellegrini S. Specific contribution of Tyk2 JH regions to the binding and the expression of the interferon alpha/beta receptor component IFNAR1. J Biol Chem. 1998;273:24723-24729.

27. Haan C, Heinrich P, Behrmann I. Structural requirements of the interleukin-6 signal transducer gp130 for its interaction with Janus kinase 1: the receptor is crucial for kinase activation. Biochem J. 2002;361:105-111.

28. Baker S, Rane S, Reddy E. Hematopoietic cytokine receptor signaling. Oncogene. 2007;26:6724-6737.

29. Watowich S, Wu H, Socolovsky M, Klingmuller U, Constantinescu S, Lodish H. Cytokine receptor signal transduction and the control of hematopoietic cell development. Annu Rev Cell Dev Biol. 1996;12:91-128.

30. Vainchenker W, Dusa A, Constantinescu S. JAKs in pathology: role of Janus kinases in hematopoietic malignancies and immunodeficiencies. Semin Cell Dev Biol. 2008;19:385-393.

31. Darnell JE, Jr., Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science. 1994;264:1415-1421.

32. Darnell JE, Jr. STATs and gene regulation. Science. 1997;277:1630-1635.

33. Kisseleva T, Bhattacharya S, Braunstein J, Schindler C. Signaling through the JAK/STAT pathway, recent advances and future challenges. Gene. 2002;285:1-24.

34. Horvath C, Darnell J. The state of the STATs: recent developments in the study of signal transduction to the nucleus. Curr Opin Cell Biol. 1997;9:233-239.

35. Shuai K, Liu B. Regulation of JAK-STAT signalling in the immune system. Nat Rev Immunol. 2003;3:900-911.

36. Murray P. The JAK-STAT signaling pathway: input and output integration. J Immunol. 2007;178:2623-2629.

37. Irie-Sasaki J, Sasaki T, Matsumoto W, et al. CD45 is a JAK phosphatase and negatively regulates cytokine receptor signalling. Nature. 2001;409:349-354.

38. Myers M, Andersen J, Cheng A, et al. TYK2 and JAK2 are substrates of protein-tyrosine phosphatase 1B. J Biol Chem. 2001;276:47771-47774.

39. Simoncic P, Lee-Loy A, Barber D, Tremblay M, McGlade C. The T cell protein tyrosine phosphatase is a negative regulator of janus family kinases 1 and 3. Curr Biol. 2002;12:446-453.

40. Yoshimura A, Ohkubo T, Kiguchi T, et al. A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. Embo Journal. 1995;14:2816-2826.

41. Starr R, Willson TA, Viney EM, et al. A family of cytokine-inducible inhibitors of signalling. Nature. 1997;387:917-921.

42. Ungureanu D, Saharinen P, Junttila I, Hilton D, Silvennoinen O. Regulation of Jak2 through the ubiquitin-proteasome pathway involves phosphorylation of Jak2 on Y1007 and interaction with SOCS-1. Mol Cell Biol. 2002;22:3316-3326.

43. Liu B, Liao J, Rao X, et al. Inhibition of Stat1-mediated gene activation by PIAS1. Proc Natl Acad Sci U S A. 1998;95:10626-10631.

44. Miyazaki T, Takaoka A, Nogueira L, et al. Pyk2 is a downstream mediator of the IL-2 receptor-coupled Jak signaling pathway. Genes Dev. 1998;12:770-775.

45. Barge R, De KJ, Pouwels K, Dong F, Loewenberg B, Touw IP. Tryptophan 650 of human granulocyte colony-stimulating factor (G-CSF) receptor, implicated in the activation of JAK2, is also required for G-CSF - Mediated activation of signaling complexes of the p21ras route. Blood. 1996;87:2148-2153.

46. Schiemann W, Bartoe J, Nathanson N. Box 3-independent signaling mechanisms are involved in leukemia inhibitory factor receptor alpha- and gp130-mediated stimulation of mitogen-activated protein kinase. Evidence for participation of multiple signaling pathways which converge at Ras. J Biol Chem. 1997;272:16631-16636.

47. Mizuguchi R, Hatakeyama M. Conditional activation of Janus kinase (JAK) confers factor independence upon interleukin-3-dependent cells. Essential role of Ras in JAK-triggered mitogenesis. J Biol Chem. 1998;273:32297-32303.

48. Watanabe S, Itoh T, Arai K. Roles

of JAK kinase in human GM-CSF receptor signals. Leukemia. 1997;11 Suppl 3:76-78.

49. Dawson M, Bannister A, Göttgens B, et al. JAK2 phosphorylates histone H3Y41 and excludes HP1alpha from chromatin. Nature. 2009;461:819-822.

50. Dameshek W. Some speculations on the myeloproliferative syndromes. Blood. 1951;6:372.

51. NOWELL P, HUNGERFORD D. Chromosome studies on normal and leukemic human leukocytes. J Natl Cancer Inst. 1960;25:85-109.

52. Rowley J. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature. 1973;243:290-293.

53. Teyssier J, Bartram C, Deville J, Potron G, Pigeon F. c-abl Oncogene and chromosome 22 "bcr" juxtaposition in chronic myelogenous leukemia. N Engl J Med. 1985;312:1393-1394.

54. Tefferi A, Gilliland G. Classification of chronic myeloid disorders: from Dameshek towards a semi-molecular system. Best Pract Res Clin Haematol. 2006;19:365-385.

55. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood. 2002;100:2292-2302.

56. Tefferi A, Thiele J, Orazi A, et al. Proposals and rationale for revision of the World Health Organization diagnostic criteria for polycythemia vera, essential thrombocythemia, and primary myelofibrosis: recommendations from an ad hoc international expert panel. Blood. 2007;110:1092-1097.

57. Vardiman JW, Thiele J, Arber DA, et al. The 2008 revision of the WHO classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 2009.

58. Thiele J. Philadelphia chromosome-negative chronic myeloproliferative disease. Am J Clin Pathol. 2009;132:261-280.

59. Adamson JW, Fialkow PJ, Murphy S, Prchal JF, Steinmann L. Polycythemia vera: stem-cell and probable clonal origin of the disease. New England Journal of Medicine. 1976;295:913-916.

60. El Kassar N, Hetet G, Li Y, Briere J, Grandchamp B. Clonal analysis of haemopoietic cells in essential thrombocythaemia. Br J Haematol. 1995;90:131-137.

61. Tsukamoto N, Morita K, Maehara T, et al. Clonality in chronic myeloproliferative disorders defined by X-chromosome linked probes: demonstration of heterogeneity in lineage involvement. Br J Haematol. 1994;86:253-258.

62. Reeder T, Bailey R, Dewald G, Tefferi A. Both B and T lymphocytes may be clonally involved in myelofibrosis with myeloid metaplasia. Blood. 2003;101:1981-1983.

63. Buschle M, Janssen J, Drexler H, Lyons J, Anger B, Bartram C. Evidence for pluripotent stem cell origin of idiopathic myelofibrosis: clonal analysis of a case characterized by a N-ras gene mutation. Leukemia. 1988;2:658-660.

64. Bellanne-Chantelot C, Chaumarel I, Labopin M, et al. Genetic and clinical implications of the Val617Phe JAK2 mutation in 72 families with myeloproliferative disorders. Blood. 2006;108:346-352.

65. Li S, Kralovics R, De Libero G,

Theocharides A, Gisslinger H, Skoda RC. Clonal heterogeneity in polycythemia vera patients with JAK2 exon12 and JAK2-V617F mutations. Blood. 2008;111:3863-3866.

66. Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med. 2005;352:1779-1790.

67. Kralovics R, Guan Y, Prchal JT. Acquired uniparental disomy of chromosome 9p is a frequent stem cell defect in polycythemia vera. Experimental Hematology. 2002;30:229-236.

68. Rege-Cambrin G, Mecucci C, Tricot G, et al. A chromosomal profile of polycythemia vera. Cancer Genetics And Cytogenetics. 1987;25:233-245.

69. Diez-Martin JL, Graham DL, Petitt RM, Dewald GW. Chromosome studies in 104 patients with polycythemia vera. Mayo Clinic Proceedings. 1991;66:287-299.

70. Mertens F, Johansson B, Heim S, Kristoffersson U, Mitelman F. Karyotypic patterns in chronic myeloproliferative disorders: report on 74 cases and review of the literature. Leukemia : Official Journal of the Leukemia Society of America, Leukemia Research Fund, UK. 1991;5:214-220.

71. Reilly JT, Snowden JA, Spearing RL, et al. Cytogenetic abnormalities and their prognostic significance in idiopathic myelofibrosis: a study of 106 cases. British Journal of Haematology. 1997;98:96-102.

72. Kanfer E, Price CM, Colman SM, Barrett AJ. Erythropoietin-independent colony growth in polycythaemia vera is not restricted to progenitor cells with trisomy of chromosome 8. British Journal Of Haematology. 1992;82:773-774.

73. Westwood NB, Gruszka-Westwood AM, Pearson CE, et al. The incidences of trisomy 8, trisomy 9 and D20S108 deletion in polycythaemia vera: an analysis of blood granulocytes using interphase fluorescence in situ hybridization. British Journal of Haematology. 2000;110:839-846.

74. Najfeld V, Montella L, Scalise A, Fruchtman S. Exploring polycythaemia vera with fluorescence in situ hybridization: additional cryptic 9p is the most frequent abnormality detected. British Journal of Haematology. 2002;119:558-566.

75. Bench AJ, Nacheva EP, Champion KM, Green AR. Molecular genetics and cytogenetics of myeloproliferative disorders. Baillieres Clin Haematol. 1998;11:819-848.

76. Gangat N, Strand J, Lasho TL, et al. Cytogenetic studies at diagnosis in polycythemia vera: clinical and JAK2V617F allele burden correlates. Eur J Haematol. 2008;80:197-200.

77. Sinclair E, Forrest E, Reilly J, Watmore A, Potter A. Fluorescence in situ hybridization analysis of 25 cases of idiopathic myelofibrosis and two cases of secondary myelofibrosis: monoallelic loss of RB1, D13S319 and D13S25 loci associated with cytogenetic deletion and translocation involving 13q14. Br J Haematol. 2001;113:365-368.

78. Prchal JF, Axelrad AA. Letter: Bone-marrow responses in polycythemia vera. N Engl J Med. 1974;290:1382.

79. Lutton JD, Levere RD. Endogenous erythroid colony formation by peripheral blood mononuclear cells from patients with myelofibrosis and polycythemia vera. Acta Haematologica. 1979;62:94-99.

80. James C, Ugo V, Le Couedic JP, et

al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature. 2005;434:1144-1148.

81. Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet. 2005;365:1054-1061.

82. Levine RL, Wadleigh M, Cools J, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell. 2005;7:387-397.

83. Dusa A, Staerk J, Elliott J, et al. Substitution of pseudokinase domain residue Val-617 by large non-polar amino acids causes activation of JAK2. J Biol Chem. 2008;283:12941-12948.

84. Scott LM, Tong W, Levine RL, et al. JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. N Engl J Med. 2007;356:459-468.

85. Ding J, Komatsu H, Wakita A, et al. Familial essential thrombocythemia associated with a dominant-positive activating mutation of the c-MPL gene, which encodes for the receptor for thrombopoietin. Blood. 2004;103:4198-4200.

86. Pikman Y, Lee BH, Mercher T, et al. MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. PLoS Med. 2006;3:e270.

87. Pardanani AD, Levine RL, Lasho T, et al. MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. Blood. 2006;108:3472-3476.

88. Wernig G, Mercher T, Okabe R, Levine RL, Lee BH, Gilliland DG. Expression of Jak2V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model. Blood. 2006;107:4274-4281.

89. Lacout C, Pisani DF, Tulliez M, Gachelin FM, Vainchenker W, Villeval JL. JAK2V617F expression in murine hematopoietic cells leads to MPD mimicking human PV with secondary myelofibrosis. Blood. 2006;108:1652-1660.

90. Bumm TG, Elsea C, Corbin AS, et al. Characterization of murine JAK2V617F-positive myeloproliferative disease. Cancer Res. 2006;66:11156-11165.

91. Tiedt R, Hao-Shen H, Sobas MA, et al. Ratio of mutant JAK2-V617F to wild-type Jak2 determines the MPD phenotypes in transgenic mice. Blood. 2008;111:3931-3940.

92. Xing S, Wanting TH, Zhao W, et al. Transgenic expression of JAK2V617F causes myeloproliferative disorders in mice. Blood. 2008;111:5109-5117.

93. Shide K, Shimoda HK, Kumano T, et al. Development of ET, primary myelofibrosis and PV in mice expressing JAK2 V617F. Leukemia. 2008;22:87-95.

94. de Boer J, Williams A, Skavdis G, et al. Transgenic mice with hematopoietic and lymphoid specific expression of Cre. Eur J Immunol. 2003;33:314-325.

95. Kiladjian JJ, Elkassar N, Cassinat B, et al. Essential thrombocythemias without V617F JAK2 mutation are clonal hematopoietic stem cell disorders. Leukemia. 2006;20:1181-1183.

96. Levine RL, Belisle C, Wadleigh M, et al. X-inactivation-based clonality analysis and quantitative JAK2V617F assessment reveal a strong association between clonality and JAK2V617F in PV but not ET/MMM, and identifies a subset of JAK2V617F-neg-

ative ET and MMM patients with clonal hematopoiesis. Blood. 2006;107:4139-4141.

97. Kralovics R, Teo SS, Li S, et al. Acquisition of the V617F mutation of JAK2 is a late genetic event in a subset of patients with myeloproliferative disorders. Blood. 2006;108:1377-1380.

98. Schaub FX, Jager R, Looser R, et al. Clonal analysis of deletions on chromosome 20q and JAK2-V617F in MPD suggests that del20q acts independently and is not one of the pre-disposing mutations for JAK2-V617F. Blood. 2008.

99. Nussenzveig RH, Swierczek SI, Jelinek J, et al. Polycythemia vera is not initiated by JAK2V617F mutation. Exp Hematol. 2007;35:32-38.

100. Campbell PJ, Baxter EJ, Beer PA, et al. Mutation of JAK2 in the myeloproliferative disorders: timing, clonality studies, cytogenetic associations, and role in leukemic transformation. Blood. 2006;108:3548-3555.

101. Theocharides A, Boissinot M, Girodon F, et al. Leukemic blasts in transformed JAK2-V617F-positive myeloproliferative disorders are frequently negative for the JAK2-V617F mutation. Blood. 2007;110:375-379.

102. Jones AV, Chase A, Silver RT, et al. JAK2 haplotype is a major risk factor for the development of myeloproliferative neoplasms. Nat Genet. 2009;41:446-449.

103. Olcaydu D, Harutyunyan A, Jager R, et al. A common JAK2 haplotype confers susceptibility to myeloproliferative neoplasms. Nat Genet. 2009;41:450-454.

104. Olcaydu D, Skoda RC, Looser R, et al. The 'GGCC' haplotype of JAK2 confers susceptibility to JAK2 exon 12 mutation-positive polycythemia vera. Leukemia. 2009.

105. Kilpivaara O, Mukherjee S, Schram A, et al. A germline JAK2 SNP is associated with predisposition to the development of JAK2(V617F)-positive myeloproliferative neoplasms. Nat Genet. 2009;41:455-459.

106. Pardanani A, Lasho T, Finke C, et al. The JAK2 46/1 haplotype confers susceptibility to essential thrombocythemia regardless of JAK2V617F mutational status-clinical correlates in a study of 226 consecutive patients. Leukemia. 2009.

107. Tefferi A, Lasho T, Patnaik M, et al. JAK2 germline genetic variation affects disease susceptibility in primary myelofibrosis regardless of V617F mutational status: nullizygosity for the JAK2 46/1 haplotype is associated with inferior survival. Leukemia. 2009.

108. Delhommeau F, Dupont S, Della Valle V, et al. Mutation in TET2 in myeloid cancers. N Engl J Med. 2009;360:2289-2301.

109. Ono R, Taki T, Taketani T, Taniwaki M, Kobayashi H, Hayashi Y. LCX, leukemia-associated protein with a CXXC domain, is fused to MLL in acute myeloid leukemia with trilineage dysplasia having t(10;11)(q22;q23). Cancer Res. 2002;62:4075-4080.

110. Lorsbach RB, Moore J, Mathew S, Raimondi SC, Mukatira ST, Downing JR. TET1, a member of a novel protein family, is fused to MLL in acute myeloid leukemia containing the t(10;11)(q22;q23). Leukemia. 2003;17:637-641.

111. Tefferi A, Levine RL, Lim KH, et al. Frequent TET2 mutations in systemic mastocytosis: clinical, KITD816V and FIP1L1-PDGFRA correlates. Leukemia.

2009;23:900-904.

112. Tefferi A, Pardanani A, Lim KH, et al. TET2 mutations and their clinical correlates in polycythemia vera, essential thrombocythemia and myelofibrosis. Leukemia. 2009;23:905-911.

113. Tefferi A, Lim KH, Abdel-Wahab O, et al. Detection of mutant TET2 in myeloid malignancies other than myeloproliferative neoplasms: CMML, MDS, MDS/MPN and AML. Leukemia. 2009;23:1343-1345.

114. Langemeijer SM, Kuiper RP, Berends M, et al. Acquired mutations in TET2 are common in myelodysplastic syndromes. Nat Genet. 2009;41:838-842.

115. Demory JL, Dupriez B, Fenaux P, et al. Cytogenetic studies and their prognostic significance in agnogenic myeloid metaplasia: a report on 47 cases. Blood. 1988;72:855-859.

116. Asimakopoulos FA, White NJ, Nacheva E, Green AR. Molecular analysis of chromosome 20q deletions associated with myeloproliferative disorders and myelodysplastic syndromes. Blood. 1994;84:3086-3094.

117. Asimakopoulos FA, Gilbert JG, Aldred MA, Pearson TC, Green AR. Interstitial deletion constitutes the major mechanism for loss of heterozygosity on chromosome 20q in polycythemia vera. Blood. 1996;88:2690-2698.

118. Bench AJ, Aldred MA, Humphray SJ, et al. A detailed physical and transcriptional map of the region of chromosome 20 that is deleted in myeloproliferative disorders and refinement of the common deleted region. Genomics. 1998;49:351-362.

119. Bench AJ, Nacheva EP, Hood TL, et al. Chromosome 20 deletions in myeloid malignancies: reduction of the common deleted region, generation of a PAC/BAC contig and identification of candidate genes. UK Cancer Cytogenetics Group (UKCCG). Oncogene. 2000;19:3902-3913.

120. Douet-Guilbert N, Basinko A, Morel F, et al. Chromosome 20 deletions in myelodysplastic syndromes and Philadelphia-chromosome-negative myeloproliferative disorders: characterization by molecular cytogenetics of commonly deleted and retained regions. Ann Hematol. 2008;87:537-544.

121. Li J, Bench AJ, Vassiliou GS, Fourouclas N, Ferguson-Smith AC, Green AR. Imprinting of the human L3MBTL gene, a polycomb family member located in a region of chromosome 20 deleted in human myeloid malignancies. Proc Natl Acad Sci U S A. 2004;101:7341-7346.

122. Pardanani A, Lasho TL, Finke C, et al. Extending Jak2V617F and MplW515 mutation analysis to single hematopoietic colonies and B and T lymphocytes. Stem Cells. 2007;25:2358-2362.

123. Lasho TL, Pardanani A, McClure RF, et al. Concurrent MPL515 and JAK2V617F mutations in myelofibrosis: chronology of clonal emergence and changes in mutant allele burden over time. Br J Haematol. 2006;135:683-687.

124. Greenman C, Stephens P, Smith R, et al. Patterns of somatic mutation in human cancer genomes. Nature. 2007;446:153-158.

125. Sjoblom T, Jones S, Wood LD, et al. The consensus coding sequences of human breast and colorectal cancers. Science. 2006;314:268-274.

126. Plo I, Nakatake M, Malivert L, et al.

JAK2 stimulates homologous recombination and genetic instability: potential implication in the heterogeneity of myeloproliferative disorders. Blood. 2008;112:1402-1412.

127. Bench AJ, Li J, Huntly BJ, et al. Characterization of the imprinted polycomb gene L3MBTL, a candidate 20q tumour suppressor gene, in patients with myeloid malignancies. Br J Haematol. 2004;127:509-518.

128. Olson A, Sheth N, Lee J, Hannon G, Sachidanandam R. RNAi Codex: a portal/database for short-hairpin RNA (shRNA) gene-silencing constructs. Nucleic Acids Res. 2006;34:D153-157.

129. Dickins R, Hemann M, Zilfou J, et al. Probing tumor phenotypes using stable and regulated synthetic microRNA precursors. Nat Genet. 2005;37:1289-1295.

130. Tuduri S, Crabbé L, Conti C, et al. Topoisomerase I suppresses genomic instability by preventing interference between replication and transcription. Nat Cell Biol. 2009.

131. Zhang X, Guo A, Yu J, et al. Identification of STAT3 as a substrate of receptor protein tyrosine phosphatase T. Proc Natl Acad Sci U S A. 2007;104:4060-4064.

132. Couronne L, Lippert E, Andrieux J, et al. Analyses of TET2 mutations in post-myeloproliferative neoplasm acute myeloid leukemias. Leukemia. 2009.

133. Kosmider O, Gelsi-Boyer V, Cheok M, et al. TET2 mutation is an independent favorable prognostic factor in myelodysplastic syndromes (MDS). Blood. 2009.

134. Mullighan CG. TET2 mutations in myelodysplasia and myeloid malignancies. Nat Genet. 2009;41:766-767.

135. Mohamedali AM, Smith AE, Gaken J, et al. Novel TET2 mutations associated with UPD4q24 in myelodysplastic syndrome. J Clin Oncol. 2009;27:4002-4006.

136. Abdel-Wahab O, Mullally A, Hedvat C, et al. Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. Blood. 2009;114:144-147.

137. Jankowska AM, Szpurka H, Tiu RV, et al. Loss of heterozygosity 4q24 and TET2 mutations associated with myelodysplastic/myeloproliferative neoplasms. Blood. 2009;113:6403-6410.

138. Gregg XT, Kralovics R, Prchal JT. A polymorphism of the X-linked gene IDS increases the number of females informative for transcriptional clonality assays. American Journal of Hematology. 2000;63:184-191.

139. Liu E, Jelinek J, Pastore YD, Guan Y, Prchal JF, Prchal JT. Discrimination of polycythemias and thrombocytoses by novel, simple, accurate clonality assays and comparison with PRV-1 expression and BFU-E response to erythropoietin. Blood. 2003;101:3294-3301.

140. Saint-Martin C, Leroy G, Delhommeau F, et al. Analysis of the ten-eleven translocation 2 (TET2) gene in familial myeloproliferative neoplasms. Blood. 2009;114:1628-1632.

141. Kralovics R, Buser AS, Teo SS, et al. Comparison of molecular markers in a cohort of patients with chronic myeloproliferative disorders. Blood. 2003;102:1869-1871.

142. Liva S, Hupe P, Neuvial P, et al. CAPweb: a bioinformatics CGH array Analysis Platform. Nucleic Acids Res. 2006;34:W477-481.

143. Jensen L, Kuhn M, Stark M, et al.

STRING 8--a global view on proteins and their functional interactions in 630 organisms. Nucleic Acids Res. 2009;37:D412-416.

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Education

Nov. 2005 - Dez.2009	PhD student at Department of Biomedicine, University Hospital Basel
Aug. 2004 - Oct. 2005	Master in Molecular Biology with Major in Cell Biology at *Biozentrum,* University of Basel and Department of Biomedicine, University Hospital Basel
Oct. 2001 - Aug. 2004	Bachelor of Science in Biology with Major in Molecular Biology at *Biozentrum,* University of Basel
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Publications

Schaub FX, Jager R, Looser R, Hao-Shen H, Hermouet S, Girodon F, Tichelli A, Gisslinger H, Kralovics R, Skoda RC. *Clonal analysis of deletions on chromosome 20q and JAK2-V617F in MPD suggests that del20q acts independently and is not one of the predisposing mutations for JAK2-V617F*. Blood. 2009;113:2022-2027.

Schmitt-Graeff A, Teo S, Olschewski M, **Schaub F**, Haxelmans S, Kirn A, Reinecke P, Germing U, Skoda RC. *JAK2V617F mutation status identifies subtypes of refractory anemia with ringed sideroblasts associated with marked thrombocytosis.* Haematologica. 2008;93:34-40.

Schaub FX, Looser R, Li S, Hao-Shen H, Lehmann T, Tichelli A, and Skoda RC. *Clonal analysis of TET2 and JAK2 Mutations suggests that TET2 can be a late event in the progression of myeloproliferative neoplasms.* Blood, 2010.

7. Curriculum Vitae

Conferences

"49th Annual Meeting of the American Society of Hematology" Atlanta, USA, December 8-11, 2007 (Poster Presentation)

"**76th Annual Meeting of the Swiss Society of Hematology**" Lausanne, Switzerland, Mai 21-23, 2008 (oral presentation)

Personal information

Starting as a master student and continuing as a PhD student in the lab of Prof. Radek Skoda, I was able to acquire a broad knowledge about the different aspects of hematology, Myeloproliferative Disorders (MPD) and the general principles of cancer. The conditions in the lab of Prof. Radek Skoda made it possible to work independently on different research topics in the competitive field of MPD research. As a PhD student I was working with a wide range of different techniques including the whole spectrum of molecular biology, handling of primary cells, eukaryotic cell culture, gene regulation using shRNA, lentiviral and retroviral transduction, sequencing including next generation sequencing, microarrays and mouse experiments (bone marrow transplantation). During my time as PhD student I was also involved in teaching activities (tutorials) for biology and medical students.
I have good skills in using a variety of computer software including standard applications (Word, Excel, Access) and graphics application (Adobe Photoshop, Illustrator).
My first language is German, but I'm also fluent in English and I have good knowledge in French.

8. APPENDIX

Clonal analysis of deletions on chromosome 20q and *JAK2*-V617F in MPD suggests that del20q acts independently and is not one of the predisposing mutations for *JAK2*-V617F

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We developed a real-time copy number polymerase chain reaction assay for deletions on chromosome 20q (del20q), screened peripheral blood granulocytes from 664 patients with myeloproliferative disorders, and identified 19 patients with del20q (2.9%), of which 14 (74%) were also positive for *JAK2*-V617F. To examine the temporal relationship between the occurrence of del20q and *JAK2*-V617F, we performed colony assays in methylcellulose, picked individual burst-forming units–erythroid (BFU-E) and colony-forming units–granulocyte (CFU-G) colonies, and genotyped each colony individually for del20q and *JAK2*-V617F. In 2 of 9 patients, we found that some colonies with del20q carried only wild-type *JAK2*, whereas other del20q colonies were *JAK2*-V617F positive, indicating that del20q occurred before the acquisition of *JAK2*-V617F. However, in colonies from 3 of 9 patients, we observed the opposite order of events. The lack of a strict temporal order of occurrence makes it doubtful that del20q represents a predisposing event for *JAK2*-V617F. In 2 patients with *JAK2*-V617F and 1 patient with *MPL*-W515L, microsatellite analysis revealed that del20q affected chromosomes of different parental origin and/or 9pLOH occurred at least twice. The fact that rare somatic events, such as del20q or 9pLOH, occurred more than once in subclones from the same patients suggests that the myeloproliferative disorder clone carries a predisposition to acquiring such genetic alterations. (Blood. 2009;113: 2022-2027)

Introduction

Deletions of the long arm of chromosome 20 (del20q) have been observed frequently in patients with myeloproliferative disorders (MPDs), myelodysplastic syndromes (MDSs), and acute myeloid leukemia (AML).[1-4] A common deleted region (CDR) of 2.7 megabases (Mb) was mapped for patients with MPD, and a 2.6-Mb CDR was defined for MDS/AML with an overlap of 1.7 Mb between the 2 CDRs.[5-10] However, to date, it was not possible to identify a gene mutation within the CDR that is functionally linked to the expansion of the del20q clone.[11] In MPD patients, del20q was found in the bone marrow from approximately 1% of patients with essential thrombocythemia (ET), up to 9% of polycythemia vera (PV) and up to 12% of primary myelofibrosis (PMF).[10,12] The del20q occurred preferentially together with the *JAK2*-V617F mutation in 28 of 29 MPD patients studied.[13] We previously analyzed 2 such patients with del20q and *JAK2*-V617F and found that the size of the del20q clone by far exceeded the size of the clone carrying *JAK2*-V617F,[14] suggesting that del20q preceded the acquisition of the *JAK2*-V617F mutation. Here we expanded these studies by screening for del20q in 664 patients with MPD and examined the temporal order of the acquisition of *JAK2*-V617F and del20q in patients carrying both mutations by analyzing single colonies grown in methylcellulose.

Methods

Patients

The collection of blood samples was performed at the study centers in Basel, Switzerland, Vienna, Austria, and Dijon and Nantes, France and was approved by the local Ethics Committees (Ethik Kommission Beider Basel, the Ethik Kommission der Universität Wien und des Allgemeinen Krankenhauses der Stadt Wien-AK, and the institutional boards of Dijon and Nantes). Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki. The diagnosis of MPD was established according to the criteria of the World Health Organization.[4]

Cells and DNA analysis

Purification of granulocytes and peripheral mononuclear cells (PBMCs) and extraction of DNA were performed as described.[15,16] The granulocyte DNA was assayed using a real-time polymerase chain reaction (PCR)–based copy number assay as previously published.[16] The following primers were used to detect the copy number of 2 different exon of *L3MBTL*: 1736-GATCCCAATCAGGACCCCC, 1737-CGCCTGGCACTGACAGGT for exon 2 and 1995-CATGAAGCTGGAGGCTGTTG, 1996-GCCACGCAGACAAGGGAC for exon 9. Primers for *LOC221154* (1283-CCATGGACGACGGGTTTCT, 1284-TGTACAGGACGTAGGAGGGTGA) were used as a diploid reference. Samples that showed a decrease in copy number in both exons were validated using primers in the neighboring genes *PTPRT*

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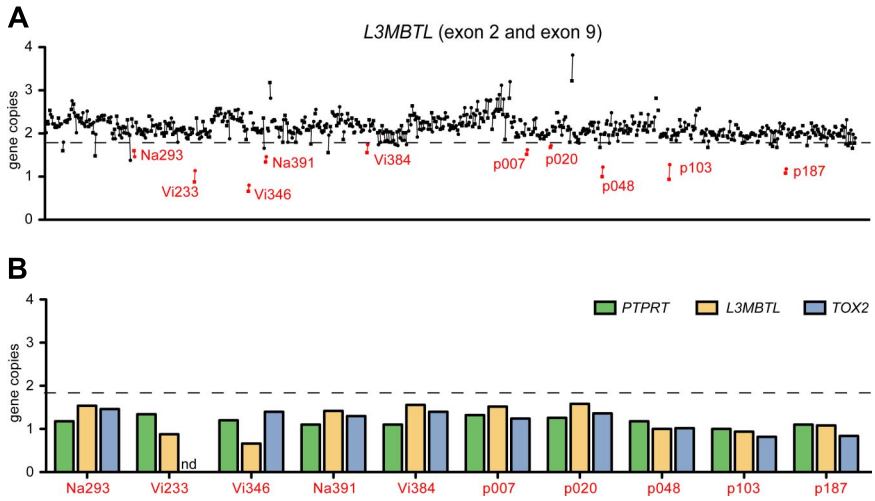

Figure 1. Screening for del20q by real-time PCR. (A) The copy number of the *L3MBTL* gene was determined in DNA from purified peripheral blood granulocytes using 2 SYBR real-time PCR assays with primer pairs located in *L3MBTL* exon 2 (quadrangles) and exon 9 (circles). Samples with gene copy values less than a threshold of 1.8 in both assays are marked in red and identified by unique patient numbers. (B) Patients with decreased *L3MBTL* gene copy number were further examined with real-time PCR assays for the neighboring genes *PTPRT* and *TOX2*. The decreased copy number was confirmed in all cases. nd indicates not determined.

(1740-TGCCCCGGAACCATGATA, 1741-GGTCCAGAGGCAGCACGT) and *TOX2* (1949-CCTGCCTACTCCTATCAGGCC, 1950-GTTGGACACCATGATGGCTG). The colony assays were performed using PBMCs from patients as published.[17] Methylcellulose-based media (no. 4431) containing erythropoietin from StemCell Technologies (Vancouver, BC) was used to grow burst-forming units–erythroid (BFU-E) and colony-forming units–granulocyte (CFU-G). To ensure that plates with an optimal density were obtained so that colonies can be picked without contamination by cells from neighboring colonies, PBMCs were plated at 3 different concentrations (50 000, 100 000, and 200 000 per mL).

Comparative genomic hybridization

The custom oligo comparative genomic hybridization (CGH) array was designed using the eArray platform from Agilent Technologies (Palo Alto, CA). The chips were processed following the instructions of the supplier. Either a pool of male whole blood DNAs (Promega, Madison, WI) or T-cell DNAs from the respective patients were used as reference DNA. The data were analyzed using Capweb software (http://bioinfo-out.curie.fr/CAPweb/).[18]

Results

Purified peripheral blood granulocyte DNAs of 664 MPD patients (320 ET, 262 PV, 82 PMF) were screened for del20q by monitoring the copy number of *L3MBTL*, a gene located in the common deleted region on chromosome 20q. Two real-time PCR assays with primers placed in exons 2 and 9 of *L3MBTL* were used in parallel (Figure 1A). Patients with decreased gene copy numbers in both assays were further examined with real-time PCR assays for *PTPRT* and *TOX2*, 2 genes flanking *L3MBTL* (Figure 1B). The del20q was confirmed in 19 of 664 (2.9%) MPD patients, or specifically in 4 of 320 (1.3%) ET, 7 of 262 (2.7%) PV, and 8 of 82 (9.8%) PMF. The *JAK2*-V617F mutation was present in 14 of 19 (74%) patients with del20q, whereas the *MPL*-W515L was present in 1 patient without *JAK2*-V617F. No mutations in *JAK2* exon 12 were found among the patients with del20q (Table 1). Cytogenetic analysis was available for 8 of the 19 patients with del20q and confirmed the results obtained by real-time PCR in all

Table 1. Characteristics of patients with del20q

UPN	Diagnosis	Sex	Age at sampling, y	Disease duration, mo	*JAK2*-V617F, % T	Complications	Treatment
p007	PV	M	76	264	92	sMF, thrombosis	Phlebotomy, IFN, hydroxyurea
p020	PMF	F	75	132	0	Thrombosis	Hydroxyurea
p048	PMF	M	77	168	95	Splenomegaly	NA
p103	PV	F	82	600	95	None	Phlebotomy, hydroxyurea, ^{32}P
p187	PMF	M	74	186	59	Splenomegaly	No treatment
Di433	PV	F	79	22	94	None	Hydroxyurea
Na293	ET	M	68	28	16	Anemia, splenomegaly	No treatment
Na391	ET	F	41	240	68	SVT and other thromboses, sMF, anemia	Hydroxyurea
Vi062	ET	F	54	257	10	Abortion (3 times)	Anagrelide
Vi102	ET	M	51	46	23	MI, melanoma	Anagrelide, hydroxyurea, IFN
Vi108	PMF	F	59	39	72	PAD, insult, splenomegaly	IFN
Vi117	PMF	M	64	43	0	Anemia, splenomegaly	IFN, lenalidomide
Vi139	PV	F	50	52	0	None	IFN, anagrelide, phlebotomy
Vi141	PV	F	79	211	11	Bleeding, TIA	Anagrelide, hydroxyurea, IFN, phlebotomy
Vi162	PMF	M	67	142	0	Anemia, splenomegaly	IFN
Vi233	PMF	M	80	53	9	Lung cancer, DVT, CAD	Hydroxyurea
Vi318	PV	F	65	30	12	PAD	Phlebotomy, hydroxyurea
Vi346	PMF	M	56	72	0	Lung cancer	IFN
Vi384	PV	M	64	197	98	None	Phlebotomy

UPN indicates unique patient number; sMF, secondary myelofibrosis; NA, not available; IFN, interferon; SVT, splanchnic vein thrombosis; TIA, transient ischemic attack; MI, myocardial infarction; PAD, peripheral artery disease; DVT, deep vein thrombosis; and CAD, coronary artery disease.

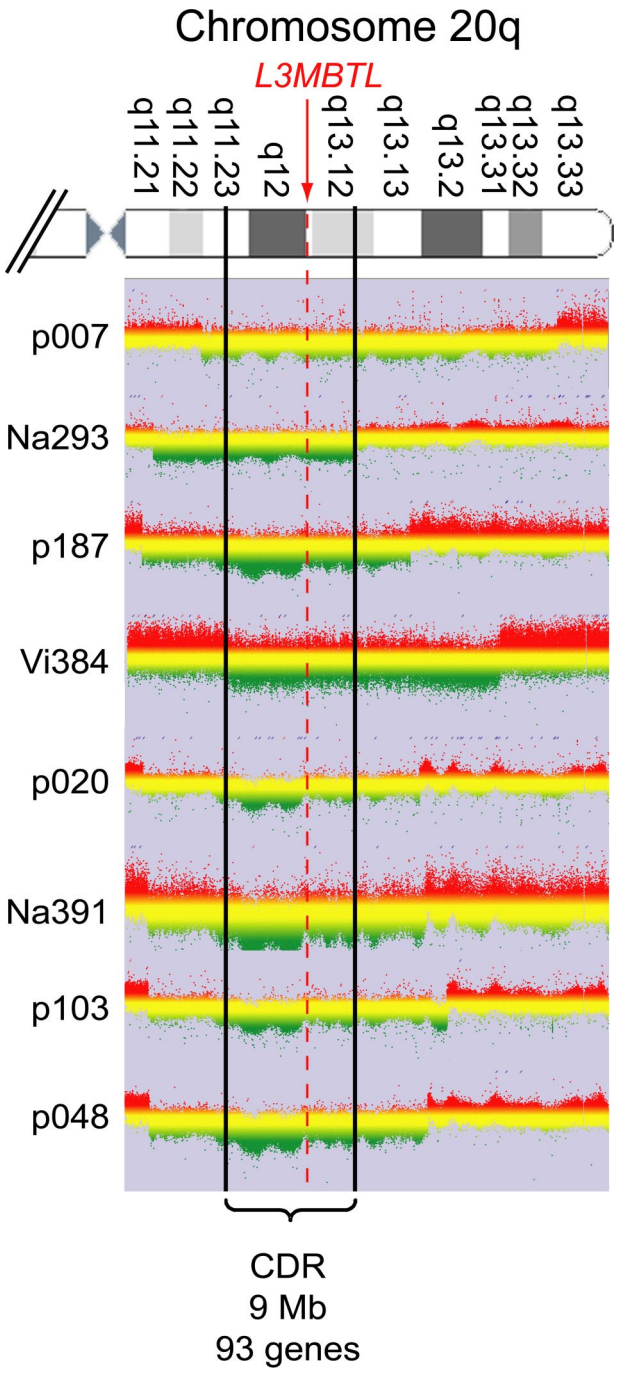

Figure 2. CGH. Granulocyte DNA from patients and a reference DNA were used to perform custom high-density oligonucleotide CGH arrays. The graphs show the log ratios for all probes located on chromosome 20q. Green represents regions deleted in the patient's DNA. The boundaries are marked by a clear drop in the ratio between the patient's DNA and the reference. Patient Vi384 delineates the centromeric and patient Na293 the telomeric border of the CDR, which spans 9 Mb and includes 93 genes.

8 cases. To map the breakpoints of the individual deletions, we performed custom high-density oligonucleotide CGH arrays on 8 del20q patients from whom DNA of sufficient quality was available. A CDR of 8.98 Mb (35.95-44.93 Mb) containing 93 genes was defined (Figure 2).

To determine the temporal relationship between the occurrence of del20q and *JAK2*-V617F, we performed colony assays in methylcellulose, picked single BFU-E and CFU-G colonies grown in the presence of erythropoietin, and genotyped each colony individually for del20q, *JAK2*-V617F, or in patient Vi346 for *MPL*-W515L (Figure 3). We observed 3 different patterns: First, in patients p187 and Na293, we found that some del20q-positive colonies carried the wild-type *JAK2*, whereas other del20q-positive colonies were positive for *JAK2*-V617F, indicating that the del20q clone is larger than the *JAK2*-V617F clone (Figure 3A). Because all colonies positive for *JAK2*-V617F also displayed del20q, we infer that del20q occurred before the *JAK2* mutation. Second, in 3 patients, we observed the reverse order, with *JAK2*-V617F preceding del20q, as illustrated by the presence of *JAK2*-V617F–positive colonies with and without del20q (Figure 3B). Patient Vi233 appears to have acquired del20q in a cell heterozygous for *JAK2*-V617F, whereas in patients p048 and p103 the transition to del20q occurred in a cell homozygous for *JAK2*-V617F. Third, in patients p007, Vi102, and Vi346, we found a more complex pattern (Figure 3C). In the first sample of p007 from February 2005, only a few CFU-G heterozygous for *JAK2*-V617F carried del20q, whereas colonies homozygous for *JAK2*-V617F already existed. Two years later, del20q was also present in a subset of colonies homozygous for *JAK2*-V617F. This pattern could be explained either by postulating 2 independent del20q events or 2 independent 9pLOH events. The del20q-positive BFU-E colonies in patient Vi102 were wild-type for *JAK2,* and the *JAK2*-V617F–positive colonies were all negative for del20q, which is compatible with originating from 2 independent clones. However, the finding of a CFU-G colony positive for both *JAK2*-V617F and del20q suggests that either del20q or JAK2-V617F occurred twice independently. Patient Vi346 was negative for *JAK2*-V617F but carried the *MPL*-W515L mutation. The del20q was only detectable in CFU-G colonies, and all of these colonies were also heterozygous for *MPL*-W515L. Some colonies were homozygous for *MPL*-W515L but negative for del20q.

In one patient (Na391) with 50% of erythroblasts in the peripheral blood, all colonies were positive for both del20q and *JAK2*-V617F, which precluded us from determining the order of events (not shown), and one patient (p020) had del20q without a mutation in the *JAK2* or *MPL* gene (Figure 3D). We analyzed p020 at 3 different time points, and we were able to detect an increase in del20q-positive colonies in CFU-Gs in 2007. However, one year later, the percentage of del20q-positive colonies was again comparable with the first time point. An increase in the percentage of del20q-positive colonies was observed in serial samples from Na293 and p007, whereas in p187 the colonies were all del20q-positive and remained so during follow-up, except for one BFU-E and one CFU-G (Figure 3A,C).

To examine the complex pattern in patients p007, Vi102, and Vi346, we performed a more detailed microsatellite analysis for regions on chromosomes 9 and 20 (Figure 4). In p007, the deletion in colony no. 185 affected the chromosome 20q of a different parental origin than in colony no. 6, indicating that 2 independent deletion events must have occurred (Figure 4A left panel). Furthermore, 4 different sizes of the latter del20q haplotype were detected when additional colonies were analyzed. This pattern could arise when a small del20q event was followed by sequential events that increased the size of the deleted region in the same cell, or alternatively, each of the del20q regions could represent a separate de novo deletion event. At present, we cannot distinguish between these possibilities. In addition, colony no. 185 also differed in the size of the 9pLOH region, suggesting the presence of 2 different 9pLOH events (Figure 4A right panel). In contrast, analysis of the deleted regions in colonies from other patients showed a unique size for each of the del20q regions (not shown), making it doubtful that the pattern observed in p007 is the result of artifacts that occurred during the methylcellulose culture. Evidence for 2 independent del20q

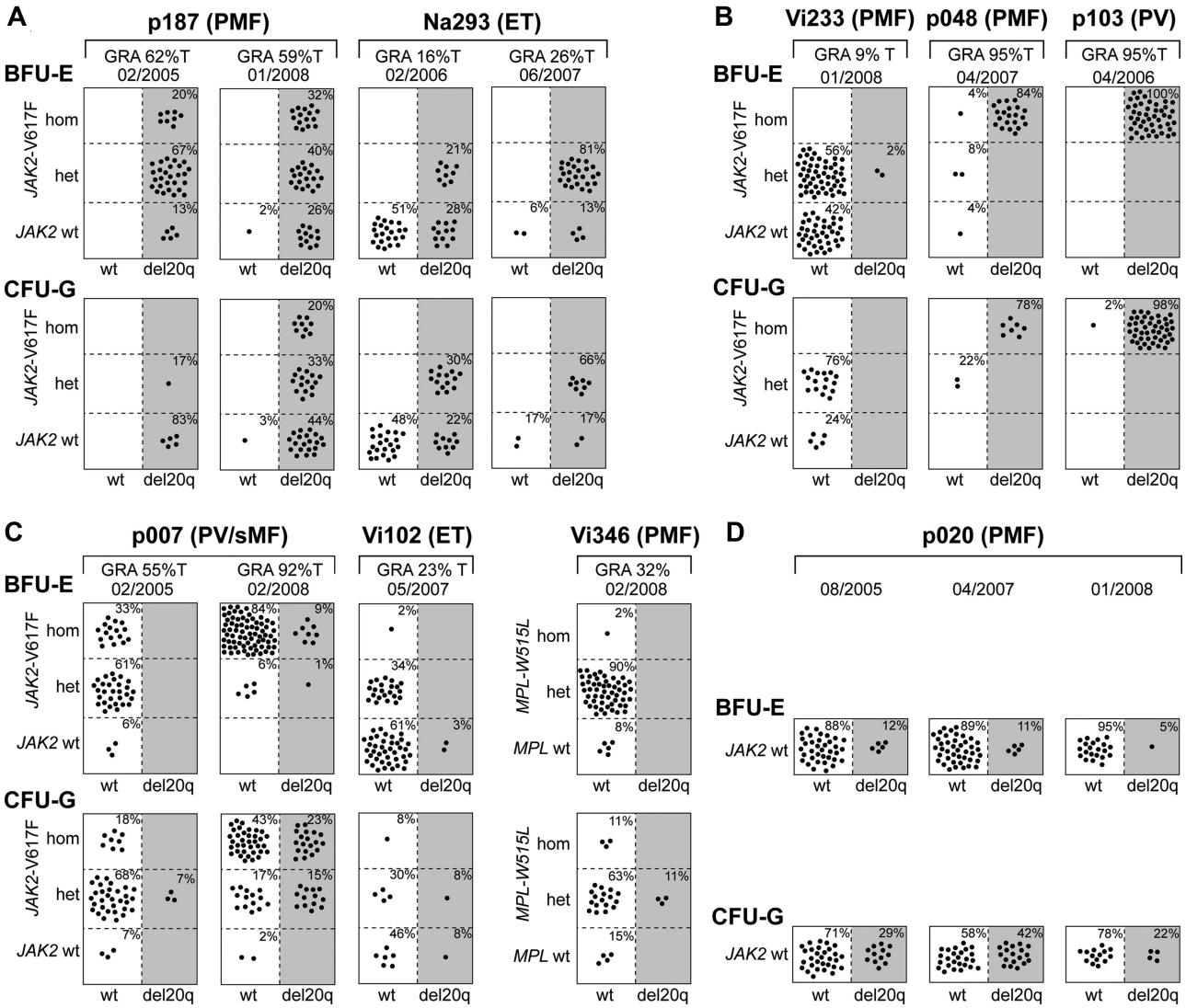

Figure 3. Single colony assays for del20q and *JAK2*-V617F. PBMCs were grown in methylcellulose in the presence of erythropoietin. Single erythroid colonies (BFU-E) and granulocytic colonies (CFU-G) were picked and analyzed individually for the presence of del20q and *JAK2*-V617F by microsatellite PCR and allele-specific PCR, respectively. Each colony is represented by a dot that is placed into one of 6 quadrangles representing the 6 possible genotypes: wild-type (wt), heterozygous (het), and homozygous (hom) for *JAK2*-V617F or *MPL*-W515L on the vertical axis, and absence (open quadrangles) or presence of del20q (gray quadrangles) on the horizontal axis. Results for BFU-Es are shown in the upper part and CFU-Gs in the lower part of the panels. The unique patient numbers, the diagnoses (PMF, ET, PV, sMF indicates secondary myelofibrosis), the allelic ratio of *JAK2*-V617F or *MPL*-W515L in purified granulocytes (%T), and the date of the sample drawing are shown above the corresponding boxes. (A) This group of patients has acquired the del20q before *JAK2*-V617F, as demonstrated by the presence of *JAK2* wt colonies carrying del20q. For both patients shown, 2 sequential samples were analyzed. (B) In this group of patients, del20q was acquired in a cell already carrying *JAK2*-V617F, as indicated by the presence of JAK2-V617F-positive colonies with or without del20q. (C) Patients with a complex pattern of del20q acquisition that does not comply with a linear temporal order of events. In patient Vi346, the del20q coexists with MPL-W515L. (D) A patient with del20q that is negative for the *JAK2*-V617F mutation.

events was also found in patients Vi102 (Figure 4B) and Vi346 (Figure 4C). In both cases, colonies were found that showed loss of heterozygosity affecting 2 different parental chromosomes 20.

Discussion

In the present study, we assessed the potential connection between *JAK2*-V617F and del20q by analyzing the temporal order of acquisition of the 2 events. Our copy number assay allowed us to screen for del20q by real-time PCR in peripheral blood from a large number of MPD patients. The *L3MBTL* gene, which we have chosen for the copy number assay, is located within the CDR shared between patients with MPD and MDS/AML.[9] This assay requires that the majority of granulocytes carry del20q and therefore selects for del20q events that dominate in granulocytes in the peripheral blood. Nevertheless, our observed frequencies in patients with ET, PV, and PMF of del20q are comparable with results obtained with cytogenetic studies of bone marrow.[10,12] Furthermore, real-time PCR and cytogenetic analysis were in agreement in all 8 patients in whom both analyses have been performed. We found no difference in the frequencies of *JAK2*-V617F in PV and PMF patients with and without del20q. Our results do not confirm a previously reported preferential association of del20q with *JAK2*-V617F-positive cases of PMF (Table 2).[13] The reason for this discrepancy is currently unclear. In ET, 4 of 4 patients with del20q were *JAK2*-V617F–positive, similar to 5 of 5 del20q-positive ET cases reported previously (Table 2).[13] Thus, by combining the studies, 9 of 9 ET patients with del20q were also *JAK2*-V617F–

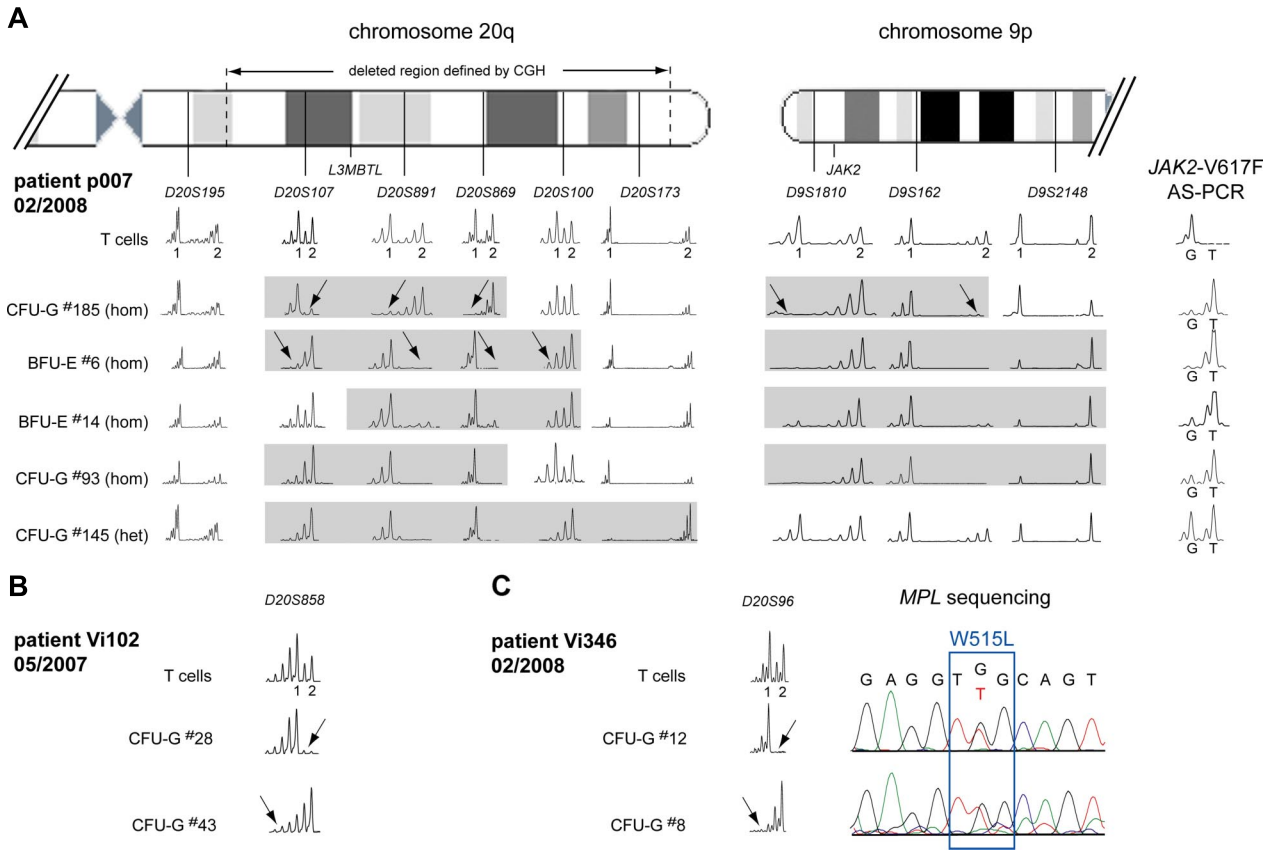

Figure 4. Multiple del20q and 9pLOH events in colonies from 3 MPD patients. (A) The deleted region on chromosome 20q was mapped in individual colonies from patient p007 using 6 microsatellites distributed along chromosome 20q. T-cell DNA was used to define the 2 alleles for each informative microsatellite. The gray boxes mark the deleted region for each individual colony. del20q in colony no. 185 created a different haplotype than the deletion in colony no. 6 (marked by arrows), indicating that 2 del20q events occurred independently and affected the chromosome 20q of different parental origin. Furthermore, 4 different sizes of the del20q haplotype were detected when additional colonies were analyzed. The results from the mapping of the 9pLOH region are shown on the right side. The 9pLOH region is smaller in colony no. 185, as shown by the heterozygosity of D9S2148 in this colony. (B) Two separate del20q events affecting the chromosome 20q of different parental origin occurred in patient Vi102. (C) Two separate del20q events affecting the chromosome 20q of different parental origin occurred in patient Vi346. The chromatograms for the MPL-W515L mutation are shown for the 2 colonies analyzed.

positive. However, this association should be examined in a larger series of patients with del20q. Mapping of the del20q region by CGH revealed that in most patients large deletions have occurred (Figure 2) and that the CDR derived from analyzing granulocytes overlaps with the published region obtained by mapping bone marrow samples.[9]

Disparity between the size of the JAK2-V617F–positive clone and the clone determined by analysis of the X-chromosome inactivation

Table 2. Relationship between del20q and *JAK2* mutations

	del20q		
	Absent (%)	**Present (%)**	**Present (%) (Campbell et al[13])**
MPD (total 664)	645	19	28
JAK2-V617F positive	449 (70)	14 (74)	27 (96)
JAK2-V617F negative	196 (30)	5 (26)	1 (4)
	$P = .805$		
ET (total 320)	316	4	5
JAK2-V617F positive	200 (63)	4 (100)	5 (100)
JAK2-V617F negative	116 (37)	0 (0)	0 (0)
	$P = .3$		
PV (total 262)	255	7	12
JAK2-V617F positive	213 (84)	6 (86)	12 (100)
JAK2 exon 12	5 (2)	0	
JAK2-V617F/ exon 12 negative	37 (14)	1 (14)	0 (0)
	$P = 1$		
PMF (total 82)	74	8	10
JAK2-V617F positive	37 (50)	4 (50)	9 (90)
JAK2-V617F negative	37 (50)	4 (50)	1 (10)
	$P = 1$		

P values represent Fisher exact test.

pattern,[14,19] presence of endogenous erythroid colonies that are negative for JAK2-V617F,[17,20,21] and coexistence of JAK2-V617F and MPL-W515L/K in the same patients,[22,23] suggested that clonal events preceding the acquisition of JAK2-V617F exist in patients with MPD. We previously found 2 patients in whom the clone carrying del20q was larger than the clone positive for *JAK2*-V617F, and we hypothesized that *JAK2*-V617F preferentially occurs on the background of clonal hematopoiesis, which in some cases may be caused by del20q.[14] Our single clone analysis confirmed the previously observed temporal order of events in 2 patients (Figure 3A), but in 3 additional patients we detected the inverse order, ie, *JAK2*-V617F preceding del20q (Figure 3B). Thus, there appears to be no strict temporal order of acquisition, making it doubtful that del20q represents a predisposing event for *JAK2*-V617F. At present, we cannot formally exclude the possibility that del20q may represent a "passenger mutation," ie, a genetic alteration without functional consequence.[24,25] The fact that in serial samples of patients Na293 and p007 we observed an expansion of the del20q subclone (Figure 3) could be interpreted in favor of a growth advantage provided by del20q and thus for a role as a "driver mutation." Similarly, in patients p048 and p103, nearly all colonies were del20q-positive, suggesting a growth advantage of the cells harboring del20q.

Unexpectedly, in 3 patients (p007, Vi102, and Vi346), we found evidence for several independent del20q events and in p007 for 2 independent 9pLOH events (Figure 4). Patient p007 was diagnosed with PV 22 years ago and for 11 years showed signs of a progression to secondary myelofibrosis. The long disease duration and/or progression to spent phase may favor accumulation of de novo mutation events. However, the disease duration in ET patient Vi102 (4 years) and PMF patient Vi346 (6 years) was shorter. Genomic instability as a consequence of JAK2-V617F was recently reported.[26] This could explain the findings in p007. However, in patient Vi102, one of the del20q events occurred in a *JAK2*-V617F–negative cell (Figure 3C) and patient Vi346 was negative for *JAK2*-V617F, suggesting that genomic instability in these patients is independent of *JAK2*.

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Authorship

Contribution: F.X.S. performed research, analyzed data, and wrote the paper; R.J., R.L., and H.H.-S. performed research; S.H., F.G., A.T., H.G., and R.K. provided essential reagents and analyzed data; and R.C.S. designed research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References

1. Rege-Cambrin G, Mecucci C, Tricot G, et al. A chromosomal profile of polycythemia vera. Cancer Genet Cytogenet. 1987;25:233-245.
2. Demory JL, Dupriez B, Fenaux P, et al. Cytogenetic studies and their prognostic significance in agnogenic myeloid metaplasia: a report on 47 cases. Blood. 1988;72:855-859.
3. Diez-Martin JL, Graham DL, Petitt RM, Dewald GW. Chromosome studies in 104 patients with polycythemia vera. Mayo Clin Proc. 1991;66:287-299.
4. Mertens F, Johansson B, Heim S, Kristoffersson U, Mitelman F. Karyotypic patterns in chronic myeloproliferative disorders: report on 74 cases and review of the literature. Leukemia. 1991;5:214-220.
5. Asimakopoulos FA, White NJ, Nacheva E, Green AR. Molecular analysis of chromosome 20q deletions associated with myeloproliferative disorders and myelodysplastic syndromes. Blood. 1994;84:3086-3094.
6. Asimakopoulos FA, Gilbert JG, Aldred MA, Pearson TC, Green AR. Interstitial deletion constitutes the major mechanism for loss of heterozygosity on chromosome 20q in polycythemia vera. Blood. 1996;88:2690-2698.
7. Bench AJ, Aldred MA, Humphray SJ, et al. A detailed physical and transcriptional map of the region of chromosome 20 that is deleted in myeloproliferative disorders and refinement of the common deleted region. Genomics. 1998;49:351-362.
8. Bench AJ, Nacheva EP, Champion KM, Green AR. Molecular genetics and cytogenetics of myeloproliferative disorders. Baillieres Clin Haematol. 1998;11:819-848.
9. Bench AJ, Nacheva EP, Hood TL, et al. Chromosome 20 deletions in myeloid malignancies: reduction of the common deleted region, generation of a PAC/BAC contig and identification of candidate genes. UK Cancer Cytogenetics Group (UKCCG). Oncogene. 2000;19:3902-3913.
10. Douet-Guilbert N, Basinko A, Morel F, et al. Chromosome 20 deletions in myelodysplastic syndromes and Philadelphia-chromosome-negative myeloproliferative disorders: characterization by molecular cytogenetics of commonly deleted and retained regions. Ann Hematol. 2008;87:537-544.
11. Li J, Bench AJ, Vassiliou GS, Fourouclas N, Ferguson-Smith AC, Green AR. Imprinting of the human L3MBTL gene, a polycomb family member located in a region of chromosome 20 deleted in human myeloid malignancies. Proc Natl Acad Sci U S A. 2004;101:7341-7346.
12. Gangat N, Strand J, Lasho TL, et al. Cytogenetic studies at diagnosis in polycythemia vera: clinical and JAK2V617F allele burden correlates. Eur J Haematol. 2008;80:197-200.
13. Campbell PJ, Baxter EJ, Beer PA, et al. Mutation of JAK2 in the myeloproliferative disorders: timing, clonality studies, cytogenetic associations, and role in leukemic transformation. Blood. 2006;108:3548-3555.
14. Kralovics R, Teo SS, Li S, et al. Acquisition of the V617F mutation of JAK2 is a late genetic event in a subset of patients with myeloproliferative disorders. Blood. 2006;108:1377-1380.
15. Kralovics R, Buser AS, Teo SS, et al. Comparison of molecular markers in a cohort of patients with chronic myeloproliferative disorders. Blood. 2003;102:1869-1871.
16. Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med. 2005;352:1779-1790.
17. Li S, Kralovics R, De Libero G, Theocharides A, Gisslinger H, Skoda RC. Clonal heterogeneity in polycythemia vera patients with JAK2 exon12 and JAK2-V617F mutations. Blood. 2008;111:3863-3866.
18. Liva S, Hupe P, Neuvial P, et al. CAPweb: a bioinformatics CGH array Analysis Platform. Nucleic Acids Res. 2006;34:W477-W481.
19. Levine RL, Belisle C, Wadleigh M, et al. X-inactivation-based clonality analysis and quantitative JAK2V617F assessment reveal a strong association between clonality and JAK2V617F in PV but not ET/MMM, and identifies a subset of JAK2V617F-negative ET and MMM patients with clonal hematopoiesis. Blood. 2006;107:4139-4141.
20. Nussenzveig RH, Swierczek SI, Jelinek J, et al. Polycythemia vera is not initiated by JAK2V617F mutation. Exp Hematol. 2007;35:32-38.
21. Pardanani A, Lasho TL, Finke C, et al. Extending Jak2V617F and MplW515 mutation analysis to single hematopoietic colonies and B and T lymphocytes. Stem Cells. 2007;25:2358-2362.
22. Pardanani AD, Levine RL, Lasho T, et al. MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. Blood. 2006;108:3472-3476.
23. Lasho TL, Pardanani A, McClure RF, et al. Concurrent MPL515 and JAK2V617F mutations in myelofibrosis: chronology of clonal emergence and changes in mutant allele burden over time. Br J Haematol. 2006;135:683-687.
24. Greenman C, Stephens P, Smith R, et al. Patterns of somatic mutation in human cancer genomes. Nature. 2007;446:153-158.
25. Sjoblom T, Jones S, Wood LD, et al. The consensus coding sequences of human breast and colorectal cancers. Science. 2006;314:268-274.
26. Plo I, Nakatake M, Malivert L, et al. JAK2 stimulates homologous recombination and genetic instability: potential implication in the heterogeneity of myeloproliferative disorders. Blood. 2008;112:1402-1412.