MEDICAL UNIVERSITY OF VIENNA

Oncogene and Tumor Suppressor Network in the Myeloproliferative Neoplasms

Doctoral thesis at the Medical University of Vienna
for obtaining the academic degree

Doctor of Philosophy

Submitted by

Mag. Thorsten Klampfl

Supervisor:
Robert Kralovics, PhD
CeMM Research Center for Molecular Medicine
of the Austrian Academy of Sciences
Lazarettgasse 14, AKH BT25.3
1090 Wien
Austria

Vienna, 05/2013

Declaration

This thesis is compiled in a cumulative format. The two manuscripts presented in this thesis were written by Thorsten Klampfl (the author of this thesis) and other coauthors. Thorsten Klampfl's detailed contribution to each of the manuscripts is outlined on the respective cover pages (pages 24 and 35). All other parts of this thesis were written solely by Thorsten Klampfl.

Figures 1 and 3 as well as Table 2 of the Introduction were taken from other publications and reprinted with permission as indicated in the respective legends**.**

Manuscript #1 was originally published in *Blood*: Klampfl T, Harutyunyan A, et al., Genome integrity of myeloproliferative neoplasms in chronic phase and during disease progression. *Blood*. 2011;118(1):167-76. © the American Society of Hematology. This manuscript is reprinted with permission.

Acknowledgements

The completion of this thesis would not have been possible without the support of numerous people. Here I want to thank the most influential ones.

Robert Kralovics – my PhD thesis supervisor. Thank you for the opportunity to work in your lab, for the delicate combination of push, direct and let-go leadership, for the possibility to follow some of my own ideas and interests, for all the discussions, the patience, the constant support. Without hesitation I am recommending Robert as a supervisor.

Giulio Superti-Furga and Heinz Gisslinger – the members of my thesis committee for all the feedback, discussion, encouragement, advice and motivation – even beyond the thesis project.

The people in the Kralovics group – for the great working atmosphere, the friendships, the scientific discussions, the personal discussions, the nonsense discussions, the helping-each-other-out, the trust, the motivation, the long pipetting nights, the feedback. In particular Ashot and Tiina, the two people who were there with me the whole time – working with you is special.

The whole community at CeMM – probably the funkiest research institute in town. I thank you for the high level of science, the flat hierarchies, the openness and support I received from so many of you, the numerous cross-labs discussions, the exchange of expertise, the whole PhD program, the retreats, parties, CeMMinars, the feedback, the helpful suggestions – the “CeMMily” style.

The collaborators outside CeMM – thank you for contributing to these projects, for feedback, input, discussions, time.

My friends – for sharing joy and pain, for excusing long working hours, for some out-of-the-box feedback and for the friendships themselves.

Mom, dad and my sister – many paths in life are influenced early on. Without your constant love and support I wouldn’t be here.

My wife and our two children – both born during the time of this PhD thesis. I thank you for all your love, support, patience, flexibility, encouragement and many more things. Coming home and having such a family waiting was one of the biggest privileges during the last years and the highlight of many days.

Jesus – for everything.

Table of contents

Abstract

Myeloproliferative neoplasms (MPN) are a group of clonal, stem cell derived disorders of hematopoiesis. The three classical *BCR-ABL1* - negative MPN are polycythemia vera, essential thrombocythemia and primary myelofibrosis. The hallmark clinical phenotype of these diseases is the overproduction of one or more fully differentiated cell types in peripheral blood. Recurrent genetic aberrations of chromosomes 1, 8, 9, 13 and 20 have been associated with the diseases. The majority of patients harbor an activating point mutation in the Janus kinase 2 (*JAK2*) gene that leads to activated JAK2 signaling. The overall genetic landscape of MPN, however, is not well understood and apart from *JAK2* on chromosome 9 no target genes of other chromosomal aberrations had been identified until the start of this PhD thesis. The projects presented here contribute to a better understanding of the genetic basis of MPN.

In the first study, we analyzed a large cohort of over 400 MPN patients for chromosomal aberrations on a genome-wide level using SNP microarray technology. We found that 37.5% of the patients had a wildtype karyotype and the remaining harbored one or more chromosomal changes including gains, losses or uniparental disomies. The number of chromosomal aberrations detected in a patient did not associate with the duration of the disease, the disease entity, or the *JAK2* mutational status. However, older patients had significantly more lesions than younger patients. The most significant association was observed between the number of genetic lesions and disease progression. Generally, MPN are chronic diseases. Some patients however develop signs of disease progression including bone marrow fibrosis and/or elevated progenitor cell levels in peripheral blood. A small fraction of patients transforms to acute myeloid leukemia (AML). We found specific aberrations of chromosomes 1, 3, 5, 6, 7, 19 and 22 to be significantly associated with the disease progression to AML. We were able to map target genes of recurrent chromosomal lesions including *FOXP1, TET2, IKZF1, CUX1, ETV6 and RUNX1* on chromosomes 3p, 4q, 7p, 7q, 12p, and 21q, respectively.

In an attempt to shed further light into the genetics of MPN, we complemented SNP microarray technology with whole-exome sequencing. In the second study presented in this thesis, we performed a detailed analysis of chromosome 11, a chromosome

frequently altered in MPN and other myeloid malignancies. We analyzed over 800 patients with different myeloid malignancies and detected frequent uniparental disomies, gains and losses of chromosome 11. Uniparental disomies were associated with mutations in the *CBL*, *MLL* and *DDB1* genes. A common deletion of chromosome 11p targeted the *LMO2* transcription factor. Another frequently deleted region on chromosome 11p was significantly associated with *de novo* AML.

The genetic basis of MPN appears to be complex. Interpretation of the data in their biological context, however, has led to the identification of common mechanisms downstream of different genetic lesions. Such a systems biology view on the data appears to be promising for a better understanding of MPN pathology and is discussed at the end of this thesis.

Zusammenfassung

Unter dem Begriff “myeloproliferative Neoplasien” (MPN) werden verschiedene Erkrankungen der Blutbildung (Hämatopoese) zusammengefasst. Die drei klassischen *BCR-ABL1* - negativen MPN sind Polycythämia vera, Essentielle Thrombozythämie und Primäre Myelofibrose. Das markanteste Kennzeichen dieser drei Erkrankungen ist die Überproduktion eines oder mehrerer ausgereifter Zelltypen im periphären Blut. Genetische Veränderungen an den Chromosomen 1, 8, 9, 13 und 20 wurden wiederholt in MPN Patienten beschrieben. Ein Großteil der Patienten trägt eine Mutation im Janus Kinase 2 Gen (*JAK2*) die zu einer Aktivierung des JAK-STAT Signaltransduktionsweges führt. Über diese Mutationen hinaus ist allerdings wenig bekannt über die genetischen Ursachen von MPN. Als mit der Arbeit zu dieser Dissertation begonnen wurde, waren abgesehen von JAK2 auf Chromosom 9 keine Zielgene der anderen chromosomalen Veränderungen bekannt. Die im Folgenden präsentierten Ergebnisse tragen zu einem besseren Verständnis der genetischen Ursachen von MPN bei.

In der ersten Studie wurden chromosomale Veränderungen in über 400 MPN Patienten mittels “single-nucleotide-polymorphism” (SNP) - Mikroarrays analysiert. Von den untersuchten Patienten hatten 37,5% einen normalen Karyotyp, der verbleibende Anteil trug eine oder mehrere genetische Veränderungen. Wir fanden Duplikationen oder Deletionen von genetischem Material sowie uniparentale Disomien. Die Anzahl der Veränderungen eines Patienten war weder abhängig von der Dauer der Krankheit, dem speziellen MPN Typ, noch davon ob der Patient Träger der JAK2 Mutation war. Allerdings war eine höhere Anzahl genetischer Veränderungen signifikant mit dem Alter der Patienten sowie mit dem Fortschreiten der Erkrankung assoziiert. Grundsätzlich sind MPN chronische Erkrankungen. Einige Patienten entwickeln jedoch eine Knochenmarksfibrose oder zeigen erhöhte Zahlen von Vorläuferzellen der Hämatopoese im peripheren Blut. Beides sind Zeichen eines Fortschritts der Erkrankung. Ein Bruchteil der Patienten entwickelt eine so genannte post-MPN akute myeloische Leukämie (AML). Wir beschrieben Veränderungen an den Chromosomen 1, 3, 5, 6, 7, 19 und 22, die signifikant mit dem Fortschreiten der Krankheit zur AML assoziiert sind. Für häufig vorkommende chromosomale

Veränderungen konnten wir folgende Zielgene identifizieren: *FOXP1* (Chromosom 3p), *TET2* (4q), *IKZF1* (7p), *CUX1* (7q), *ETV6* (12p) und *RUNX1* (21q).

In der zweiten hier präsentierten Studie haben wir Daten von SNP Mikroarrays mit “whole-exome” Sequenzierdaten kombiniert um eine detaillierte Analyse von Veränderungen auf Chromosom 11 durchzuführen. Wir analysierten Mikroarrays von über 800 Patienten mit MPN oder anderen myeloiden Erkrankungen bei denen Veränderungen am Chromosom 11 ebenfalls häufig vorkommen. Wiederum haben wir Veränderungen in Form von Deletionen, Duplikationen und uniparentalen Disomien gefunden. Die uniparentalen Disomien von Chromosom 11 waren mit Mutationen in den *CBL*, *MLL* und *DDB1* Genen assoziiert. Das Zielgen von Deletionen auf Chromosom 11p war *LMO2*. Der Verlust eines spezifischen Stücks des kurzen Armes von Chromosom 11 war signifikant mit *de novo* AML assoziiert.

Die genetischen Ursachen der MPN erscheinen komplex. Interpretiert man die zur Verfügung stehenden Daten allerdings in ihrem biologischen Kontext, so lässt sich zeigen, dass verschiedene Mutationen zu gemeinsamen Mechanismen beitragen. Ein solcher systembiologischer Ansatz erscheint vielversprechend für ein besseres Verständnis der Pathogenese von MPN und wird im Detail am Ende dieser Doktorarbeit diskutiert.

1. INTRODUCTION

1.1 Hematopoiesis

Blood is composed of two major components, the blood plasma and the corpuscular elements. The latter include erythrocytes (red blood cells), leukocytes (white blood cells) and thrombocytes (platelets). Mature blood cells are short lived therefore constant reproduction is needed. This reproduction process is termed hematopoiesis. During development hematopoiesis happens at different sites inside and outside of the embryo. Primitive hematopoiesis is first detected in the yolk sac where mainly red blood cells are produced. Later on, blood cell production takes place at the aorta-gonad-mesonephros region (AGM), the placenta, the liver, the thymus, spleen and eventually the bone marrow (Orkin & Zon, 2008). In adults hematopoiesis takes place in the red bone marrow of the vertebra bodies, the sternum, clavicles, ribs, pelvis, scapula, cranium and the proximal parts of humerus and femur.

Pluripotent hematopoietic stem cells (HSCs) are at the top of the hematopoietic hierarchy (Figure 1). They are defined by their capability to reconstitute all cells of the blood system. Following the differentiation path HSCs give rise to multipotent progenitors that are capable of producing multiple types of differentiated cells. At this level the hematopoietic tree is divided into two branches – the myeloid lineage with the common myeloid progenitor (CMP) at the hierarchical top and the lymphoid lineage with the common lymphoid progenitor (CLP) at the top (Figure 1). Further differentiation via committed precursors leads to the generation of mature erythrocytes, leukocytes (including granulocytes, monocytes and B- and T-lymphocytes) and thrombocytes.

Hematopoiesis is regulated by extrinsic factors including the bone marrow niche, cytokines and hormones (Smith, 1990). They trigger the expression of transcription factors within the hematopoietic cells that in turn orchestrate transcriptional programs for differentiation (Orkin & Zon, 2008). Different transcription factors are involved at different levels of differentiation (Figure 1). Hematopoiesis is a tightly regulated process to ensure blood homeostasis. Genetic alterations in hematopoietic stem cells can lead to the distortion of these processes and to the over- or underproduction of

terminally differentiated cells thereby resulting in the different types of myeloid and lymphoid malignancies.

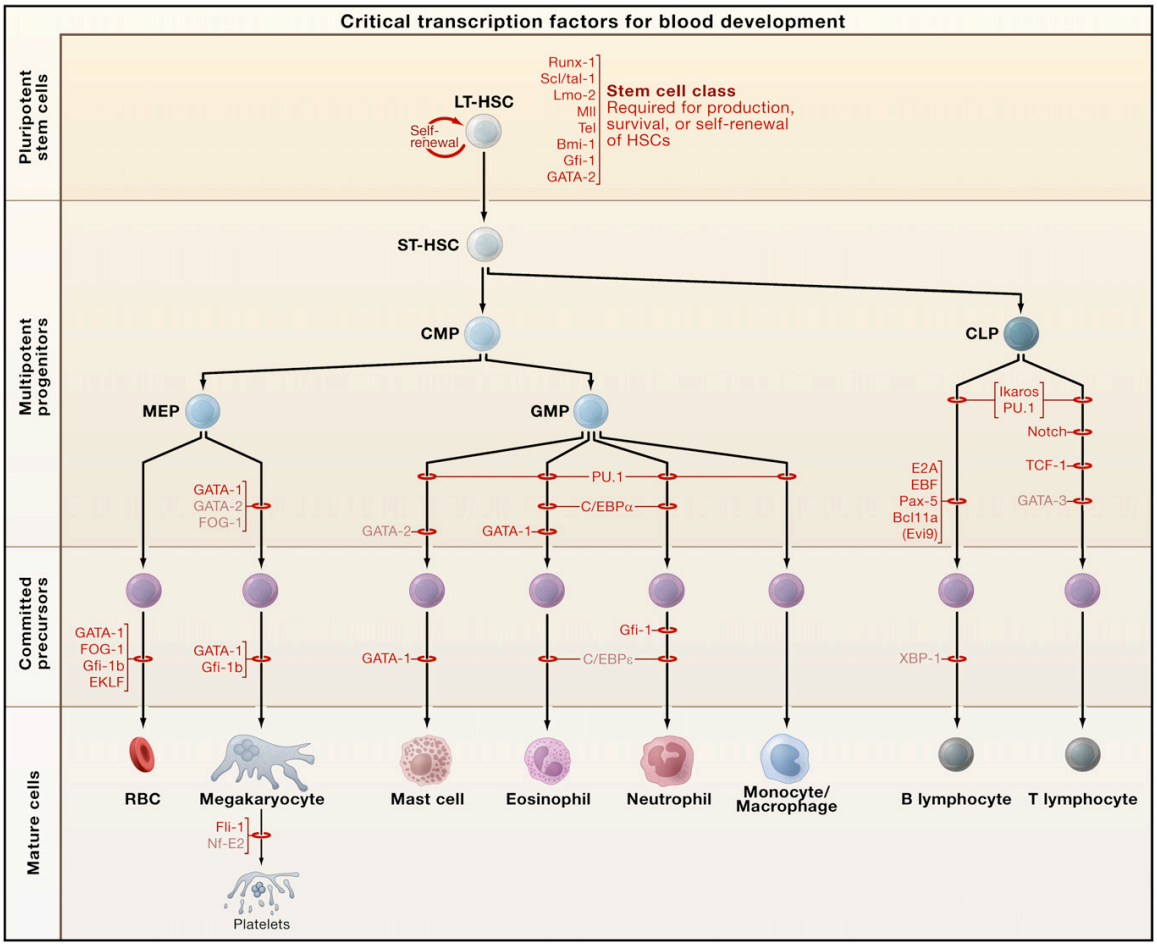


Figure 1. Requirements of Transcription Factors in Hematopoiesis

The stages at which hematopoietic development is blocked in the absence of a given transcription factor, as determined through conventional gene knockouts, are indicated by red bars. The factors depicted in black have been associated with oncogenesis. Those factors in light font have not yet been found translocated or mutated in human/mouse hematologic malignancies. Abbreviations: LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte/macrophage progenitor; RBCs, red blood cells. (Figure and Legend reprinted from Cell, 132(4), Orkin SH & Zon LI, Hematopoiesis: an evolving paradigm for stem cell biology, p631-44, Copyright 2008, with permission from Elsevier)

1.2 Myeloproliferative neoplasms – clinical aspects

In 2008 the World Health Organisation (WHO) published the latest revision of a standard of classification of hematological malignancies, the "WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues" (Sverdlow et al, 2008). Table 1 lists the five major disease categories that are introduced by the WHO for myeloid malignancies.

1. Myeloproliferative neoplasms
1.1. Chronic myelogenous leukaemia, BCR-ABL1 positive
1.2. Chronic neutrophilic leukaemia
1.3. Polycythaemia vera
1.4. Primary myelofibrosis
1.5. Essential thrombocythaemia
1.6. Chronic eosinophilic leukaemia, not otherwise specified
1.7. Mastocytosis
1.8. Myeloproliferative neoplasm, unclassifiable
2. Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of PDGFRA, PDGFRB or FGFR1
3. Myelodysplastic/myeloproliferative neoplasms
4. Myelodysplastic syndromes
5. Acute myeloid leukemia and related precursor neoplasms

Table 1: Classification of the myeloid malignancies according to the WHO

The major focus of this PhD thesis was to study the genetic basis of the three so-called "classical, *BCR-ABL1* negative" myeloproliferative neoplasms (MPNs) Polycythemia vera (PV), Essential thrombocythemia (ET) and Primary myelofibrosis (PMF) (Tefferi & Vardiman, 2008) (Table 1). The aim of this chapter is therefore to outline the clinical aspects of these three disease entities. Brief introductions are given to acute myeloid leukemia, chronic myeloid leukemia and myelodysplastic syndrome in chapter 1.2.4. Patients with the latter diseases have been analyzed in comparison to MPN in the second study presented in this thesis.

1.2.1 Polycythemia vera

The clinical hallmark of PV is an increased red blood cell count in peripheral blood (erythrocytosis) (Campbell & Green, 2006). Patients present with hypertension or vascular abnormalities associated with the elevated numbers of erythrocytes (Sverdlow et al, 2008). Constitutional symptoms include headache, dizziness, visual disturbances, parpesthesial, pruritus, or gout (Sverdlow et al, 2008). In a large cohort study on PV performed in Italy 34% of the patients had at least one thrombotic event before or at diagnosis of PV (Policitemia, 1995). The same study reported a median patient age at PV diagnosis of 60 and 62 years for men and women, respectively (Policitemia, 1995). In PV 87% of the patients show low erythropoietin levels (Mossuz et al, 2004). According to the WHO, the disease can be distinguished into three stages: A prepolycythemic phase, an overt polycytemic phase and a post-polycytemic myelofibrosis phase (post-PV MF) (Sverdlow et al, 2008). The diagnostic criteria for PV are summarized in Table 2.

Prepolycythemic phase. In the prepolycythemic phase there is no detectable increase in red blood cells or hemoglobin, however thorough examination of the patient's bone marrow allows for the diagnosis of this prodromal stage of PV (Kvasnicka & Thiele, 2010). A marked increase in platelets (thrombocythosis) in this stage of the disease might mimic essential thrombocythemia. Again, bone marrow biopsy investigation helps to distinguish early PV from ET (Thiele et al, 2005). The most prominent features in the bone marrow are found around megakaryocytes that *"exhibit not only an increase in number, but also loose clusters and many giant forms with hyperlobulated nuclei lying adjacent to medium-sized and small megakaryocytes, thus generating a pleomorphous aspect"* [SIC!] (Thiele et al, 2005). In general the morphology of megakaryocytes in the bone marrow is one of the most important aspects for differential diagnosis between MPNs especially in the early stages of the diseases (Kvasnicka & Thiele, 2010).

The overt polycythemic phase. This phase is characterized by proliferation in all myeloid lineages (panmyelosis) (Sverdlow et al, 2008). In the peripheral blood, elevated levels of red blood cells are observed (Campbell & Green, 2006). In the full blown disease stage 70% of the patients have splenomegaly and 40% hepatomegaly (Sverdlow et al, 2008). Bone marrow biopsies are usually hypercellular whereby

mainly megakaryocyte and erythroid precursor levels are elevated (Georgii et al, 1996). Erythropoiesis and granulopoiesis appears morphologically normal, while megakaryocytes show characteristic morphological abnormalities that are typical for PV (Sverdlow et al, 2008).

Post-polycytemic myelofibrosis phase (post-PV MF). A subset of PV patients progress to post-PV myelofibrosis. The 15-year risk for such a progression was estimated to 6% (Passamonti et al, 2004). In this stage of the disease, erythropoiesis decreases in the bone marrow (Thiele & Kvasnicka, 2005) as well as the erythrocyte counts in peripheral blood. In the bone marrow reticulin and collagen fibrosis is observed which is the hallmark of post-PV myelofibrosis (Thiele & Kvasnicka, 2005). Myeloid metaplasia of the spleen associated with extra medullary hematopoiesis is an associated feature (Thiele & Kvasnicka, 2005). Patients with a high grade of myelofibrosis associated with splenomegaly are often categorized as in “spent-phase” of the disease (Georgii et al, 1996), however no unique definition of “spent-phase” is available (Spivak, 2002). Between 5 to 15 % of PV patients have been reported to transform to AML as discussed by Thiele and Kvasnicka (Thiele & Kvasnicka, 2005)

1.2.2 Essential Thrombocythemia

The phenotypic hallmark of essential thrombocythemia are elevated platelet levels in peripheral blood (Campbell & Green, 2006). More than 50% of the patients are asymptomatic and the diagnosis is often made during a routine blood count. The remaining half of the patients usually present initially with thrombosis and/or hemorrhage (Sverdlow et al, 2008). In rare cases patients have mild splenomegaly (Sverdlow et al, 2008). Therefore the diagnosis has to be made carefully based on the WHO criteria listed in Table 2 (Tefferi & Vardiman, 2008). ET presents with the least severe phenotype of all the three classical *BCR-ABL1* - negative MPNs. The most obvious abnormality is thrombocytosis in the peripheral blood. Features seen in PV and PMF such as leukoerythroblastosis or poikilocytosis are not found in ET (Sverdlow et al, 2008). Granulopoiesis and erythropoiesis in the bone marrow are normal in early ET and can be slightly elevated during the course of the disease (Georgii et al, 1996). The bone marrow appears usually normocellular, sometimes

slightly hypercellular (Sverdlow et al, 2008). A clear abnormality in the bone marrow of ET patients is the marked proliferation of megakaryocytes (Georgii et al, 1996). Clustering of enlarged megakaryocytes displaying multilobulated nuclei is a specific feature of the disease (Georgii et al, 1996). Bone marrow fibrosis is virtually absent in ET (Kreft et al, 2005). In a retrospective cohort study of 891 patients with ET only 4% of the patients developed post-ET myelofibrosis and 1% of the patients transformed to AML within a follow-up time of up to 27 years from the time of diagnosis (median follow-up time was 6.2 years) (Barbui et al, 2011).

1.2.3 Primary Myelofibrosis

Fibrosis of the bone marrow is the clinical hallmark of this disease entity, albeit not the decisive diagnostic feature (Campbell & Green, 2006).

Prefibrotic stage. A prefibrotic phase of the disease often makes it difficult to identify PMF (Sverdlow et al, 2008). As with PV, bone marrow examination is a crucial tool to identify prefibrotic PMF and again the morphology of megakaryocytes is an important feature (Kvasnicka & Thiele, 2010). In general the bone marrow appears hypercellular and fibrosis is either fully absent or minimally detectable as reticulin fibrosis (Thiele et al, 1999). The characteristic appearance of megakaryocytes includes conspicuous clustering, dysplastic features and differences in size and shape (Thiele et al, 1999). According to Georgii et al, there are characteristic cloud-like nuclei of megakaryocytes found in PMF that are almost never detected in ET and PV (Georgii et al, 1996).

Fibrotic stage. In the full-blown fibrotic stage the bone marrow presents with clear reticulin or collagen fibrosis and often osteosclerosis (Thiele et al, 1999). The bone marrow is normocellular or even hypocellular. Blood vessel proliferation and intra-vascular hematopoiesis are observed (Thiele et al, 1992). Similar to post-PV myelofibrosis leukoerythroblastosis is present in the peripheral blood together with teardrop-shaped red cells (Tefferi, 2000). Patients present with fatigue, weight loss, dyspnoea, night sweats, low-grade fever and bleeding episodes (Sverdlow et al, 2008). Splenomegaly is present in up to 90% of the patients, with up to 50% having hepatomegaly (Sverdlow et al, 2008). Anemia, leukocytosis or leukocytopenia,

thrombocytosis or thrombycytopenia and elevated LDH levels are found in peripheral blood (Tefferi, 2000). The detection of thrombocytosis in the early phase of the disease can mimic ET therefore the diagnosis has to be made carefully (Sverdlow et al, 2008). Some patients present with elevated levels of blasts in bone marrow and/or peripheral blood. Patients with up to 19% blasts and an increase of CD34+ stem cells in the bone marrow are said to be in an “accelerated phase”. If more than 20% blasts are detected, patients have transformed to AML (Sverdlow et al, 2008). Barbui et al showed that from 180 patients with prefibrotic PMF, 8% developed overt bone marrow fibrosis and 5% transformed to AML within a median follow-up time of 6.2 years from diagnosis (Barbui et al, 2011). The WHO criteria for diagnosis of PMF are listed in Table 2.

	Polycythemia vera[a]	*Essential thrombocythemia*[a]	*Primary myelofibrosis*[a]
Major criteria	1 Hgb $>18.5\,g\,dl^{-1}$ (men) $>16.5\,g\,dl^{-1}$ (women) or Hgb or Hct >99th percentile of reference range for age, sex or altitude of residence or Hgb $>17\,g\,dl^{-1}$ (men), or $>15\,g\,dl^{-1}$ (women) if associated with a sustained increase of $\geqslant 2\,g\,dl^{-1}$ from baseline that cannot be attributed to correction of iron deficiency or Elevated red cell mass >25% above mean normal predicted value	1 Platelet count $\geqslant 450\times10^{9}\,l^{-1}$	1 Megakaryocyte proliferation and atypia[b] accompanied by either reticulin and/or collagen fibrosis, or In the absence of reticulin fibrosis, the megakaryocyte changes must be accompanied by increased marrow cellularity, granulocytic proliferation and often decreased erythropoiesis (i.e. pre-fibrotic PMF).
	2 Presence of *JAK2*V617F or similar mutation	2 Megakaryocyte proliferation with large and mature morphology. No or little granulocyte or erythroid Proliferation.	2 Not meeting WHO criteria for CML, PV, MDS, or other myeloid neoplasm
		3 Not meeting WHO criteria for CML, PV, PMF, MDS or other myeloid neoplasm	3 Demonstration of *JAK2*V617F or other clonal marker or no evidence of reactive marrow fibrosis
		4 Demonstration of *JAK2*V617F or other clonal marker or no evidence of reactive thrombocytosis	
Minor criteria	1 BM trilineage myeloproliferation 2 Subnormal serum Epo level 3 EEC growth		1 Leukoerythroblastosis 2 Increased serum LDH 3 Anemia 4 Palpable splenomegaly

Abbreviations: CML, chronic myelogenous leukemia; EEC, endogenous erythroid colony; Epo, erythropoietin; Hct, hematocrit; Hgb, hemoglobin; LDH, lactate dehydrogenase; MDS, myelodysplastic syndrome; WHO, World Health Organization.
[a]Diagnosis of polycythemia vera (PV) requires meeting either both major criteria and one minor criterion or the first major criterion and 2 minor criteria. Diagnosis of essential thrombocythemia requires meeting all four major criteria. Diagnosis of primary myelofibrosis (PMF) requires meeting all three major criteria and two minor criteria.
[b]Small to large megakaryocytes with an aberrant nuclear/cytoplasmic ratio and hyperchromatic and irregularly folded nuclei and dense clustering.

Table 2: The 2008 World Health Organization diagnostic criteria for polycythemia vera, essential thrombocythemia, and primary myelofibrosis*. (Reprinted by permission from Macmillan Publishers Ltd: Leukemia 22(1), p. 14-22, Tefferi A & Vardiman JW, Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms, Leukemia, copyright 2008)*

1.2.4 Other myeloid malignancies

Acute myeloid leukemia (AML). The genetic basis of AML has been well studied and a large number of aberrations have been identified. The WHO currently classifies several sub-entities of AML based on these genetic lesions (Sverdlow et al, 2008). Although there are differences between these entities, the key clinical feature for the diagnosis of AML is a blast count of > 20% in bone marrow or peripheral blood. This is associated with anemia in the majority of cases (Estey, 2011). Other blood lineages are affected at different proportions in the different subtypes of AML (Sverdlow et al, 2008).

Myelodysplastic syndrome (MDS). MDS constitute a group of diseases commonly identified by cytopenias of one or more cell types in peripheral blood and abnormal maturation of progenitor cells (dysplasia) in the bone marrow (Sverdlow et al, 2008). The bone marrow often appears hypercellular however hematopoiesis is not effective (Sverdlow et al, 2008). An increase in blast counts can be observed up to < 20%, depending on the subtype of MDS (Sverdlow et al, 2008). Evolution to AML is more frequent in MDS than in MPN but incidences differ between subtypes of the disease (Sverdlow et al, 2008).

Chronic myeloid leukemia (CML). In CML three disease phases can be distinguished: a chronic phase, an accelerated phase and a blast phase (Jabbour & Kantarjian, 2012). In 20-40% of the patients the diagnosis of CML is made during a routine blood count. These patients are asymptomatic whereas others present with fatigue, weight loss, malaise, night sweats (Jabbour & Kantarjian, 2012; Sverdlow et al, 2008). The hallmark feature in the chronic phase of the disease is marked leukocytosis (Spiers et al, 1977). The key genetic aberration is the presence of the t(9;22) translocation which creates the *BCR-ABL1* fusion gene (Rowley, 1973; Groffen et al, 1984; Shtivelman et al, 1985). Splenomegaly is present in 50-60% of the cases, hepatomegaly in 10-20% (Jabbour & Kantarjian, 2012). The bone marrow appears hypercellular with less then 5% blasts. More than 19% of blasts in PB or bone marrow or alternatively extramedullary blast proliferation require the diagnosis of CML in blast phase (Sverdlow et al, 2008). CML is the prototype example of a disease treated with targeted therapy. In a study of over 800 CML patients treated

with imatinib the estimated 3-year survival for patients with chronic-phase CML was 95%, and for patients with CML blast-phase was 18% (Cortes et al, 2006).

1.3 Myeloproliferative neoplasms – the genetic basis

1.3.1 Genetic lesions identified by classical cytogenetics

The study of chromosomal changes in myeloid malignancies dates back to the early 1960s (Hungerford & Nowell, 1962; Nowell & Hungerford, 1961; Nowell & Hungerford, 1962). In 1960 Nowell and Hungerford presented the identification of a recurrent shortage of a chromosome in patients with CML (Nowell & Hungerford, 1960). Thirteen years after the initial discovery Rowley and colleagues reported that the appearance of a shortened chromosome 22q (the so-called "Philadelphia Chromosome") is associated with a gain on chromosome 9. They thereby identified the translocation t(9,22) in CML (Rowley, 1973). This translocation leads to the creation of a fusion gene between the breakpoint cluster region gene (*BCR*) on chromosome 22 and the c-Abl oncogene 1 (*ABL1*) on chromosome 9 (Groffen et al, 1984; Shtivelman et al, 1985). The synthesis of a small molecule inhibitor against BCR-ABL1 is the hallmark example of a targeted therapy in myeloid malignancies and in cancer in general (Capdeville et al, 2002).

Detection of common lesions of chromosomes 13, 20, 1, 8 and 9 in MPN. The finding of the *BCR-ABL1* fusion gene as the hallmark genetic aberration in CML led to the formal spin-off of CML from the four classical MPN disease entities. The remaining three - PV, ET and PMF - are therefore termed "the three classical *BCR-ABL1* - negative MPNs". Early cytogenetic studies in these three diseases identified recurrent chromosomal aberrations of chromosomes 13 (Borgstrom et al, 1984) and 20 (Reeves et al, 1972). These studies, however, included only few patients and larger studies were needed to investigate frequencies and the prognostic relevance of these lesions. Studies that used classical cytogenetics or fluorescence in-situ hybridization technology (FISH) showed that trisomies of chromosomes 8 and 9 as well as duplications of chromosome 1q are other frequent genetic events in MPN (Amiel et al, 1995; Bacher et al, 2005; Reilly et al, 1997; Swolin et al, 1988; Tefferi et al, 2001).

Association of chromosomal lesions with clinical features. The overall karyotypic complexity in MPN patients was shown to depend on a number of factors. Generally, cytogenetic aberrations were found to be most frequent in PMF with 40% in one study (Bacher et al, 2005) or 48% in another (Tefferi et al, 2001) followed by PV (35%) and rare in ET (3%) (Bacher et al, 2005). However, the incidence of chromosomal aberrations is dependent on the stage of the disease. Andrieux et al. showed that patients with post-PV MF express aberrant karyotypes in 90% of the cases (Andrieux et al, 2003) a number much higher than observed in chronic phase PV (Andrieux et al, 2003; Bacher et al, 2005; Swolin et al, 1988). In particular chromosome 1q trisomies were observed in 70% of these cases (Andrieux et al, 2003). Interestingly chromosome 1q lesions were absent in 24 post-PV MF cases investigated by Tefferi and colleagues (Tefferi et al, 2001). Not only disease progression, but also therapy with hydroxyurea, 32P, or alkylating agents was shown to increase incidence of chromosomal lesions in PV (Swolin et al, 1988).

It is a matter of debate if overall karyotypic abnormality is associated with worse survival in MPN. In PMF Reilly and colleagues found a significant association of an abnormal karyotype with worse survival (Reilly et al, 1997). Tefferi et al. on the other hand, did not observe an association of a generally abnormal karyotype with worse prognosis, but associated specific chromosomal aberrations including trisomy 8 and deletion of chromosome 12p with an adverse prognosis (Tefferi et al, 2001). Other common lesions such as deletions of chromosomes 13q and 20q did not show an influence on survival in PMF. Similar associations were found in a study of post-PV MF and post-ET MF (Dingli et al, 2006).

Attempts to identify target genes of the common lesions. Alongside the identification of common chromosomal aberrations and their association with clinical parameters like disease progression and survival, researchers tried to find target genes of these genetic lesions. In an attempt to find the minimal deleted region of chromosome 20q Bench et al combined data from FISH and microsatellite PCR analyses (Bench et al, 2000). They identified a 2.7 Mb deleted region on chromosome 20q that is shared by all MPN patients analyzed. Interestingly they were able create a slightly different minimal deleted region for patients with MDS, where deletions of chromosome 20q are common as well (Bench et al, 2000). The overlap of the two common deleted regions (CDRs) contained 1.7 Mb of DNA (Bench et al,

2000). Within this region they described five genes that were expressed in CD34 positive cells as candidate target genes of chromosome 20q deletions (Bench et al, 2000). Very recently the same group described genomic imprinting of the genes in this region and the consistent loss of expression of *L3MBTL1* and *SGK2*, two genes within the CDR (Aziz et al, 2013). They were able to link the loss of expression of both genes to dysregulation of hematopoiesis, most likely by deregulation of MYC expression (Aziz et al, 2013).

Deletions of chromosome 13q were studied across several myeloid malignancies. Pastore et al. focused on *RB1* as a potential target gene of 13q deletions based on the fact that this gene is often mutated in other cancers (Pastore et al, 1995). In their analysis, they showed that the remaining *RB1* allele in patients with 13q deletions was wildtype (Pastore et al, 1995). This was in contrast to studies from other cancers, where *RB1* had been described to follow the classical two-hit inactivation model of tumor suppressor genes where both alleles get disrupted. Another study mapped the CDR of chromosome 13q to the chromosomal bands q14-q21 by cytogenetic analysis (La Starza et al, 1998). La Starza and colleagues also analyzed three patients that had translocations involving chromosome 13q using fluorescence in-situ hybridization. They detected a translocation-associated loss of a minimal chromosomal region within the afore-mentioned CDR. This small stretch of DNA still contained the *RB1* gene (La Starza et al, 1998). It is still a matter of investigation if *RB1* is the target gene of 13q deletions in myeloid malignancies.

Amplifications of chromosome 1q have been found to involve the minimum amplified region from 1q21 to 1q32 (Andrieux et al, 2003). No specific gene within this region had been investigated as a potential target.

Two main types of targets have been suggested for trisomies of chromosome 8. The *MYC* gene which is commonly amplified in various cancers lies on chromosome 8 and has been discussed as the target for hematologic malignancies as well (La Starza et al, 1998). A study on micro RNA expression in AML revealed that elevated expression of two microRNAs, miR-124a and miR-30d, both located on chromosome 8, was associated with trisomy 8 (Garzon et al, 2008).

Another commonly aberrant chromosome in MPN is chromosome 9. The detection of amplifications involving chromosome 9 was greatly improved by the use of FISH

analysis in addition to classical cytogenetics. Not only did the detection of trisomy 9 become more sensitive (Amiel et al, 1995) the technology also allowed the detection of gains affecting only short arm of chromosome 9 as reported in PV (Blij-Philipsen et al, 1997; Chen et al, 1998). Further investigations showed that chromosome 9p is the most frequently affected chromosomal region in PV (Najfeld et al, 2002). The importance of this genomic region for the pathogenesis of PV was further strengthened when Kralovics et al. described the occurrence of chromosome 9p uniparental disomies (UPDs) in one third of PV patients (Kralovics et al, 2002). A uniparental disomy arises during mitotic recombination when a region of one chromosome is replaced by a copy of the same region from the homologous chromosome (Figure 2). The group had studied loss-of-heterozygosity on a genome-wide level using microsatellite analysis. UPD was identified as a frequent chromosomal aberration in PV, mainly associated with chromosome 9p and previously undetectable by classical cytogenetics or FISH (Kralovics et al, 2002). These findings around chromosome 9p paved the way for the following hallmark discovery in MPN.

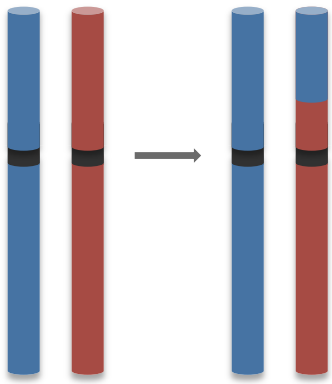


Figure 2: Uniparental disomy (UPD). *UPD is the result of a mitotic recombination event in which a region of one chromosome (the red one) gets replaced by a copy of the identical region from the homologous chromosome (the blue one). In a daughter cell both chromosomes carry this region from the same parent (therefore "uniparental" disomy). Red and blue bars represent the chromosomes, black bands the centromeres.*

1.3.2 The identification of mutations in the JAK2 pathway

JAK2-V617F. Following up on the insights from classical cytogenetics and microsatellite analysis of chromosome 9 a breakthrough discovery in 2005 pushed

the understanding of MPN pathogenesis forward. Four groups published the identification of a single point mutation in the Janus Kinase 2 (*JAK2*) gene on chromosome 9p affecting the majority of cases with PV and more than half of the patients diagnosed for ET or PMF (Baxter et al, 2005; James et al, 2005; Kralovics et al, 2005; Levine et al, 2005). *JAK2* is a member of the Janus kinase family of proteins including *JAK1*, *JAK2*, *JAK3* and Tyrosine kinase 2 (*TYK2*). The *JAK2* gene is located on the short arm of chromosome 9 at the cytoband 9p24.1 and spans almost 143 kb. The protein is composed of 1132 aminoacids. The reported mutation found in the three MPN disease entities affects cDNA position 1849 (G -> T) leading to a V617F substitution in the protein (Baxter et al, 2005; James et al, 2005; Kralovics et al, 2005; Levine et al, 2005). The JAK2-V617F mutation was detected in granulocytes (Baxter et al, 2005; James et al, 2005; Kralovics et al, 2005; Levine et al, 2005) as well as in progenitor colonies (Baxter et al, 2005) or different peripheral blood cell populations (James et al, 2005) of the myeloid lineage indicating its origin in a multipotent progenitor. The mutation was somatic in all cases. In most of the patients the mutation was found to be heterozygous however a fraction of mainly PV patients showed homozygous JAK2-V617F. All four groups showed that the homozygosity arises from mitotic recombination leading to uniparental disomy of chromosome 9p and not from a deletion event (Baxter et al, 2005; James et al, 2005; Kralovics et al, 2005; Levine et al, 2005). This data is in line with the previously described association of 9p UPDs with PV (Kralovics et al, 2002). In cell culture models of cytokine dependent Ba/F3 and FDCP cell lines JAK2-V617F induced cytokine independence and constitutive activation of STAT, ERK and AKT signaling pathways (James et al, 2005; Kralovics et al, 2005) (compare Figure 3). Several mouse models tried to assess the effect of JAK2-V617F on hematopoiesis and to functionally link the mutation to the MPN disease phenotype. Studies using bone marrow transplant models showed that JAK2-V617F induces a PV like phenotype in mice with a majority of features present in the human disease including disease progression to myelofibrosis (Bumm et al, 2006; Lacout et al, 2006; Wernig et al, 2006; Zaleskas et al, 2006). Interestingly, two of the groups observed slightly different phenotypes when comparing C57Bl/6 and BALB/c mice with increased leukocytosis, neutrophilia, splenomegaly and reticulin fibrosis in the latter (Wernig et al, 2006; Zaleskas et al, 2006). That was a first indication that germline factors, i.e. the genetic background could influence the disease phenotype upon *JAK2* mutations.

Transgenic mouse models have demonstrated that JAK2-V617F is capable of inducing PV, ET and PMF and that the gene expression level of the transgene correlated with disease phenotype (Shide et al, 2008; Tiedt et al, 2008; Xing et al, 2008). In these models high expression levels of JAK2-V617F correlated with PV and low expression levels with ET. This resembles the situation in humans where it was shown that myeloid progenitors of PV patients more frequently harbor mutant *JAK2* in homozygous state, compared to progenitors of ET patients (Scott et al, 2006). A third series of mouse models for JAK2-V617F used a targeted knock-in approach. These models largely confirmed previous findings and are comprehensively reviewed by Li et al. (Li et al, 2011a). In summary all mouse models showed that JAK2-V617F is sufficient to introduce a myeloproliferative phenotype largely resembling the human disease.

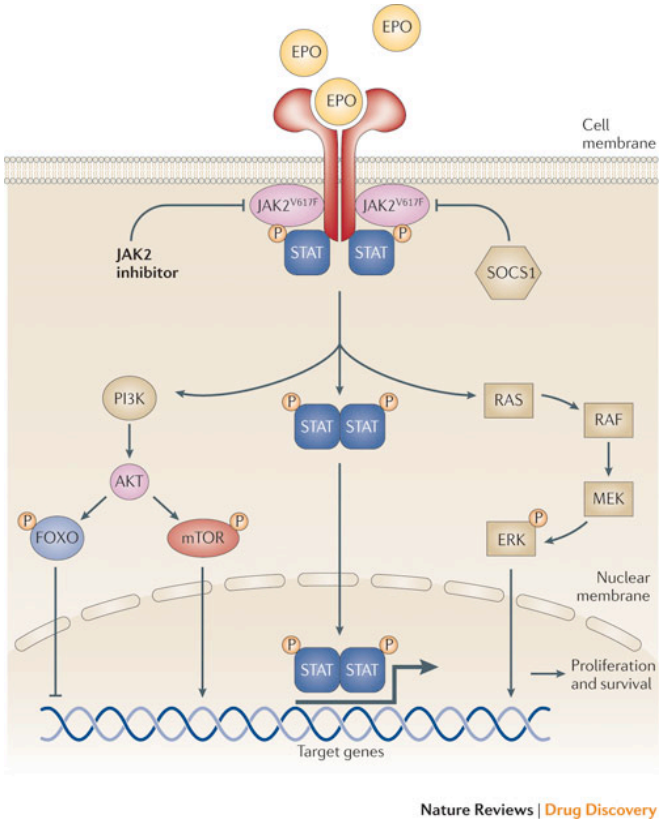


Figure 3: JAK2-V617F signaling pathways in myeloproliferative neoplasms *(Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery, 10(2), p. 127-140, Quintas-Cardama A, et al., Janus kinase inhibitors for the treatment of myeloproliferative neoplasias and beyond, copyright 2008)*

JAK2 exon 12. In 2007 different mutations affecting *JAK2* exon 12 were identified in PV patients without the JAK2-V617F mutation (Scott et al, 2007). JAK2-exon 12 mutations have not been detected in ET of PMF in this study. Comparing JAK2-V617F and JAK2-exon 12 phenotypes in cell culture models, the authors reported similar cytokine independent growth properties and stronger signaling via the ERK pathway as reflected by *ERK1* and *ERK2* phosphorylation (compare Figure 3). Patients with JAK2-exon 12 mutations exhibited higher levels of hemoglobin and lower counts of white blood cells and platelets compared to JAK2-V617F harboring patients. In a bone marrow transplant mouse model of one of the JAK2-exon 12 mutations the authors reported an erythroproliferative phenotype displaying features comparable to human patients harboring the mutation (Scott et al, 2007).

The thrombopoietin receptor. After the JAK2-V617F discovery, research groups were investigating other proteins of the JAK2 signaling pathway for potential aberrations in MPN patients. In a study focused on cytokine receptors upstream of JAK2 Pikman et al reported the finding of a recurrent mutation of the thrombopoietin receptor *(MPL)* in patients with JAK2-V617F primary myelofibrosis (Pikman et al, 2006). The MPL-W515L mutation showed constitutive JAK2 pathway activation as well as induction of a myeloproliferative phenotype in cell culture and bone marrow transplant mouse models (Pikman et al, 2006). Following up on this study Pardanani et al screened a large cohort of patients with different myeloid malignancies and detected MPL-W515L and MPL-W515K mutations in 5% of patients with PMF and 1% of patients with ET. No MPL-W515 mutations were detected in the other myeloid diseases studied (Pardanani et al, 2006). Since then, a variety of additional mutations were described in MPL including S505N, A506T, L510P, A519T, W515A (Beer et al, 2008; Chaligne et al, 2008). However, only mutations of amino acid 515 were able to introduce cytokine independent growth and activate JAK/PI3K/AKT signaling in BaF/3 cells. Only MPL-W515K or L transduced Ba/F3 cells were able to introduce subcutaneous tumors and metastasis in nude mice (Chaligne et al, 2008). MPL-W515L mutations have been shown to associate with uniparental disomies of chromosome 1p leading to homozygosity of the mutant allele (Buxhofer-Ausch et al, 2009; Szpurka et al, 2009).

1.3.3 Other genes involved in the disease pathogenesis of MPN

The development of single nucleotide polymorphism (SNP) microarrays allowed the genome wide detection of uniparental disomies that were undetectable with classical cytogenetics due to the copy-number neutral nature of this type of aberration (Figure 2).

CBL (Cbl proto-oncogene, E3 ubiquitin ligase). Dunbar et al. applied SNP microarrays to detect aberrations in different myeloid malignancies including MPN (Dunbar et al, 2008). In their study they found frequent UPDs of chromosome 11q and associated somatic mutations of the *CBL* gene – among others in one patient with PMF (Dunbar et al, 2008). *CBL* encodes a ubiquitin ligase. Among other targets it attaches ubiquitin to JAK2 or MPL which leads to their degradation (Schmidt & Dikic, 2005; Thien & Langdon, 2001). Mutations in *CBL* cluster in exons 8 and 9 within a linker-RING domain of the protein and have been mainly detected in PMF (6%) and rarely in PV or ET (Dunbar et al, 2008; Grand et al, 2009; Sanada et al, 2009). Mutant *CBL* has a dominant negative function (Sanada et al, 2009) and in a bone marrow transplant mouse model mutant *CBL* induced myeloproliferative features in recipient mice (Bandi et al, 2009).

TET2 (tet methylcytosine dioxygenase 2). SNP microarrays also allow the detection of chromosomal aberrations at much higher resolution than classical cytogenetics. This enabled Delhommeau and colleagues to identify a 325 kilobase deletion on chromosome 4 in a PV patient, using a combination of SNP microarrays and comparative-genomic-hybridization arrays (Delhommeau et al, 2009). This deletion contained only the *TET2* gene. The authors reported a variety of mutations in *TET2* in patients with MDS, MPN, secondary AML and chronic myelomonocytic leukemia with frequencies of 19%, 12%, 24% and 22%, respectively (Delhommeau et al, 2009). Abdel-Wahab and colleagues found mutations of *TET2* clearly associated with post-MPN AML (Abdel-Wahab et al, 2010). Analyzing 14 paired samples in MPN chronic-phase and post-MPN AML they found *TET2* mutations in none of the chronic phase samples, but in 43% of the post-MPN samples (Abdel-Wahab et al, 2010). Delhommeau et al reported 26 chronic-phase MPN samples out of a total of 203 MPN samples to harbor mutations in *TET2*. However no paired samples were analyzed in this study (Delhommeau et al, 2009). Further studies are needed to

clarify the proposed association of *TET2* mutations with disease progression. TET2 has been shown to convert 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) and other derivates and is discussed to have a role in DNA demethylation (He et al, 2011; Ito et al, 2011). In a *TET2* knockout mouse model bone marrow derived DNA had reduced 5-hmC levels coupled with elevated 5-mC levels and the mice developed different myeloid malignancies (Li et al, 2011b).

SH2B3 (SH2B adaptor protein 3), LNK. Following up on findings that SH2B3 alias LNK (LNK is more frequently used in MPN literature) is a negative regulator of thrombopoietin signaling (Bersenev et al, 2008; Gery et al, 2007) Oh and colleagues reported somatic mutations in *LNK* in patients with JAK2-wildtype ET or PMF associated with JAK-STAT pathway activation (Oh et al, 2010). LNK knockout mice showed an MPN-like phenotype (Velazquez et al, 2002). Comparing MPN patient samples from the chronic phase of the disease with blast samples *LNK* was shown to associate with the disease progression (Pardanani et al, 2010).

ASXL1 (additional sex combs like 1 (Drosophila). Mutations in *ASXL1* were first described in MDS (Gelsi-Boyer et al, 2009). They were also frequently found in MPN (36%) where they were primarily associated with PMF as well as post-PV MF and post-ET MF (Stein et al, 2011). *ASXL1* encodes a histone modifying enzyme and is involved in regulation of transcription for example of retinoic acid receptor and p53 (Cho et al, 2006; Kim et al, 2008). *ASXL1* knockout in the mouse was reported to interfere with hematopoiesis in myeloid and lymphoid lineages, however, the associated phenotypes were rather mild and did not reflect human myeloid malignancies (Fisher et al, 2010).

IDH1 and 2 (isocitrate dehydrogenase 1 and 2). Mutations in IDH1 and 2 are rarely reported in chronic phase MPN but have been detected in more than 20% of post-MPN AML patients (Tefferi et al, 2010). The IDH enzymes catalyze the generation of α-ketogluterate (α-KG) from isocitrate, whereby IDH1 is localized in the cytoplasm and IDH2 in the mitochondria, both performing a similar function. Mutations of *IDH1* and *2* were also observed in AML (Mardis et al, 2009) and they have been shown to exert a gain-of-function effect in generating 2-hydroxyglutarate (2-HG) from α-KG (Dang et al, 2009; Ward et al, 2010). The direct effects of 2-HG are still under discussion. A knock-in mouse model of the IDH1-R132H mutation, which is

frequently found in AML, showed features associated with myeloid malignancies including elevated numbers of progenitors in the bone marrow, splenomegaly and anemia (Sasaki et al, 2012).

DNMT3A. First described in AML (Ley et al, 2010; Yamashita et al, 2010), mutations in *DNMT3A* were reported with frequencies around 10% in MPN, depending on the disease entity (Abdel-Wahab et al, 2011; Stegelmann et al, 2011). DNMT3A is a DNA methyl transferase involved in de novo DNA methylation. DNA hypomethylation has been associated with a variety of cancers and has been causally related to tumor generation in mice (Gaudet et al, 2003). The exact role of *DNMT3A* mutations in the pathogenesis of MPN is however a matter of further investigations.

2. AIMS

The general aim of this thesis was to contribute to a better understanding of the genetic basis of myeloproliferative neoplasms. To accomplish this, specific aims were formulated:

- Using high-resolution genome-wide SNP microarrays a large cohort of patients diagnosed for MPN was to be analyzed for chromosomal aberrations including chromosomal gains, losses and uniparental disomies.
- We aimed to identify specific target genes of large-scale aberrations as this knowledge was largely missing in MPN genetics.
- We also aimed to correlate findings of the genetic analysis to clinical parameters of the disease pathogenesis.
- With the upcoming of next-generation sequencing technology we planned to complement data generated by SNP microarray analysis with whole-exome sequencing data.
- In a larger cohort including other myeloid malignancies we tried to elucidate genetic mechanisms that are either shared by or specific for different disease entities.

3. RESULTS

3.1 Manuscript #1: Genome integrity of myeloproliferative neoplasms in chronic phase and during disease progression

Thorsten Klampfl*, Ashot Harutyunyan*, et al.

** equal contribution*

published in ***Blood*** 2011 Jul 7;118(1):167-76

The author of this PhD thesis contributed to the manuscript as follows:

The author performed microarray experiments in the lab together with Ashot Harutyunyan and Tiina Berg. Thorsten Klampfl and Ashot Harutyunyan analyzed all 444 microarray data. The author of this thesis collected all clinical data from the clinical collaborators and calculated the statistics that contributed to Figures 1 and 3 of the manuscript. Also, the data summary and presentation that led to Figure 2 were done by him. Ashot Harutyunyan calculated the statistics for JAK2 association and disease progression presented in Tables 1-3. The data presented in Table 4 were generated by both first authors with contribution of other coauthors. Figure 4 was assembled by Ashot Harutyunyan. All data were regularly discussed and interpreted among the two first authors and the last author with helpful contribution from the other authors. The manuscript was written by the two first authors at equal contribution together with Robert Kralovics, the last author.

MYELOID NEOPLASIA

Genome integrity of myeloproliferative neoplasms in chronic phase and during disease progression

*Thorsten Klampfl,[1] *Ashot Harutyunyan,[1] Tiina Berg,[1] Bettina Gisslinger,[2] Martin Schalling,[1] Klaudia Bagienski,[1] Damla Olcaydu,[1] Francesco Passamonti,[3] Elisa Rumi,[4] Daniela Pietra,[4] Roland Jäger,[1] Lisa Pieri,[5] Paola Guglielmelli,[5] Ilaria Iacobucci,[6] Giovanni Martinelli,[6] Mario Cazzola,[4] Alessandro M. Vannucchi,[5] Heinz Gisslinger,[2] and Robert Kralovics[1,2]

[1]Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria; [2]Department of Internal Medicine I, Division of Hematology and Blood Coagulation, Medical University of Vienna, Vienna, Austria; [3]Division of Hematology, Azienda Ospedaliera Universitaria Fondazione Macchi, Varese, Italy; [4]Department of Hematology, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Policlinico San Matteo, University of Pavia, Pavia, Italy; [5]Section of Hematology, University of Florence, Florence, Italy; and [6]Department of Hematology and Oncological Sciences L. and A. Seragnoli, University of Bologna, Bologna, Italy

Philadelphia chromosome–negative myeloproliferative neoplasms (MPNs) are clonal myeloid disorders with increased production of terminally differentiated cells. The disease course is generally chronic, but some patients show disease progression (secondary myelofibrosis or accelerated phase) and/or leukemic transformation. We investigated chromosomal aberrations in 408 MPN samples using high-resolution single-nucleotide polymorphism microarrays to identify disease-associated somatic lesions. Of 408 samples, 37.5% had a wild-type karyotype and 62.5% harbored at least 1 chromosomal aberration. We identified 25 recurrent aberrations that were found in 3 or more samples. An increased number of chromosomal lesions was significantly associated with patient age, as well as with disease progression and leukemic transformation, but no association was observed with MPN subtypes, Janus kinase 2 (*JAK2*) mutational status, or disease duration. Aberrations of chromosomes 1q and 9p were positively associated with disease progression to secondary myelofibrosis or accelerated phase. Changes of chromosomes 1q, 7q, 5q, 6p, 7p, 19q, 22q, and 3q were positively associated with post-MPN acute myeloid leukemia. We mapped commonly affected regions to single target genes on chromosomes 3p (forkhead box P1 [*FOXP1*]), 4q (tet oncogene family member 2 [*TET2*]), 7p (IKAROS family zinc finger 1 [*IKZF1*]), 7q (cut-like homeobox 1 [*CUX1*]), 12p (ets variant 6 [*ETV6*]), and 21q (runt-related transcription factor 1 [*RUNX1*]). Our data provide insight into the genetic complexity of MPNs and implicate new genes involved in disease progression. (*Blood*. 2011;118(1):167-176)

Introduction

The 3 classic BCR-ABL–negative myeloproliferative neoplasms (MPNs) are polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF).[1] The 3 forms share several clinical characteristics, such as clonal hematopoiesis, bone marrow hypercellularity, and a propensity to thrombosis and hemorrhage. Distinct hallmarks include elevated erythrocyte counts in PV, elevated platelet levels in ET, and bone marrow fibrosis in PMF.[1] In general, MPNs are chronic diseases, but some patients exhibit clinical signs of disease progression. Progression stages include post-PV or post-ET secondary myelofibrosis (sMF) associated with an increase in reticulin fibrosis in the bone marrow and extramedullary hematopoiesis.[1] Another progression stage often referred to as the "accelerated phase" (AP) is characterized by variable degrees of pancytopenia and the presence of blasts in up to 20% in bone marrow.[2] In some of the patients, the disease transforms to post-MPN acute myeloid leukemia (AML), which is a major complication with a poor prognosis and a mean survival of around 5 months. The clinical hallmark is a blast count of $> 20\%$ in the bone marrow. The yearly risk for transformation is 0.38%, 0.37%, and 1.09% for PV, ET, and PMF, respectively.[3]

The genetic causes of MPN initiation and progression have been studied extensively in the last decades. Numerous reports have been published investigating chromosomal abnormalities using conventional cytogenetic technologies.[4-7] Abnormal cytogenetics were found to be frequent in PMF but less prevalent in PV and ET.[8] Some aberrations have been observed recurrently, most notably, deletions of chromosomes 20q, 13q, and 12p; trisomy 8 and 9; gains of 9p; and various translocations.[4-7] The karyotyping methods used usually detect large-scale genomic changes, but these studies did not succeed in the identification of target genes. Microsatellite studies on chromosome 9 identified acquired uniparental disomy (UPD) of the short arm (9pUPD) as a common defect in MPN.[9] In-depth investigation of this region resulted in the identification of the V617F mutation in the Janus kinase 2 gene (*JAK2*),[10-13] which is present in $\sim 95\%$ of PV and $> 50\%$ of ET and PMF patients. Studies on other members of the JAK2 pathway led to the identification of different mutations of the thrombopoietin receptor *MPL*, which were often associated with 1pUPDs.[14-16]

After high-resolution DNA microarrays became available, it became possible to study cytogenetic changes in great detail. Consequently, new target genes have been identified, such as the tet oncogene family member 2 (*TET2*), which is associated with deletions and UPDs of chromosome 4q,[17,18] and the Cas-Br-M (murine) ecotropic retroviral transforming sequence (*CBL*), which

Submitted January 20, 2011; accepted April 15, 2011. Prepublished online as *Blood* First Edition paper, April 29, 2011; DOI 10.1182/blood-2011-01-331678.

*T.K. and A.H. contributed equally to this study.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

is associated with aberrations of chromosome 11q.[19,20] Different mutations have a variety of roles in MPN pathogenesis; some, such as *JAK2* or *MPL* mutations, define the MPN phenotype, whereas mutations in additional sex combs like 1 (*ASXL1*)[21] and isocitrate dehydrogenase 1/2 (*IDH1/2*)[22] or deletions of IKAROS family zinc finger 1 (*IKZF1*)[23] are associated with transformation to post-MPN AML. The exact influence of mutations in *TET2* and *CBL* on the disease course is still not well understood. A few recent studies have assessed the cytogenetic profiles of MPN patients in the chronic phase and in transformation to AML; however, the number of studied samples has been rather limited.[24,25] In the present study, we present the results of a study on a large MPN cohort using high-resolution single-nucleotide polymorphism (SNP) arrays to detect chromosomal aberrations. We investigated chromosomal changes, together with clinical parameters such as post-MPN AML transformation, and describe genes so far unknown for their role in MPN pathogenesis.

Methods

Patient samples

Peripheral blood samples were collected from MPN patients after written informed consent at the Medical University of Vienna, Austria, the University of Pavia, Italy, and the University of Florence, Italy, according to the regulations of the ethics committees of each participating institution. Genomic DNA was isolated from either granulocyte or mononuclear cell fractions of these blood samples according to standard procedures.

Microarray analysis

Patient DNA was processed and hybridized to Genome-Wide Human SNP 6.0 arrays (Affymetrix) according to the manufacturer's instructions. The raw data of 444 samples were processed using Genotyping Console Version 3.0.2 software (Affymetrix). The data were first analyzed for quality, and samples showing high background signal were excluded. In addition, several mononuclear cell samples showing deletions at the TCR locus on chromosome 14 in > 50% of the cells, which is suggestive of a significant number of T cells in the sample, were excluded from further analysis. In total, we excluded 36 samples and used the remaining 408 (representing 398 patients) for further analysis. For 10 patients, we had 2 samples each from 2 different disease stages (supplemental Table 2, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Chromosomal aberrations such as deletions, gains, and UPDs were annotated after loss of heterozygosity and copy number regions were identified (as implemented in the Genotyping Console software). Our criteria for UPDs were a terminal location (at the ends of chromosomal arms) and a size of at least 1 Mb. Terminal UPDs in patients with numerous extensive (> 10Mb) interstitial runs of homozygosity were not included. In general, we cannot exclude that some chromosomal aberrations were germline, because we did not test constitutional DNA for all samples. We also did not annotate aberrations that mapped to known copy number variation loci according to the Database of Genomic Variants (DGV version 5, human reference genome assembly hg18).

The microarray raw data (CEL files) and the processed data (CNCHP files) are available at ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under the accession number E-MTAB-608.

Single-gene mutational analysis

All primer sequences and PCR conditions are provided in supplemental Table 1. *JAK2* mutational status was determined using allele-specific PCR for the JAK2-V617F mutation, as described previously.[26] Exon sequencing of runt-related transcription factor 1 (*RUNX1*), tumor protein p53 (*TP53*), *IDH1*, *IDH2,* cut-like homeobox 1 (*CUX1*), and SH2B adaptor protein 2 (*SH2B2*) was performed using the BigDye Terminator version 3.1 cycle-sequencing kit and the 3130xl Genetic Analyzer (Applied Biosystems). Sequence analysis was performed using Sequencher Version 4.9 software (Gene Codes). Nucleophosmin (*NPM1*) exon 12 was analyzed for duplications. The exon was amplified with dye-labeled primers, and PCR product length differences were detected on the Genetic Analyzer. The data were analyzed using Gene Mapper Version 4.0 software (Applied Biosystems). We also screened for fms-related tyrosine kinase 3 internal tandem duplications (FLT3-ITD) and FLT3-D835 mutations, as described previously.[27]

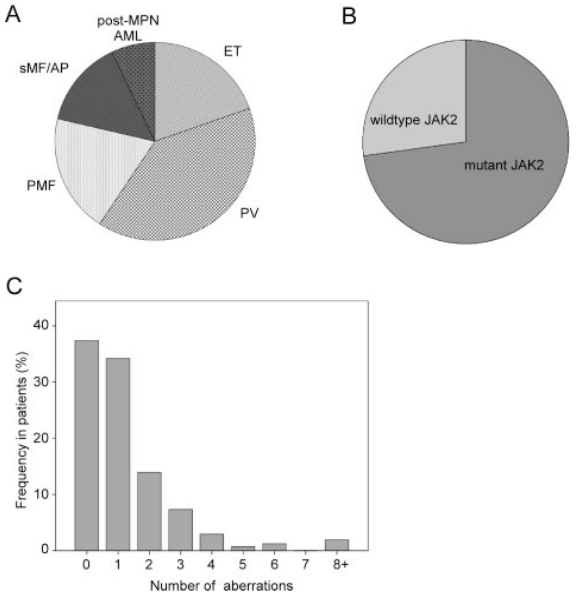


Figure 1. Cohort descriptives. (A) Diagnosis distribution. (B) Fraction of the samples with mutant or wild-type *JAK2*. (C) Distribution of chromosomal aberrations in the entire MPN cohort.

Statistical analysis

Differences in the distributions of chromosomal aberrations between different sample groups were tested statistically using the Kruskal-Wallis test (in the case of multiple comparisons) or the Mann-Whitney test (in the case of 2-sample comparisons). Differences in frequencies of individual chromosomal aberrations between chronic-phase MPN and sMF/AP or post-MPN AML, as well as between JAK2-V617F–positive and -negative samples were tested for significance using the Fisher exact test.

Results

Whole-genome analysis of MPN patients

We investigated 408 MPN samples for chromosomal aberrations using high-resolution SNP microarrays that deliver 1.8 million copy number measurements and 0.9 million SNP genotypes per genome. The patients were diagnosed as chronic-phase PV (n = 162), chronic-phase ET (n = 80), chronic-phase PMF (n = 79), or post-MPN AML (n = 29) at the time of sampling (Figure 1A). The remaining 58 patients showed clear signs of disease progression (they were not in chronic phase), but did not fulfill the clinical criteria for post-MPN AML diagnosis. Diagnoses for these patients included post-PV or post-ET (secondary) myelofibrosis, as well as the clinical stage of AP, which was defined by variable degrees of pancytopenia and blasts in bone marrow up to 20%. This group of 58 patients will be referred to as sMF/AP

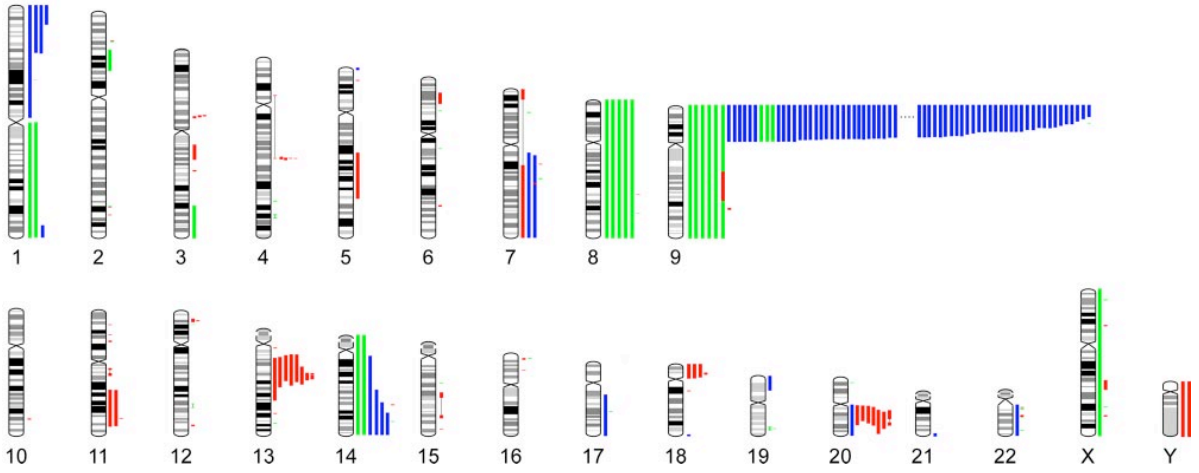


Figure 2. Karyoview of all chromosomal aberrations detected by Affymetrix SNP array analysis in 321 chronic-phase MPN patients. Bars depict the physical position and the size of the aberration; green indicates gains; red, deletions; and blue, UPDs. Thin black lines that connect 2 bars indicate multiple aberrations in the same patient.

(Figure 1A). The whole cohort included 199 male and 209 female samples. Of the total 408 samples, 297 (72.8%) harbored mutations in the *JAK2* gene (Figure 1B). The analysis for chromosomal aberrations revealed 153 samples (37.5%) with a normal karyotype, whereas 255 (62.5%) harbored at least 1 chromosomal aberration (Figure 1C). All samples with UPD on chromosome 9p were positive for the JAK2-V617F mutation, whereas 6 of 7 samples with UPD on chromosome 1p were positive for the MPL-W515L mutation. All detected chromosomal aberrations are provided in supplemental Table 2.

Correlation of genomic aberrations with clinical parameters

To investigate whether chromosomal aberrations cluster in a certain patient group defined by clinical criteria, we first focused on the patients in the chronic phase of the disease (n = 321). We detected a total of 50 gains, 76 deletions, and 147 UPDs, with sizes ranging from 0.1-146.3 Mb distributed across the genome (Figure 2). We compared the distribution of chromosomal aberrations between the 3 MPN disease entities (Figure 3A) and found that PV patients harbored significantly more chromosomal aberrations than ET ($P < .001$) or PMF ($P = .004$) patients. Because 92% of the PV patients were positive for the *JAK2* mutation and 68% of these patients harbored the associated 9pUPD, we examined whether the observed difference was mainly based on the high prevalence of 9pUPDs in PV patients. We reanalyzed the data excluding 9pUPDs and could no longer observe a significant difference in chromosomal aberrations between the disease entities (Figure 3A).

To investigate the relationship between *JAK2* mutational status and chromosomal aberrations, we compared the frequency of aberrations in patients with and without *JAK2* mutations (V617F and exon 12 mutations combined; Figure 3B). We observed a significant difference ($P < .001$), which was again lost after the exclusion of 9pUPDs from the analysis ($P = .392$). We also compared the distribution of individual recurrent chromosomal abnormalities (found in at least 3 patients) between *JAK2* mutation–positive and -negative patients. The only significant difference was the clustering of 9pUPD in *JAK2*-positive MPN patients (Table 1). This result indicates that patients positive for *JAK2* mutations do not carry more chromosomal aberrations compared with *JAK2*-negative patients. Furthermore, we did not detect an aberration specifically associated with JAK2-V617F–negative MPN.

We further examined the correlation between the cytogenetic complexity of patients and disease duration and age. The median disease duration of the chronic-phase patients was 4 years (range 0-26), and the median age at sample was 65 years (range 22-92). As shown in Figure 3C, patients without chromosomal lesions and patients with increasing numbers of lesions did not significantly differ in their disease duration ($P = .273$). Conversely, we observed a significant difference related to the patient age at sampling. Patients with normal karyotypes were younger than those with complex karyotypes ($P = .009$; Figure 3D).

As a next step, we compared the distributions of chromosomal aberrations in the 3 stages of disease progression (Figure 3E). Samples diagnosed with sMF/AP harbored significantly more chromosomal aberrations than samples in the chronic phase of the disease ($P < .001$) and significantly fewer aberrations compared with samples diagnosed with post-MPN AML ($P < .001$; Figure 3E).

Association of specific chromosomal aberrations with sMF/AP and post-MPN AML

To identify specific chromosomal lesions associated with disease progression, we compared the distribution of recurrent chromosomal abnormalities (found at least in 3 patients) between samples in chronic phase, sMF/AP, and post-MPN AML. Gains of chromosomes 1q and UPDs of 9p were significantly associated with sMF/AP compared with chronic phase (Table 2). Chromosome 1q and 3q amplifications; deletions of 7q, 5q, 6p, and 7p; and UPDs of 19q and 22q showed significant association with post-MPN AML compared with chronic phase (Table 3). We did not observe any associations of recurrent aberrations comparing sMF/AP with post-MPN AML. Common chromosomal aberrations in MPN, such as deletions of 4q (*TET2* gene deletions), 13q, and 20q, were evenly distributed among the groups.

The chromosomal defects most significantly associated with sMF/AP and post-MPN AML transformation were gains of chromosome 1q (Tables 2 and 3). We recently reported that the minimal amplified region on chromosome 1q harbors the *MDM4* gene, a potent inhibitor of p53. We also showed that p53 mutations are associated with post-MPN AML.[28] In the present study, we extended our analysis on this finding and sequenced the *TP53* gene in a total of 129 chronic-phase and the 29 post-MPN AML samples. We found

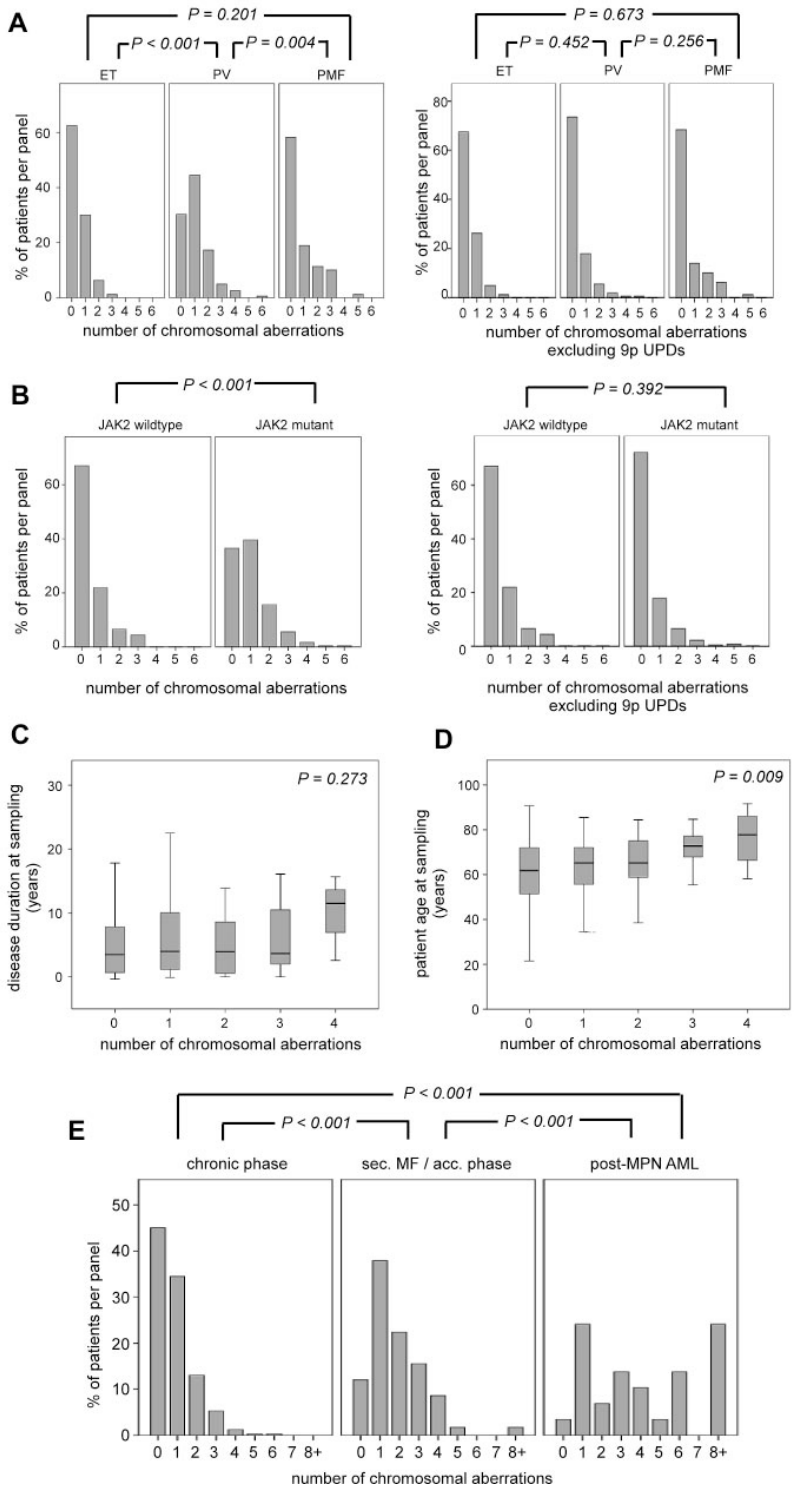


Figure 3. Correlation of chromosomal aberrations with clinical parameters. *P* values refer to differences between the indicated distribution patterns. Shown are the distributions of chromosomal aberrations in the 3 MPN entities PV, ET, and PMF (A) and in patients positive or negative for *JAK2* mutations (B). Also shown are the associations of the number of chromosomal aberrations with disease duration (C) and patient age (D); outliers are not shown. (E) Distribution of chromosomal aberrations comparing patients in the chronic phase of the disease, patients with sMF/AP, and patients who transformed to post-MPN AML.

2 samples in the chronic phase (1.6%) and 6 samples with post-MPN AML (20%) carrying mutations in *TP53*. This result confirms the reported association of *TP53* mutations with post-MPN AML ($P < .001$). The chromosome 9p UPDs, which were also associated with sMF/AP, amplified JAK2-V617F mutations.

The role of other de novo AML-specific mutations in post-MPN AML

To gain deeper insight into other pathways involved in the transformation to AML, we screened all post-MPN AML patients for point mutations common in de novo AML affecting the genes *RUNX1, FLT3, NPM1, IDH1*, and *IDH2* (Table 4). Sample 393 carried 2 nonsense

Table 1. Association of individual chromosomal aberrations with *JAK2* mutational status

Aberration type		JAK2 mutant (n = 297)	JAK2 wild-type (n = 111)	*P*	*P**
UPD	**9p**	**169 (56.90%)**	**0 (0.00%)**	**1.35×10^{-32}**	**3.38×10^{-31}**
UPD	1p	2 (0.67%)	6 (5.41%)	.0062	.1548
deletion	12p	2 (0.67%)	4 (3.60%)	.0492	1
gain	9p	6 (2.02%)	0 (0.00%)	.1959	1
trisomy	9	9 (3.03%)	1 (0.90%)	.2983	1
UPD	7q	2 (0.67%)	2 (1.80%)	.2990	1
deletion	3p	5 (1.68%)	0 (0.00%)	.3293	1
deletion	4q	11 (3.70%)	2 (1.80%)	.5278	1
deletion	11q	4 (1.35%)	0 (0.00%)	.5783	1
deletion	5q	3 (1.01%)	2 (1.80%)	.6164	1
deletion	6p	3 (1.01%)	2 (1.80%)	.6164	1
UPD	22q	3 (1.01%)	2 (1.80%)	.6164	1
deletion	12q	4 (1.35%)	2 (1.80%)	.6653	1
UPD	14q	7 (2.36%)	1 (0.90%)	.6888	1
gain	1q	11 (3.70%)	3 (2.70%)	.7668	1
deletion	13q	11 (3.70%)	3 (2.70%)	.7668	1
deletion	20q	11 (3.70%)	5 (4.50%)	.7752	1
gain	3q	3 (1.01%)	1 (0.90%)	1	1
deletion	7q	7 (2.36%)	3 (2.70%)	1	1
deletion	7p	3 (1.01%)	1 (0.90%)	1	1
trisomy	8	6 (2.02%)	2 (1.80%)	1	1
UPD	11q	2 (0.67%)	1 (0.90%)	1	1
deletion	18p	4 (1.35%)	1 (0.90%)	1	1
UPD	19q	2 (0.67%)	1 (0.90%)	1	1
trisomy	21	2 (0.67%)	1 (0.90%)	1	1

*After Bonferroni correction for multiple testing; significant associations are bold.

mutations in *RUNX1* and also tested positive for somatic trisomy 21. Sample 396 had a hemizygous single-gene deletion of *RUNX1*. Sample 373 carried a L29S missense mutation and sample 381 harbored 1 missense mutation and a 5-bp insertion. Three patients harbored mutations in *FLT3* and 1 patient carried a *NPM1* insertion. IDH1-R132G, IDH2-R140W, and IDH2-R140Q mutations were detected in samples 407, 304, and 381, respectively. All point mutations and chromosomal defects of post-MPN AML samples are summarized in Table 4.

CDRs that mapped to single genes

Common deleted regions (CDRs) were mapped as the minimum overlap of all deletions detected for a particular chromosome in our

Table 2. Association of individual chromosomal aberrations with progression to secondary myelofibrosis/accelerated phase

Aberration type		sMF/AP (n = 58)	Chronic MPN (n = 321)	*P*	*P**
gain	**1q**	**7 (12.07%)**	**2 (0.62%)**	**3.98×10^{-5}**	**.0010**
UPD	**9p**	**38 (65.52%)**	**120 (37.38%)**	**8.15×10^{-5}**	**.0020**
deletion	12q	3 (5.17%)	1 (0.31%)	.0122	.3053
UPD	14q	4 (6.90%)	4 (1.25%)	.0216	.5393
UPD	11q	2 (3.45%)	0 (0.00%)	.0231	.5770
deletion	20q	5 (8.62%)	7 (2.18%)	.0239	.5963
deletion	7q	3 (5.17%)	2 (0.62%)	.0272	.6798
deletion	4q	4 (6.90%)	5 (1.56%)	.0344	.8605
deletion	12p	2 (3.45%)	2 (0.62%)	.1125	1
deletion	7p	1 (1.72%)	0 (0.00%)	.1530	1
trisomy	21	1 (1.72%)	0 (0.00%)	.1530	1
UPD	1p	2 (3.45%)	4 (1.25%)	.2299	1
UPD	22q	1 (1.72%)	1 (0.31%)	.2830	1
trisomy	9	2 (3.45%)	5 (1.56%)	.2916	1
UPD	7q	1 (1.72%)	2 (0.62%)	.3933	1
deletion	13q	3 (5.17%)	9 (2.80%)	.4051	1
deletion	3p	1 (1.72%)	3 (0.93%)	.4869	1
gain	9p	1 (1.72%)	4 (1.25%)	.5662	1
gain	3q	0 (0.00%)	1 (0.31%)	1	1
deletion	5q	0 (0.00%)	1 (0.31%)	1	1
deletion	6p	0 (0.00%)	1 (0.31%)	1	1
trisomy	8	1 (1.72%)	5 (1.56%)	1	1
deletion	11q	0 (0.00%)	3 (0.93%)	1	1
deletion	18p	0 (0.00%)	4 (1.25%)	1	1
UPD	19q	0 (0.00%)	0 (0.00%)	1	1

*After Bonferroni correction for multiple testing; significant associations are bold.

Table 3. Association of individual chromosomal aberrations with progression to post-MPN AML

Aberration type		Post-MPN AML (n = 29)	Chronic MPN (n = 321)	*P*	*P**
gain	**1q**	**5 (17.24%)**	**2 (0.62%)**	**5.20×10^{-5}**	**.0013**
deletion	**7q**	**5 (17.24%)**	**2 (0.62%)**	**5.20×10^{-5}**	**.0013**
deletion	**5q**	**4 (13.79%)**	**1 (0.31%)**	**1.82×10^{-4}**	**.0046**
deletion	**6p**	**4 (13.79%)**	**1 (0.31%)**	**1.82×10^{-4}**	**.0046**
deletion	**7p**	**3 (10.34%)**	**0 (0.00%)**	**5.16×10^{-4}**	**.0129**
UPD	**19q**	**3 (10.34%)**	**0 (0.00%)**	**5.16×10^{-4}**	**.0129**
gain	**3q**	**3 (10.34%)**	**1 (0.31%)**	**.0019**	**.0487**
UPD	**22q**	**3 (10.34%)**	**1 (0.31%)**	**.0019**	**.0487**
deletion	4q	4 (13.79%)	5 (1.56%)	.0036	.0904
trisomy	21	2 (6.90%)	0 (0.00%)	.0066	.1662
deletion	20q	4 (13.79%)	7 (2.18%)	.0084	.2102
deletion	12q	2 (6.90%)	1 (0.31%)	.0189	.4728
trisomy	9	3 (10.34%)	5 (1.56%)	.0217	.5413
deletion	12p	2 (6.90%)	2 (0.62%)	.0359	.8970
UPD	1p	2 (6.90%)	4 (1.25%)	.0808	1
UPD	11q	1 (3.45%)	0 (0.00%)	.0829	1
trisomy	8	2 (6.90%)	5 (1.56%)	.1073	1
deletion	13q	2 (6.90%)	9 (2.80%)	.2289	1
UPD	7q	1 (3.45%)	2 (0.62%)	.2291	1
deletion	3p	1 (3.45%)	3 (0.93%)	.2936	1
deletion	11q	1 (3.45%)	3 (0.93%)	.2936	1
gain	9p	1 (3.45%)	4 (1.25%)	.3528	1
deletion	18p	1 (3.45%)	4 (1.25%)	.3528	1
UPD	9p	11 (37.93%)	120 (37.38%)	1	1
UPD	14q	0 (0.00%)	4 (1.25%)	1	1

*After Bonferroni correction for multiple testing; significant associations are bold.

cohort. We found 6 such regions that mapped to single-target genes. The CDR of chromosome 4q harbors only the known target gene *TET2* (Figure 4A), and the CDR of chromosome 7p contained only the *IKZF1* gene (Figure 4B). Mutations of both genes have been reported previously.[17,18,23] The deleted regions of chromosome 7q mapped to *CUX1* (Figure 4B). Chromosome 7p and 7q deletions were among the lesions most significantly associated with post-MPN AML. Two patients tested positive for an acquired 7qUPD in the same chromosomal region. Sequence analysis of *CUX1* in these 2 patients did not reveal any mutations. When we sequenced the enhancer of zeste homolog 2 gene (*EZH2*), a described target of 7qUPDs,[29,30] we identified mutations in both patients. A third patient harboring a 7qUPD had wild-type *EZH2* but harbored a homozygous single-gene deletion of *CUX1* within the UPD region. A close neighboring gene of *CUX1* is *SH2B2*, a homolog of the SH2B adaptor protein 3 gene (*SH2B3 LNK*). Aberrations in *SH2B3* have been associated with myeloid malignancies.[31,32] Because chromosome 7q aberrations were associated with post-MPN AML, we sequenced *SH2B2* in the 29 leukemic patients of our cohort, and identified a single somatic missense mutation (G113C) in 1 patient.

The CDR on chromosome 3p (71.2-71.3 Mb) mapped to the forkhead box P1 (*FOXP1*) gene (Figure 4C). We found 6 patients with overlapping deletions on chromosome 12p. The smallest of these deletions mapped to the ets variant 6 gene (*ETV6*; Figure 4D). Three patients harbored chromosome 21 trisomies. In another patient with post-MPN AML, a deletion containing only the *RUNX1* gene on chromosome 21q was detected (Figure 4E).

Discussion

Classic metaphase cytogenetics is widely used to detect chromosomal aberrations in hematologic malignancies and has emerged as a standard diagnostic tool.[4,5,7] Recently, SNP array technology has been intensively discussed as a valuable complement to metaphase cytogenetics.[8,33] We report here on the analysis of a large cohort of classic MPN patients at high resolution using Affymetrix 6.0 SNP arrays containing more than 1.8 million genomic markers. We found a highly diverse pattern of chromosomal aberrations among individual patients. Whereas studies of MPN using classic metaphase cytogenetics reported around 30% patients with aberrant karyotype,[4,5,7] we found chromosomal changes in > 50% of the patients. This was clearly due to the fact that SNP arrays allow the detection of small aberrations as well as UPDs, both of which are undetectable by metaphase cytogenetics. It has been widely reported that mitotic recombination resulting in UPD often amplifies oncogene or tumor suppressor gene mutations. In MPN, chromosome 9pUPD amplifies the JAK2-V617F mutation,[12] 1pUPD amplifies *MPL* mutations,[14-16] and chromosome 11qUPD has been shown to amplify various mutations of *CBL*.[19] We detected chromosome 9pUPDs in 169 patients, all of whom tested positive for *JAK2* mutations. Of the 7 1pUPD cases, only 6 were associated with mutations in the *MPL* gene. The remaining case raises the possibility that chromosome 1p harbors an unknown gene mutation relevant for MPN pathogenesis.

We did not observe any association of the number of chromosomal aberrations with any of the 3 MPN disease entities investigated in this study. This is in contrast to previous reports showing that PMF patients have a higher rate of chromosomal abnormalities.[8] A recent publication suggested that the JAK2-V617F mutation induces genomic instability and might account for chromosomal lesions observed in MPN.[34] Our results show that JAK2-V617F–positive patients do not have a higher frequency of chromosomal aberrations. We expected to observe accumulation of chromosomal aberrations over the course of the disease as the malignant clone evolved. Disease duration was not associated with the frequency of chromosomal aberrations in the studied patients. However, the age of the patients at the time of sample was

Table 4. Summary of point mutations and recurrent chromosomal aberrations in post-MPN AML patients

Sample ID	*JAK2*	*RUNX1*	*FLT3*	*NPM1*	*IDH1*	*IDH2*	*TP53*	gain 1q	del 7q	del 5q	del 6p	del 7p	UPD 19q	gain 3q	UPD 22q
sample_281	V617F	wt	wt	wt	wt	wt	wt					Yes			
sample_289	V617F	wt	wt	wt	wt	wt	wt					Yes			
sample_304	V617F	wt	wt	wt	wt	R140W	wt								
sample_373	V617F	L29S	wt	mut	wt	wt	wt								
sample_374	wt	wt	wt	wt	wt	wt	wt				Yes		Yes		Yes
sample_375	wt	wt	wt	wt	wt	wt	wt								
sample_381	wt	R135K/442insTGCCT_T150PfsX1	ITD	wt	wt	R140Q	wt								
sample_382	wt	wt	wt	wt	wt	wt	wt								
sample_383	wt	wt	wt	wt	wt	wt	wt	Yes	Yes	Yes	Yes				
sample_389	V617F	wt	wt	wt	wt	wt	wt								
sample_390	V617F	wt	wt	wt	wt	wt	wt	Yes							
sample_391	V617F	wt	wt	wt	wt	wt	wt								
sample_392	V617F	wt	D835	wt	wt	wt	c.994–2 A > G (17pUPD)			Yes	Yes				
sample_393	V617F	Q235X/S373X	wt	wt	wt	wt	wt	Yes	Yes						
sample_394	V617F	wt	wt	wt	wt	wt	C135S / M246K			Yes	Yes			Yes	
sample_395	V617F	wt	wt	wt	wt	wt	wt								
sample_396	V617F	del 21q†	wt	wt	wt	wt	wt	Yes	Yes				Yes		
sample_397	V617F	wt	wt	wt	wt	wt	wt								
sample_398	V617F	wt	wt	wt	wt	wt	wt								
sample_399	V617F	wt	wt	wt	wt	wt	N239D (17pUPD)								
sample_400	V617F	wt	wt	wt	wt	wt	c.560–1 G > A / Y220H			Yes				Yes	
sample_401	V617F	wt	wt	wt	wt	wt	wt								
sample_402	V617F	wt	wt	wt	wt	wt	K132E						Yes		
sample_403	V617F	wt	wt	wt	wt	wt	S261T / N239D								Yes
sample_404	V617F	wt	D835	wt	wt	wt	wt								Yes
sample_405	V617F	wt	wt	wt	wt	wt	wt								
sample_406	V617F	wt	wt	wt	wt	wt	wt		Yes					Yes	
sample_407	V617F	wt	wt	wt	R132G	wt	wt		Yes*			Yes*			
sample_408	wt	wt	wt	wt	wt	wt	wt	Yes							

wt indicates wild-type; mut, mutant; ITD, internal tandem duplication; and del, deletion.
*Monosomy 7.
†Single-gene deletion of *RUNX1*.

positively correlated with the number of defects. In agreement with this finding, there have been reports showing that aging stem cells acquire chromosomal defects[35]; a study in PV showed a similar correlation.[7]

MPN patients diagnosed for sMF/AP had significantly more chromosomal aberrations than patients in the chronic phase of the disease. Patients with post-MPN AML harbored more aberrations than both chronic-phase and sMF/AP patients, which has been reported previously.[5] Chromosome 9pUPDs were more closely associated with sMF/AP than with chronic-phase disease, indicating that a high burden of JAK2-V617F (located on chromosome 9p) predisposes for disease progression. JAK2-V617F homozygosity had already been associated with a higher risk for secondary myelofibrosis in PV and ET.[36]

Chromosome 1q amplifications were more closely associated with both sMF/AP and post-MPN AML compared with chronic phase, but no significant difference was observed between sMF/AP and post-MPN AML. All observed chromosome 1q gains amplified the *MDM4* gene, which is a potent inhibitor of p53.[37,38] *TP53* itself has been shown to be involved in the leukemic transformation process in MPN,[28,39] which was confirmed in the present study. It is also known that an impairment of the p53 pathway and altered cell-cycle regulation are associated with the accumulation of mutations. Whether chromosome 1q gains could set the stage for the acquisition of further aberrations that eventually induce leukemic transformation is a matter for further investigations. We also show herein that lesions of chromosomes 7, 5, 6, 19, 22, and 3 significantly cluster in post-MPN AML compared with chronic phase. It remains to be seen if the transformation-associated aberrations identified here will have prognostic value.

Six CDRs in our cohort mapped to single-target genes. We identified a new putative tumor suppressor gene on chromosome 7 that encodes CUX1, a DNA-binding protein that plays a role in gene transcription and cell-cycle regulation and is involved in hematopoiesis[40,41]; however, its precise role in MPN pathogenesis needs to be addressed in functional studies. Recently, Thoennissen et al reported an MPN patient harboring a 0.88-Mb deletion on chromosome 7q that only included the 2 genes *CUX1* and *SH2B2*.[24] Our data conclusively show that *CUX1* is a major target of chromosome 7q deletions. It remains to be seen whether deletions of *SH2B2* play a role in a smaller subset of patients. We also mapped 2 known tumor suppressor genes on chromosome 4 (*TET2*) and on chromosome 7p (*IKZF1*), which were reported previously.[17,23] The CDR of chromosome 3p mapped to *FOXP1*, a member of the large family of forkhead transcription factors that are involved in various cellular processes. Differential expression of *FOXP1* has been observed in several types of tumors, including both overexpression and loss of expression.[42] Loss of heterozygosity of the region on chromosome 3p14 that harbors *FOXP1* is common in cancer.[43] *FOXP1* has been shown to play important roles in B-cell development.[44] Its role in myeloid malignancies is so far poorly understood. *ETV6* is a transcription factor of the ets

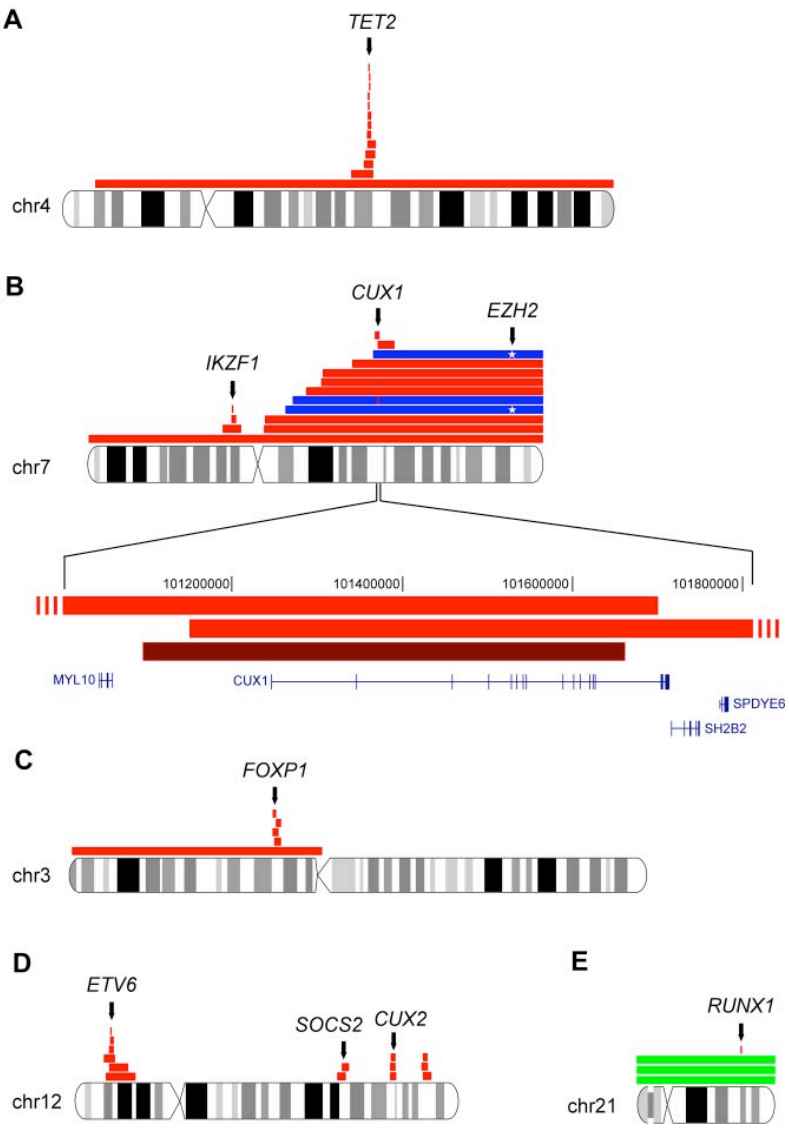


Figure 4. Commonly deleted chromosomal regions in MPN that map to single-target genes. Red horizontal bars indicate deletions; dark red, homozygous deletion; and blue, UPDs. (A) Recurrent deletions on chromosome 4q target the *TET2* gene. (B) Complexity of chromosome 7 aberrations in MPN. The CDR on 7p (*IKZF1*) and 7q (*CUX1*) are shown. Two events of 7qUPD associated with an *EZH2* mutation (*) are shown in blue. Another 7qUPD is negative for *EZH2* mutations but has homozygous *CUX1* deletion. Two overlapping deletions in the *CUX1* gene locus and the homozygous deletion of *CUX1* are shown at higher magnification. (C) Five overlapping deletion events on chromosome 3p define the target gene as *FOXP1*. (D) Recurrent deletions on chromosome 12. The target of 12p deletions is *ETV6*, whereas 1 of the commonly deleted regions on 12q contains *CUX2*. (E) Aberrations on chromosome 21q affecting *RUNX1*: single-gene deletion of *RUNX1* and trisomy 21 with 2 nonsense mutations in *RUNX1*. chr indicates chromosome and Mbp, mega base pairs.

family and the only gene in the CDR of chromosome 12p. It was originally identified in a translocation between chromosomes 5 and 12 in a patient with chronic myelomonocytic leukemia,[45] and was then shown to be involved in >40 translocations in several different hematologic malignancies.[46] Different fusion genes of *ETV6* with other genes, including *ABL1*, *JAK2*, and *RUNX1*, have also been described.[47] In addition to translocations, deletions of *ETV6* are often reported, which may affect the remaining allele not involved in the translocations.[48] Common deleted regions have been mapped to a small region including *ETV6* and *CDKN1B*.[47] To our knowledge, the present study is the first report of a single-gene deletion of *ETV6* in MPN.

RUNX1 (*AML1*) has been implicated previously in leukemic transformation of MPNs.[39,49] Both mutations and translocations of *RUNX1/AML1* are frequently encountered in various leukemias. *RUNX1* is a heterodimeric transcription factor that binds to the core element of many enhancers and promoters and is thought to be involved in the development of normal hematopoiesis.[50] Inactivating mutations in *RUNX1* result in differentiation arrest. Our observation of both point mutations and chromosomal aberrations at the *RUNX1* locus in post-MPN AML samples confirms its role in leukemic transformation. With *FOXP1*, *IKZF1*, *CUX1*, *ETV6*, and *RUNX1*, 5 of the 6 target genes of CDRs are transcription factors, indicating that transcription factor networks may play crucial roles in MPN pathogenesis.

In this study, we have described several chromosomal regions with recurrent deletions in which the CDRs were small and contained only few genes. We identified a "hot spot" of complex deletions on chromosome 12q that contains *CUX2*, a homolog of *CUX1*. In a recent study, *CUX2* was found in 1 MPN patient to be homozygously deleted,[25] suggesting that both *CUX* family members play a role in MPN pathogenesis (Figure 4D). Our CDR on chromosome 17q includes the *NF1* gene that has been mapped as the target of this chromosomal region.[25] The pathogenesis of de novo AML and post-MPN AML involve different genetic mechanisms, because we found mutations of *FLT3*, *IDH1*, and *IDH2* at

lower frequencies than reported in de novo AML. Interestingly, 6 of 29 post-MPN AML patients carried no chromosomal aberrations except 9pUPDs, and were negative for all leukemia-associated mutations tested. This indicates that there are as-yet-unknown pathways of leukemic transformation in MPN.

The results of the present study suggest that the chromosomal aberrations detected in MPN can be separated into 2 groups: the first is compatible with the chronic phase of the disease and the second with disease progression. Understanding the modifications that lead to MPN disease progression has important implications for therapy. Prevention of the leukemic transformation and prolongation of the chronic phase of MPN could be associated with fewer clinical complications and a more favorable prognosis for MPN patients.

Acknowledgments

This study was supported by the Austrian Academy of Sciences, the Austrian Science Fund (FWF, P20033-B11), and the MPN Research Foundation. Studies in Pavia (M.C.) and Florence (A.M.V.) were funded by the Associazione Italiana per la Ricerca sul Cancro (AIRC), Special Program Molecular Clinical Oncology 5 per mille, Milan, Italy; progetto AGIMM, http://www.progettoagimm.it). M.C. was also supported by Fondazione Cariplo (Milan, Italy).

Authorship

Contribution: T.K. and A.H. designed and performed research, analyzed and interpreted data, and wrote the manuscript; T.B., M.S., K.B., D.O., and R.J. performed research; B.G. and D.P. performed DNA extractions and biobanking; F.P. and E.R. managed patient accrual and collected clinical data; L.P. and P.G. managed patient accrual and analyzed data; I.I. and G.M. analyzed data; M.C. and H.G. collected samples and acquired and analyzed clinical data; A.M.V. designed the study, managed patient accrual, and analyzed data; and R.K. designed the research, interpreted data, and wrote the manuscript.

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

Correspondence: Robert Kralovics, PhD, Center for Molecular Medicine (CeMM) of the Austrian Academy of Sciences, Lazarettgasse 14, BT25.3, 1090 Vienna, Austria; e-mail: robert.kralovics@cemm.oeaw.ac.at.

References

1. Campbell PJ, Green AR. The myeloproliferative disorders. *N Engl J Med.* 2006;355(23):2452-2466.
2. Tam CS, Kantarjian H, Cortes J, et al. Dynamic model for predicting death within 12 months in patients with primary or post-polycythemia vera/essential thrombocythemia myelofibrosis. *J Clin Oncol.* 2009;27(33):5587-5593.
3. Abdulkarim K, Girodon F, Johansson P, et al. AML transformation in 56 patients with Ph- MPD in two well defined populations. *Eur J Haematol.* 2009;82(2):106-111.
4. Hussein K, Van Dyke DL, Tefferi A. Conventional cytogenetics in myelofibrosis: literature review and discussion. *Eur J Haematol.* 2009;82(5):329-338.
5. Gangat N, Tefferi A, Thanarajasingam G, et al. Cytogenetic abnormalities in essential thrombocythemia: prevalence and prognostic significance. *Eur J Haematol.* 2009;83(1):17-21.
6. Panani AD. Cytogenetic and molecular aspects of Philadelphia negative chronic myeloproliferative disorders: clinical implications. *Cancer Lett.* 2007;255(1):12-25.
7. 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(3):197-200.
8. Reilly JT. Pathogenetic insight and prognostic information from standard and molecular cytogenetic studies in the BCR-ABL-negative myeloproliferative neoplasms (MPNs). *Leukemia.* 2008;22(10):1818-1827.
9. Kralovics R, Guan Y, Prchal JT. Acquired uniparental disomy of chromosome 9p is a frequent stem cell defect in polycythemia vera. *Exp Hematol.* 2002;30(3):229-236.
10. Baxter EJ, Scott LM, Campbell PJ, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet.* 2005;365(9464):1054-1061.
11. James C, Ugo V, Le Couedic JP, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature.* 2005;434(7037):1144-1148.
12. Kralovics R, Passamonti F, Buser AS, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med.* 2005;352(17):1779-1790.
13. 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(4):387-397.
14. Pardanani AD, Levine RL, Lasho T, et al. MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. *Blood.* 2006;108(10):3472-3476.
15. Szpurka H, Gondek LP, Mohan SR, Hsi ED, Theil KS, Maciejewski JP. UPD1p indicates the presence of MPL W515L mutation in RARS-T, a mechanism analogous to UPD9p and JAK2 V617F mutation. *Leukemia.* 2009;23(3):610-614.
16. Buxhofer-Ausch V, Gisslinger H, Berg T, Gisslinger B, Kralovics R. Acquired resistance to interferon alpha therapy associated with homozygous MPL-W515L mutation and chromosome 20q deletion in primary myelofibrosis. *Eur J Haematol.* 2009;82(2):161-163.
17. Delhommeau F, Dupont S, Della Valle V, et al. Mutation in TET2 in myeloid cancers. *N Engl J Med.* 2009;360(22):2289-2301.
18. Tefferi A, Pardanani A, Lim KH, et al. TET2 mutations and their clinical correlates in polycythemia vera, essential thrombocythemia and myelofibrosis. *Leukemia.* 2009;23(5):905-911.
19. Dunbar AJ, Gondek LP, O'Keefe CL, et al. 250K single nucleotide polymorphism array karyotyping identifies acquired uniparental disomy and homozygous mutations, including novel missense substitutions of c-Cbl, in myeloid malignancies. *Cancer Res.* 2008;68(24):10349-10357.
20. Sanada M, Suzuki T, Shih LY, et al. Gain-of-function of mutated C-CBL tumour suppressor in myeloid neoplasms. *Nature.* 2009;460(7257):904-908.
21. Carbuccia N, Murati A, Trouplin V, et al. Mutations of ASXL1 gene in myeloproliferative neoplasms. *Leukemia.* 2009;23(11):2183-2186.
22. Green A, Beer P. Somatic mutations of IDH1 and IDH2 in the leukemic transformation of myeloproliferative neoplasms. *N Engl J Med.* 2010;362(4):369-370.
23. Jäger R, Gisslinger H, Passamonti F, et al. Deletions of the transcription factor Ikaros in myeloproliferative neoplasms. *Leukemia.* 2010;24(7):1290-1298.
24. Thoennissen NH, Krug UO, Lee DH, et al. Prevalence and prognostic impact of allelic imbalances associated with leukemic transformation of Philadelphia chromosome-negative myeloproliferative neoplasms. *Blood.* 2010;115(14):2882-2890.
25. Stegelmann F, Bullinger L, Griesshammer M, et al. High-resolution single-nucleotide polymorphism array-profiling in myeloproliferative neoplasms identifies novel genomic aberrations. *Haematologica.* 2010;95(4):666-669.
26. 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(4):1377-1380.
27. Murphy KM, Levis M, Hafez MJ, et al. Detection of FLT3 internal tandem duplication and D835 mutations by a multiplex PCR and capillary electrophoresis assay. *J Mol Diagn.* 2003;5(2):96-102.
28. Harutyunyan A, Klampfl T, Cazzola M, Kralovics R. p53 lesions in leukemic transformation. *N Engl J Med.* 2011;364(5):488-490.
29. Ernst T, Chase AJ, Score J, et al. Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. *Nat Genet.* 2010;42(8):722-726.
30. Nikoloski G, Langemeijer SM, Kuiper RP, et al. Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. *Nat Genet.* 2010;42(8):665-667.
31. Oh ST, Simonds EF, Jones C, et al. Novel mutations in the inhibitory adaptor protein LNK drive JAK-STAT signaling in patients with myeloproliferative neoplasms. *Blood.* 2010;116(6):988-992.
32. Pardanani A, Lasho T, Finke C, Oh ST, Gotlib J, Tefferi A. LNK mutation studies in blast-phase myeloproliferative neoplasms, and in chronic-phase disease with TET2, IDH, JAK2 or MPL mutations. *Leukemia.* 2010;24(10):1713-1718.
33. Maciejewski JP, Mufti GJ. Whole genome scanning as a cytogenetic tool in hematologic malignancies. *Blood.* 2008;112(4):965-974.
34. 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(4):1402-1412.

35. Rossi DJ, Bryder D, Seita J, Nussenzweig A, Hoeijmakers J, Weissman IL. Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature.* 2007; 447(7145):725-729.
36. Vannucchi AM, Antonioli E, Guglielmelli P, et al. Clinical profile of homozygous JAK2 617V>F mutation in patients with polycythemia vera or essential thrombocythemia. *Blood.* 2007;110(3): 840-846.
37. Laurie NA, Donovan SL, Shih CS, et al. Inactivation of the p53 pathway in retinoblastoma. *Nature.* 2006;444(7115):61-66.
38. Riemenschneider MJ, Knobbe CB, Reifenberger G. Refined mapping of 1q32 amplicons in malignant gliomas confirms MDM4 as the main amplification target. *Int J Cancer.* 2003;104(6):752-757.
39. Beer PA, Delhommeau F, LeCouedic JP, et al. Two routes to leukemic transformation after a JAK2 mutation-positive myeloproliferative neoplasm. *Blood.* 2010;115(14):2891-2900.
40. Cadieux C, Fournier S, Peterson AC, Bedard C, Bedell BJ, Nepveu A. Transgenic mice expressing the p75 CCAAT-displacement protein/Cut homeobox isoform develop a myeloproliferative disease-like myeloid leukemia. *Cancer Res.* 2006; 66(19):9492-9501.
41. Truscott M, Harada R, Vadnais C, Robert F, Nepveu A. p110 CUX1 cooperates with E2F transcription factors in the transcriptional activation of cell cycle-regulated genes. *Mol Cell Biol.* 2008; 28(10):3127-3138.
42. Banham AH, Beasley N, Campo E, et al. The FOXP1 winged helix transcription factor is a novel candidate tumor suppressor gene on chromosome 3p. *Cancer Res.* 2001;61(24):8820-8829.
43. Kok K, Naylor SL, Buys CH. Deletions of the short arm of chromosome 3 in solid tumors and the search for suppressor genes. *Adv Cancer Res.* 1997;71:27-92.
44. Hu H, Wang B, Borde M, et al. Foxp1 is an essential transcriptional regulator of B cell development. *Nat Immunol.* 2006;7(8):819-826.
45. Golub TR, Barker GF, Lovett M, Gilliland DG. Fusion of PDGF receptor beta to a novel ets-like gene, tel, in chronic myelomonocytic leukemia with t(5;12) chromosomal translocation. *Cell.* 1994;77(2):307-316.
46. Odero MD, Carlson K, Calasanz MJ, Lahortiga I, Chinwalla V, Rowley JD. Identification of new translocations involving ETV6 in hematologic malignancies by fluorescence in situ hybridization and spectral karyotyping. *Genes Chromosomes Cancer.* 2001;31(2):134-142.
47. Bohlander SK. ETV6: a versatile player in leukemogenesis. *Semin Cancer Biol.* 2005;15(3):162-174.
48. Cavé H, Cacheux V, Raynaud S, et al. ETV6 is the target of chromosome 12p deletions in t(12; 21) childhood acute lymphocytic leukemia. *Leukemia.* 1997;11(9):1459-1464.
49. Ding Y, Harada Y, Imagawa J, Kimura A, Harada H. AML1/RUNX1 point mutation possibly promotes leukemic transformation in myeloproliferative neoplasms. *Blood.* 2009;114(25):5201-5205.
50. Tanaka T, Tanaka K, Ogawa S, et al. An acute myeloid leukemia gene, AML1, regulates hemopoietic myeloid cell differentiation and transcriptional activation antagonistically by two alternative spliced forms. *EMBO J.* 1995;14(2):341-350.

3.2 Manuscript #2: Complex Patterns of Chromosome 11 Aberrations in Myeloid Malignancies Target *CBL, MLL, DDB1* and *LMO2*

Thorsten Klampfl et al.

submitted for publication

The author of this PhD thesis contributed to the manuscript as follows:

The author contributed to the generation of microarray data, analyzed raw data of around 400 of the microarrays (half of the microarray dataset) in this study and collected microarray analysis results for the remaining samples from the collaborators. Parts of the exome-sequencing analysis pipeline (downstream of GATK) were assembled by the author. The whole-exome sequencing analysis was done by the author. The coverage analysis pipeline, which led to the detection of the *MLL* duplication, was designed and assembled by the author. He also performed the coverage analysis. The author generated and analyzed large amounts of the Sanger sequencing data presented in this manuscript especially validating whole-exome sequencing hits, and most of the data around *CBL*, *MLL*, and *LMO2*. Data interpretation was done in close collaboration with Jelena Milosevic and Ana Puda with the help of Robert Kralovics. The author of this thesis assembled all Figures and Tables of the manuscript and wrote the manuscript together with the senior author. Only the part of the methods section explaining the use of the Genome Analysis toolkit was written by Andreas Schönegger. Helpful comments from the other authors were included.

Complex Patterns of Chromosome 11 Aberrations in Myeloid Malignancies Target *CBL, MLL, DDB1* and *LMO2*

Short title: Genetics of Chromosome 11 in Myeloid Malignancies

Thorsten Klampfl[1], Jelena D. Milosevic[1], Ana Puda[1], Andreas Schönegger[1], Klaudia Bagienski[1], Tiina Berg[1], Ashot S. Harutyunyan[1], Bettina Gisslinger[2], Elisa Rumi[3], Luca Malcovati[3], Daniela Pietra[3], Chiara Elena[3], Matteo Giovanni Della Porta[3,4], Lisa Pieri[5], Paola Guglielmelli[5], Christoph Bock[1], Michael Doubek[6,7], Dana Dvorakova[6,7], Nada Suvajdzic[8], Dragica Tomin[8], Natasa Tosic[9], Zdenek Racil[6,7], Michael Steurer[10], Sonja Pavlovic[9], Alessandro M. Vannucchi[5], Mario Cazzola[3,4], Heinz Gisslinger[2], Robert Kralovics[1,2]

[1]CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria; [2]Department of Internal Medicine I, Division of Hematology and Blood Coagulation, Medical University of Vienna, Vienna, Austria; [3]Department of Hematology Oncology, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy [4]Department of Molecular Medicine, University of Pavia, Pavia, Italy; [5]Section of Hematology, University of Florence, Florence, Italy; [6]Department of Internal Medicine Hematology and Oncology, University Hospital Brno, Masaryk University Brno, Brno, Czech Republic; [7]CEITEC - Central European Institute of Technology, Masaryk University Brno, Brno, Czech Republic; [8]Clinic of Hematology, Clinical Center of Serbia, University of Belgrade, School of Medicine, Belgrade, Serbia; [9]Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia; [10]Division of Hematology and Oncology, Innsbruck University Hospital, Innsbruck, Austria.

Corresponding author:

Robert Kralovics, Ph.D.

CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, BT25.3 AKH, Lazarettgasse 14, 1090 Vienna, Austria.

Phone: +43-1-40160-70027

Fax: +43-1-40160-970000

robert.kralovics@cemm.oeaw.ac.at

The authors have no relevant conflicts of interests to disclose.

Abstract

Exome sequencing of primary tumors identifies complex somatic mutation patterns. Assignment of relevance of individual somatic mutations is difficult and poses the next challenge for interpretation of next generation sequencing data. Here we present an approach how exome sequencing in combination with SNP microarray data may identify targets of chromosomal aberrations in myeloid malignancies. The rationale of this approach is that hotspots of chromosomal aberrations might also harbor point mutations in the target genes of deletions, gains or uniparental disomies (UPDs). Chromosome 11 is a frequent target of lesions in myeloid malignancies. Therefore, we studied chromosome 11 in a total of 813 samples with different myeloid malignancies by SNP microarrays and complemented the data with exome sequencing in selected cases exhibiting chromosome 11 defects. We found gains, losses and UPDs of chromosome 11 in 52 of the 813 samples (6.4%). Chromosome 11q UPDs frequently associated with mutations of *CBL*. In one patient the 11qUPD amplified somatic mutations in both *CBL* and the DNA repair gene *DDB1*. A duplication within *MLL* exon 3 was detected in another patient with 11qUPD. We identified several common deleted regions (CDR) on chromosome 11. One of the CDRs associated with *de novo* acute myeloid leukemia (P=0.013). One patient with a deletion at the *LMO2* locus harbored an additional point mutation on the other allele indicating that *LMO2* might be a tumor suppressor frequently targeted by 11p deletions. Our chromosome-centered analysis indicates that chromosome 11 contains a number of tumor suppressor genes and that the role of this chromosome in myeloid malignancies is more complex than previously recognized.

Author Summary

Current high-throughput technologies provide means to study genetic changes contributing to cancer on a genome-wide level. However, it remains challenging to distinguish genetic findings that are relevant for the disease from unassociated lesions. In an attempt to overcome this problem, we combined data of chromosomal aberrations indentified by microarray analysis in over 800 patients suffering from hematological cancers. Recurrence of large chromosomal aberrations at specific regions in the genome suggests an importance of one or more genes in these regions for cancer development. In particular, we focused our analysis on a frequently altered chromosome in hematological cancers, chromosome 11. We found large aberrations in 52 out of 813 analyzed samples. Intersection of these data with findings from whole-exome sequencing allowed the identification of *CBL, DDB1, MLL* and *LMO2* genes as potential targets of these aberrations. We showed that mutations in *CBL* associate with disease progression in a subtype of hematological cancers, and that deletions of a distinct region of chromosome 11 specifically associated with another subtype. The combination of microarray and whole exome sequencing data therefore allowed the identification of genetic lesions that are likely to contribute to hematological malignancies.

Introduction

Hematological malignancies are broadly categorized into myeloid and lymphoid malignancies, depending on the hematopoietic lineage involved. This study focused on myeloid malignancies, in particular the disease entities acute myeloid leukemia (AML), chronic myeloid leukemia (CML), myelodysplastic syndromes (MDS) as well as the three classical myeloproliferative neoplasms (MPNs) polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). MDS and MPN are in most cases stable, chronic diseases. A fraction of patients, however, develop signs of disease progression such as myelofibrosis or elevated numbers of hematopoietic progenitors in peripheral blood referred to as “accelerated phase”. A transformation to post-MPN or post-MDS AML marks the final stage of the disease and is associated with a very bad prognosis [1]. Genetic aberrations involving chromosome 11 have been widely reported across all hematological malignancies. Translocations of chromosome 11q affecting the 11q23 region have been intensely studied since the late 1970s when the first translocation between chromosomes 11 and 4 was described in acute lymphoblastic leukemia (ALL) [2]. In 1991 the gene that was affected by these translocations on chromosome 11 was identified to be *MLL* (myeloid/lymphoid or mixed-lineage leukemia) [3]. These translocations t(4;11) led to the formation of a fusion gene of *MLL* and *AF4* (ALL1-fused gene from chromosome 4; current official symbol AFF1) on chromosome 4 [4]. Since then a variety of translocations involving *MLL* and more than 60 fusion gene partners have been identified. They are found both, in ALL and AML with a high prevalence in infants [5]. In addition to translocations, partial tandem duplications of *MLL* have also been described in AML [6,7]. The internal tandem duplications of *MLL* most often span between exon 3 and exons 9-11, [8] and show a strong association with chromosome

11q trisomies [7]. Classical karyotyping has revealed chromosomal deletions as common genetic changes in chronic lymphoid leukemia (CLL), AML, MDS and other hematological malignancies. A frequently deleted region mapped to 11q23 [9]. In recent years the upcoming of single nucleotide polymorphism (SNP) microarrays has allowed the detection of chromosomal gains and losses at a much higher resolution than with classical cytogenetics. Acquired copy number neutral loss of heterozygosity (LOH) associated with uniparental disomies (UPD), which were previously undetectable by classical cytogenetics, are now recurrently found in hematological malignancies. The first large study in AML using SNP microarrays identified chromosomal aberrations of all three types across the whole genome [10]. We, alongside others, reported such studies in the myeloproliferative neoplasms (MPN) [11-14]. All of these studies observed frequent aberrations on chromosome 11 including gains, losses and UPDs. UPDs were shown to somatically amplify mutant alleles of genes on various chromosomal arms such as 9p (*JAK2*), 1p (*MPL*) or 4q (*TET2*) [15-23]. On chromsome 11p, mutant alleles of *WT1* were associated with UPDs in AML [24], while *CBL* mutations were associated with UPDs on chromosome 11q in several hematological malignancies[25-27]. *CBL* encodes an E3 ubiquitin ligase that attaches ubiquitin to a number of membrane-associated and cytosolic proteins (such as Flt3, Kit, Jak2 and Mpl) and targets them for degradation [28,29]. In this study, we present a systematic analysis of chromosome 11 in a set of 813 samples across different myeloid malignancies. We used the latest technologies including high resolution SNP microarrays and whole exome sequencing to identify novel genetic aberrations of chromosome 11 in myeloid malignancies. We were able to detect commonly aberrant regions on this chromosome and to identify potential target genes of large aberrations.

Results and Discussion

Chromosome 11 aberrations in myeloid malignancies

In order to systematically analyze chromosome 11 aberrations in myeloid malignancies, we combined data from a total of 813 blood samples that were genotyped at high-resolution with Affymetrix Genome-Wide Human SNP 6.0 microarrays. This cohort included 180 *de novo* acute myeloid leukemia (AML), 62 chronic myeloid leukemia (CML), 101 myelodysplastic syndrome (MDS), 244 polycythemia vera (PV), 118 essential thrombocythemia (ET) and 108 primary myelofibrosis (PMF) samples (Table 1, Figure 1A). For PV, ET, PMF and MDS, the majority of samples were in chronic phase of the disease, some samples were taken when patients showed signs of disease progression or had transformed to post-chronic phase AML as outlined in Table 1. Chromosome 11 aberrations were detected in 52 of 813 samples (6.4%) (Table 1 and Figure 1B). The 52 samples were from 50 patients, for 2 patients we had 2 samples from different disease stages (Supplementary Table 1). The samples harbored between 1 to 3 genetic changes on chromosome 11, except for sample 42 which had a complex chromosome 11 (Supplementary Table 1). Excluding sample 42, we detected a total of 30 deletions, 11 gains and 17 UPDs (Figure 2 and Supplementary Table 1). In MPN, aberrations of chromosome 11 significantly associate with post-MPN AML compared to chronic phase MPN ($P<0.0001$, Fisher's exact test, Figure 1B). MPN patients that exhibited myelofibrosis or were in the accelerated phase of the disease but had not fully transformed to post-MPN AML were regarded as chronic phase patients in this analysis. This finding indicates that genes located on chromosome 11 contribute to disease progression if mutated. Associations of chromosome 11q losses of

heterozygosity with disease progression or poor prognosis have been described previously in B cell chronic lymphatic leukemia [30] or neuroblastoma [31]. Chromosome 11q23 abnormalities were associated with a poor outcome in infant acute lymphoblastic leukemia (ALL) [32].

CBL is a frequent target of chromosome 11q aberrations

We found that UPDs of chromosome 11q are the most recurrent defects in our dataset. A number of studies have shown that 11q UPDs are associated with mutations of the *CBL* gene (ensembl gene ID: ENSG00000110395) [25-27]. Mutations of *CBL* have been described to cluster within exons 8 and 9 or their exon-intron junctions [25-27]. Therefore, we sequenced these two exons of *CBL* in all samples that harbored chromosomal aberrations overlapping the *CBL* locus. Of the 14 patients that had 11q UPDs, we detected SNVs in 9 patients (Supplementary Table 1). One patient (sample 45) harbored a 6 bp tandem duplication (Figure 3A). Out of 6 patients that had 11q gains overlapping *CBL*, one had a somatic mutation in *CBL* (C384Y in sample 44). PCR subcloning revealed that the gain amplifies the mutant allele (data not shown). No mutations were detected in the 7 patients with deletions overlapping *CBL*. For the patients where we had control tissue available, the somatic origin of the variants detected in *CBL* was confirmed (Supplementary Table 1). In order to identify mutations in other exons of *CBL* or in other genes that potentially associate with 11q aberrations we performed whole exome sequencing on 3 samples with 11q uniparental disomies (samples 30, 36 and 50) and two samples with 11q gains (samples 42 and 43) which did not have mutations in exons 8 and 9 of *CBL*. Only one of these samples (36) showed a mutation in *CBL* at the 3' splice site

of exon 7 (Supplementary Table 1). The variant was somatic and independently validated by Sanger sequencing.

CBL mutations associate with leukemic transformation of MPN

As shown in Figure 1B, we associated chromosome 11 defects with leukemic transformation of MPN. In order to test if *CBL* mutations distinctly associate with disease progression, we sequenced *CBL* exons 8 and 9 in all 44 post-MPN AML samples and 274 chronic phase MPN samples. *CBL* mutations were present in 1.4% of chronic phase and 15.9% of post-MPN leukemic patients, respectively. Thus, *CBL* mutations are significantly associated with post-MPN AML (P=0.0001; Fisher's exact test). A particularly interesting case in this set of patients had two mutations of *CBL* affecting exon 8 (W408C) and the 5' splice site of exon 9 (Figure 3B). Both mutations were somatic and PCR subcloning revealed that these two mutations were on independent DNA strands. As all the bacterial clones analyzed contained only one of the mutations and no clones with wild type *CBL* were detected, we concluded that the patient harbors a compound heterozygous progenitor clone with distinct mutations of both *CBL* alleles (Figure 3B).

Mechanisms that increase mutant CBL dosage

Our data on *CBL* suggest that there are several different genetic mechanisms for how the malignant clone can increase the mutant *CBL* allele dosage (Figure 3C). The first mechanism is via mitotic recombination resulting in UPD. The second mechanism is amplification of the mutant allele by duplication. Another possibility is the inactivation of wild type alleles by two independent point mutations (compound heterozygosity). Interestingly, it seems possible that loss of a single *CBL* allele

(haploinsufficiency) might be oncogenic as 7 patients in our cohort carried hemizygous *CBL* deletions (Figure 2). In support of this hypothesis, heterozygous *Cbl* deficiency in mice showed accelerated blast crisis compared to *Cbl* wild type animals in a BCR-ABL transgenic murine model [27]. In addition, hemizygous deletions of *CBL* have been shown by others in MDS and related disorders [33].

Mutation of DDB1 associated with 11q UPD

Recently, mutations in the splicing factor 1 gene *SF1* (ensembl gene ID: ENSG00000168066) and a member of the polycomb complex 2 (*EED* – ensembl gene ID: ENSG00000074266) were found in myeloid malignancies [34,35]. Both genes are located on chromosome 11q (Figure 2). We did not find any mutations in these two genes by either whole exome or Sanger sequencing of *EED* and the C-terminal proline-rich region of *SF1* that was found to be the mutational hotspot of the gene[34]. All samples that had aberrations spanning the two loci were analyzed (Figure 2). We performed whole exome sequencing of samples 30, 36, 42, 43 and 50 and attempted to identify genes other than *CBL* that might be associated with aberrations of chromosome 11q (Supplementary Table 2). In two of the patients (samples 30 and 36), we performed a paired analysis as whole exome sequenced T lymphocyte DNA was available as germline control (samples 30c and 36c). In sample 30 we did not find any somatic mutations with an allelic frequency > 50%, which is expected for variants within the fully clonal 11qUPD region (data not shown). In addition to the somatic mutation in *CBL* described above, sample 36 also harbored another somatic mutation in *DDB1* (ensembl gene ID: ENSG00000167986) (Figure 4A). The *CBL* and *DDB1* mutations in sample 36 were validated by Sanger sequencing and shown to be homozygous and fully clonal (Figure 4A). Both

mutations were also detected in an earlier sample of the same patient (sample 23). Sample 23 harbored an 11qUPD in a subclone and accordingly, the mutations in *CBL* and *DDB1* were not fully clonal (data not shown). The *Polyphen2* tool used to predict functional effects of human non-synonymous single nucleotide variants estimated the variant in *DDB1* to be “probably damaging” with the highest probability score of 1. *DDB1* was originally identified in patients suffering from *Xeroderma pigmentosum*, with inherited deficiency in nucleotide excision repair (NER). The gene was cloned together with its binding partner *DDB2,* with which it forms the DDB protein complex[36]. Later, *DDB1* was found to form an E3 ubiquitin ligase complex together with *CUL4A*, *ROC1* and a variable fourth protein that determines the target specificity of the E3 ligase. Overall, more than 30 different proteins have been identified as binding partners[37]. The ubiquitination activity of DDB1-CUL4A-ROC1 complexes has been shown to not only play important roles in NER[38] but also in regulating the expression of the tumor suppressor *CDKN2A*[39]. *CDKN2A* gene expression is associated with histone 3 – lysine 4 (H3K4) trimethylation mediated by the MLL-RBBP5-WDR5 complex. RBBP5 and WDR5 are two of the binding partners of the DDB1-CUL4A-ROC1 complex. *DDB1* expression is required, together with *MLL,* for proper *CDKN2A* transcriptional activation[39]. Thus, inactivating mutations of *DDB1* are likely to contribute to cancer not only by impairing NER, but also by preventing the transcription of tumor suppressor genes. It remains to be seen if the described example of a concerted action of *DDB1* and *MLL* is unique or if there is a systematic relationship between these two genes that might play a role in hematologic malignancies.

In the remaining three samples that were whole exome sequenced (samples 42, 43 and 50) we identified a number of SNVs and small indels that we could validate by

Sanger sequencing (Supplementary Table 3). Only one gene appeared recurrent in this dataset, *HEPHL1*. Two patients (samples 42 and 50) harbored both an SNV in the *HEPHL1* gene as indicated in Supplementary Table 3. The function of *HEPHL1* is not known. As we did not have control tissue available from these patients, we were unable to identify the somatic or germline origin of these variants.

Tandem duplication in exon 3 of MLL associated with 11q UPD

In order to find small scale genetic alterations that are either too small to be detected by Affymetrix microarrays or too large to be detected by standard exome sequencing pipelines, we analyzed exome coverage data that we gained after alignment of the short sequence reads to the human reference genome. We compared the coverage data of each of the five exome datasets to a set of control samples to identify regions of focal deletions or gains on chromosome 11q. In sample 50, we were able to detect a focal amplification in exon 3 of *MLL* (ensembl gene ID: ENSG00000118058) (Figure 4B). Independent analysis by Sanger sequencing revealed a 513 bp tandem duplication in *MLL* exon 3. This duplication translates to an in-frame duplication of 171 amino acids from position 528 to 698 of the MLL protein (uniprot ID Q03164-1) (Figure 4B). We did not have control tissue of this patient available to confirm the somatic origin of this duplication. However, the duplication was not present in 196 control subjects ruling out the possibility of a common germline polymorphism. Tandem duplications in *MLL* have been described but usually affect the region from exon 3 to exon 9, 10 or 11 [8]. Small tandem duplications such as the 513 bp within exon 3 detected in our study have not been reported so far.

Chromosome 11p defects associate with de novo AML or target LMO2

On chromosome 11p, we identified a total of 4 CDRs (Figure 2). The most telomeric CDR contained 14 genes. Interestingly, we found a significant association of aberrations spanning this CDR with de novo AML compared to secondary AML (P = 0.013). It is likely that one or more of the genes in this region play a particular role in *de novo* AML pathogenesis. The most centromeric CDR on chromsome 11p, defined by a deletion in sample 39 contains the *LMO2* gene (ensembl gene ID: ENSG00000135363). In sample 32, where we detected a deletion spanning the *LMO2* locus (Supplementary Table 1), we also found an SNV in *LMO2* in the remaining allele (c.G388A; p.G130S, Uniprot ID P25791-3) that was hemizygous in Sanger sequencing traces (Figure 4C). The *Polyphen2* tool estimated the variant to be “probably damaging” with the highest probability score of 1. Due to lack of control tissue in this patient we could not analyze the somatic or germline origin of this SNV. Based on the available data we postulate that there is a full loss of *LMO2* activity in this patient. We tested all other patients with aberrations overlapping the *LMO2* locus, but were unable to find any mutations in the coding region or at splice sites of *LMO2* (data not shown). The deletions were detected across several different pathologies. *LMO2* is frequently involved in translocations in T-cell leukemia [40]. It is expressed in different fetal tissues [41] and the full knockout in the mouse is known to be embryonic lethal [42]. Warren et al. showed that *LMO2* is essential for erythroid development in the mouse. Deficiency in erythropoiesis was detected at E9.75. The group confirmed that this defect is intrinsic to the hematopoietic system and specific for the erythroid lineage by in vitro differentiation assays [42]. Interestingly the patient in our study showed anemia with an hemoglobin level of 97 g/L at the time of sampling.

Concluding remarks and perspectives

In this study we applied a chromosome centered genetic analysis of myeloid malignancies. The rationale of this approach is that those chromosomes that exhibit frequent chromosomal defects might also harbor point mutations in the target genes of deletions, gains or UPD. Combining SNP microarray analysis and exome sequencing may increase the likelihood of identification of novel tumor suppressor genes or oncogenes. Applying this approach we systematically analyzed chromosome 11 in myeloid malignancies and detected a large complexity of genetic aberrations especially in patients with AML (*de novo* or secondary to MPN and MDS). The various genetic lesions of chromosome 11 in myeloid malignancies target *CBL, MLL, DDB1, LMO2* and possibly other tumor suppressor genes that we could not identify in this study. The marked cytogenetic complexity associated with AML points towards a highly individual course of disease progression in each patient and might explain the current difficulty in treating patients that have transformed to AML.

Our data indicates that genetic stratification of patients into comparable groups at advanced disease stage will be extremely challenging or impossible due to highly individual mutagenesis profiles. Despite individual mutagenesis profiles, it is possible that common molecular features may emerge (based on gene expression and/or protein phosphorylation profiles). Systems level approaches may help in overcoming this obstacle of genetic heterogeneity, opening up the possibility of targeted therapies in the future. Based on current knowledge, treatment efforts in the chronic phase of myeloid malignancies should not only focus on correction of blood counts but also focus on prevention of disease progression as therapeutic intervention in advance disease stages are predicted to be difficult as the genetic complexity of tumors reach an immense scale.

Material and Methods

Ethics statement

Peripheral blood samples were collected from patients after written informed consent. Sample collection was approved by local ethics committees. These were the “Ethik Kommission der Medizinischen Universität Wien” for samples collected in Austria, the “Comitato di Bioetica” for samples collected at the Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Policlinico San Matteo, Pavia, Italy, the “Local Ethical Committee of Azienda Ospedaliera-Universitaria Careggi, Firenze” for samples collected at the University of Florence, Italy, the Ethics Committee of University Hospital Brno for samples collected at the Masaryk University Brno, Czech Republic, and the ”Eticki odbor Klinickog centra Srbije” for samples collected at the University of Belgrade, Serbia.

Patient samples

We analyzed a total of 813 samples from 773 patients. For 40 patients we had two samples available from different disease stages. Detailed information on the studied cohort is provided in Table 1. Genomic DNA was isolated from whole blood, granulocytes or mononuclear cell fractions according to standard procedures. For a subset of patients we had control tissue DNA available, extracted from either buccal mucosa cells, T lymphocyte fractions of peripheral blood or cultured skin fibroblasts.

Microarray analysis and whole exome sequencing

The genomic DNA was processed and hybridized to Genome-Wide Human SNP 6.0 arrays (Affymetrix, Santa Clara, CA) according to the manufacturer’s instructions.

Chromosomal copy number changes and UPDs were detected using the Genotyping Console version 3.0.2 software (Affymetrix).

Five tumor samples (30, 36, 42, 43 and 50) and two matched control samples (30c and 36c) were analyzed by whole exome sequencing (Supplementary Table 1). Genomic DNA libraries were generated either by using the NEBNext DNA Sample Prep Reagent Set 1 (New England Biolabs, Ipswich, MA) for sample 30 or the TruSeq DNA Sample Prep-Kit v2 (Illumina, San Diego, CA) for samples 36, 42, 43, 50, 30c and 36c. Whole exome enrichment was performed using the Sure Select Human All Exon Kit (Agilent, Santa Clara, CA) for sample 30 or the TruSeq Exome Enrichment Kit (Illumina) for the six other samples. The exome - enriched libraries were hybridized to Illumina flowcells V1 (sample 30) or V3 (other samples) and sequenced using the Illumina HiSeq 2000 instrument. A summary of all samples and sequencing parameters is provided in Supplementary Table 2. The sequence reads were aligned against the human reference genome (hg18) using BWA v0.5.9 [43]. Subsequently, the aligned samples were post processed using GATK v1.5 [44] following their best practices guidelines (v3). Briefly, this comprises marking PCR-duplicate reads, recalibrating the base quality scores and local realignment around insertions/deletions (indels). Variant discovery was performed on the post-processed alignment files using GATK's Unified Genotyper [45]. The final variant lists were generated using GATK's Variant Quality Score Recalibrator using the suggested filtering parameters.

For samples 30 and 36 where control tissue DNA was whole exome sequenced (samples 30c and 36c) we performed an analysis for somatic mutations by using the *VarScan2* software with default parameters[46] starting from the post – processed alignment files generated by GATK.

For samples 42, 43 and 50 the final variant lists of the GATK Unified Genotyper were filtered for single nucleotide variants (SNVs) and indels on chromosome 11 that were passing filter criteria according to the GATK best practice guidelines v3 and that were not annotated in dbSNP137. Gene annotation was done using the *ANNOVAR* tool version 2012-02-23 [47]

Coverage analysis from whole exome sequencing data

The analysis was performed for the five tumor samples, which had been whole exome sequenced. *Samtools 0.1.18* [48] was used with the "depth" option to retrieve coverage data for chromosome 11 from the post – processed alignment files generated by the GATK analysis pipeline. The coverage for each base on chromosome 11 in a particular patient was normalized by the summarized coverage of all bases of chromosome 11 in that particular patient. The normalized coverage of sample 30 was compared to the median normalized coverage of a set of 5 independent control samples that had been processed and whole exome sequenced with similar chemistry and instrumentation as sample 30. A similar adequate control set of 8 independent control samples was generated for samples 36, 42, 43 and 50. All of the control samples used showed wild-type chromosome 11 as analyzed by Genome-Wide Human SNP 6.0 arrays (Affymetrix, data not shown).

PCR, Sanger sequencing, PCR subcloning

Primers for PCR were designed using the *Primer 3* tool (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) or the *ExonPrimer* tool (http://ihg.gsf.de/ihg/ExonPrimer.html) except for the primers amplifying *CBL* exons 8 and 9 which were taken from a publication by Sanada et al [27]. Primer

sequences and PCR conditions are listed in Supplementary Table 4. PCRs were performed using the AmpliTaq Gold DNA Polymerase with Gold Buffer and MgCl2 solution (Applied Biosystems / Life Technologies, Paisley, UK) or the AmpliTaq Gold 360 Mastermix (Applied Biosystems). Sanger sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit and the 3130xl Genomic Analyzer (Applied Biosystems). Sequence analysis was done using the Sequencher Software 4.9 (Gene Codes, Ann Arbor, MI). For PCR product subcloning the TOPO Cloning Kit (Invitrogen / Life Technologies, Paisley, UK) was used according to manufacturer's instructions. PCR products derived from single bacterial clones were sequenced as described above.

Statistical analysis and plots

Fisher's exact tests were performed using Graphpad QuickCalcs (www.graphpad.com/quickcalcs). The plots depicting cohort distributions in Figure 1 were done using R version 2.8.1 (2008-12-22) [49]. The coverage plot in Figure 4B and the signal intensity plot in Figure 4C were done using GraphPad Prism version 5.0d for Mac OS X, GraphPad Software (San Diego, CA), www.graphpad.com.

References

1. Sverdlow S, Campl E, Harris N, Jaffe E, Pileri S, et al. (2008) WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon: International Agency for Research on Cancer.

2. Oshimura M, Freeman AI, Sandberg AA (1977) Chromosomes and causation of human cancer and leukemia. XXVI. Binding studies in acute lymphoblastic leukemia (ALL). Cancer 40: 1161-1172.

3. Ziemin-van der Poel S, McCabe NR, Gill HJ, Espinosa R, 3rd, Patel Y, et al. (1991) Identification of a gene, MLL, that spans the breakpoint in 11q23 translocations associated with human leukemias. Proc Natl Acad Sci U S A 88: 10735-10739.

4. Gu Y, Nakamura T, Alder H, Prasad R, Canaani O, et al. (1992) The t(4;11) chromosome translocation of human acute leukemias fuses the ALL-1 gene, related to Drosophila trithorax, to the AF-4 gene. Cell 71: 701-708.

5. Liu H, Cheng EH, Hsieh JJ (2009) MLL fusions: pathways to leukemia. Cancer Biol Ther 8: 1204-1211.

6. Schichman SA, Caligiuri MA, Gu Y, Strout MP, Canaani E, et al. (1994) ALL-1 partial duplication in acute leukemia. Proc Natl Acad Sci U S A 91: 6236-6239.

7. Caligiuri MA, Strout MP, Schichman SA, Mrozek K, Arthur DC, et al. (1996) Partial tandem duplication of ALL1 as a recurrent molecular defect in acute myeloid leukemia with trisomy 11. Cancer Res 56: 1418-1425.

8. Schnittger S, Kinkelin U, Schoch C, Heinecke A, Haase D, et al. (2000) Screening for MLL tandem duplication in 387 unselected patients with AML identify a prognostically unfavorable subset of AML. Leukemia 14: 796-804.

9. Monni O, Knuutila S (2001) 11q deletions in hematological malignancies. Leuk Lymphoma 40: 259-266.

10. Gupta M, Raghavan M, Gale RE, Chelala C, Allen C, et al. (2008) Novel regions of acquired uniparental disomy discovered in acute myeloid leukemia. Genes Chromosomes Cancer 47: 729-739.

11. Thoennissen NH, Krug UO, Lee DH, Kawamata N, Iwanski GB, et al. (2010) Prevalence and prognostic impact of allelic imbalances associated with leukemic transformation of Philadelphia chromosome-negative myeloproliferative neoplasms. Blood 115: 2882-2890.

12. Stegelmann F, Bullinger L, Griesshammer M, Holzmann K, Habdank M, et al. (2010) High-resolution single-nucleotide polymorphism array-profiling in myeloproliferative neoplasms identifies novel genomic aberrations. Haematologica 95: 666-669.

13. Klampfl T, Harutyunyan A, Berg T, Gisslinger B, Schalling M, et al. (2011) Genome integrity of myeloproliferative neoplasms in chronic phase and during disease progression. Blood 118: 167-176.

14. Rumi E, Harutyunyan A, Elena C, Pietra D, Klampfl T, et al. (2011) Identification of genomic aberrations associated with disease transformation by means of high-resolution SNP array analysis in patients with myeloproliferative neoplasm. Am J Hematol 86: 974-979.

15. Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, et al. (2005) A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med 352: 1779-1790.

16. Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, et al. (2005) Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential

thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell 7: 387-397.

17. James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, et al. (2005) A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature 434: 1144-1148.

18. Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, et al. (2005) Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet 365: 1054-1061.

19. Pardanani AD, Levine RL, Lasho T, Pikman Y, Mesa RA, et al. (2006) MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. Blood 108: 3472-3476.

20. Szpurka H, Gondek LP, Mohan SR, Hsi ED, Theil KS, et al. (2009) UPD1p indicates the presence of MPL W515L mutation in RARS-T, a mechanism analogous to UPD9p and JAK2 V617F mutation. Leukemia 23: 610-614.

21. Buxhofer-Ausch V, Gisslinger H, Berg T, Gisslinger B, Kralovics R (2009) Acquired resistance to interferon alpha therapy associated with homozygous MPL-W515L mutation and chromosome 20q deletion in primary myelofibrosis. Eur J Haematol 82: 161-163.

22. Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, et al. (2009) Mutation in TET2 in myeloid cancers. N Engl J Med 360: 2289-2301.

23. Tefferi A, Pardanani A, Lim KH, Abdel-Wahab O, Lasho TL, et al. (2009) TET2 mutations and their clinical correlates in polycythemia vera, essential thrombocythemia and myelofibrosis. Leukemia 23: 905-911.

24. Fitzgibbon J, Smith LL, Raghavan M, Smith ML, Debernardi S, et al. (2005) Association between acquired uniparental disomy and homozygous gene mutation in acute myeloid leukemias. Cancer Res 65: 9152-9154.

25. Dunbar AJ, Gondek LP, O'Keefe CL, Makishima H, Rataul MS, et al. (2008) 250K single nucleotide polymorphism array karyotyping identifies acquired uniparental disomy and homozygous mutations, including novel missense substitutions of c-Cbl, in myeloid malignancies. Cancer Res 68: 10349-10357.

26. Grand FH, Hidalgo-Curtis CE, Ernst T, Zoi K, Zoi C, et al. (2009) Frequent CBL mutations associated with 11q acquired uniparental disomy in myeloproliferative neoplasms. Blood 113: 6182-6192.

27. Sanada M, Suzuki T, Shih LY, Otsu M, Kato M, et al. (2009) Gain-of-function of mutated C-CBL tumour suppressor in myeloid neoplasms. Nature 460: 904-908.

28. Thien CB, Langdon WY (2001) Cbl: many adaptations to regulate protein tyrosine kinases. Nat Rev Mol Cell Biol 2: 294-307.

29. Schmidt MH, Dikic I (2005) The Cbl interactome and its functions. Nat Rev Mol Cell Biol 6: 907-918.

30. Dohner H, Stilgenbauer S, James MR, Benner A, Weilguni T, et al. (1997) 11q deletions identify a new subset of B-cell chronic lymphocytic leukemia characterized by extensive nodal involvement and inferior prognosis. Blood 89: 2516-2522.

31. Luttikhuis ME, Powell JE, Rees SA, Genus T, Chughtai S, et al. (2001) Neuroblastomas with chromosome 11q loss and single copy MYCN comprise a biologically distinct group of tumours with adverse prognosis. Br J Cancer 85: 531-537.

32. Chen CS, Sorensen PH, Domer PH, Reaman GH, Korsmeyer SJ, et al. (1993) Molecular rearrangements on chromosome 11q23 predominate in infant acute lymphoblastic leukemia and are associated with specific biologic variables and poor outcome. Blood 81: 2386-2393.

33. Makishima H, Cazzolli H, Szpurka H, Dunbar A, Tiu R, et al. (2009) Mutations of e3 ubiquitin ligase cbl family members constitute a novel common pathogenic lesion in myeloid malignancies. J Clin Oncol 27: 6109-6116.

34. Yoshida K, Sanada M, Shiraishi Y, Nowak D, Nagata Y, et al. (2011) Frequent pathway mutations of splicing machinery in myelodysplasia. Nature 478: 64-69.

35. Score J, Hidalgo-Curtis C, Jones AV, Winkelmann N, Skinner A, et al. (2012) Inactivation of polycomb repressive complex 2 components in myeloproliferative and myelodysplastic/myeloproliferative neoplasms. Blood 119: 1208-1213.

36. Dualan R, Brody T, Keeney S, Nichols AF, Admon A, et al. (1995) Chromosomal localization and cDNA cloning of the genes (DDB1 and DDB2) for the p127 and p48 subunits of a human damage-specific DNA binding protein. Genomics 29: 62-69.

37. Angers S, Li T, Yi X, MacCoss MJ, Moon RT, et al. (2006) Molecular architecture and assembly of the DDB1-CUL4A ubiquitin ligase machinery. Nature 443: 590-593.

38. Iovine B, Iannella ML, Bevilacqua MA (2011) Damage-specific DNA binding protein 1 (DDB1): a protein with a wide range of functions. Int J Biochem Cell Biol 43: 1664-1667.

39. Kotake Y, Zeng Y, Xiong Y (2009) DDB1-CUL4 and MLL1 mediate oncogene-induced p16INK4a activation. Cancer Res 69: 1809-1814.

40. Royer-Pokora B, Loos U, Ludwig WD (1991) TTG-2, a new gene encoding a cysteine-rich protein with the LIM motif, is overexpressed in acute T-cell leukaemia with the t(11;14)(p13;q11). Oncogene 6: 1887-1893.

41. Foroni L, Boehm T, White L, Forster A, Sherrington P, et al. (1992) The rhombotin gene family encode related LIM-domain proteins whose differing expression suggests multiple roles in mouse development. J Mol Biol 226: 747-761.

42. Warren AJ, Colledge WH, Carlton MB, Evans MJ, Smith AJ, et al. (1994) The oncogenic cysteine-rich LIM domain protein rbtn2 is essential for erythroid development. Cell 78: 45-57.

43. Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25: 1754-1760.

44. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, et al. (2010) The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 20: 1297-1303.

45. DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, et al. (2011) A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet 43: 491-498.

46. Koboldt DC, Zhang Q, Larson DE, Shen D, McLellan MD, et al. (2012) VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Res 22: 568-576.

47. Wang K, Li M, Hakonarson H (2010) ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res 38: e164.

48. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, et al. (2009) The Sequence Alignment/Map format and SAMtools. Bioinformatics 25: 2078-2079.

49. R Development Core Team (2008) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Figure legends

Figure 1: Cohort distribution.

A: Distribution of the 813 samples analyzed by Affymetrix microarrays according to diagnosis. **B:** Fraction of samples that harbor chromosome 11 aberrations (black bars) for each disease entity in percent. The P-value indicates an association of chromosome 11 aberrations with disease progression in MPN. MPN, myeloproliferative neoplasm; CML, chronic myeloid leukemia; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome.

Figure 2: Summary of chromosome 11 aberrations.

Large chromosomal aberrations are indicated with colored bars around the ideogram of chromosome 11. Green – gains; red – deletions; blue – uniparental disomies. The position of the bars relative to the chromosome ideogram indicates the position and size of the aberration. For the two patients of which two samples were analyzed (UPN 23 and UPN 42 – see Supplementary Table 1) recurrent aberrations are depicted only once. The positions of *CBL, MLL, EED, SF1*, *DDB1* and *LMO2* are indicated by vertical lines. Mutations in these genes are depicted by orange circles along these lines. Common deleted regions are indicated at the bottom of Figure 2 listing the genes they cover.

Figure 3: Mutational patterns in *CBL*.

A: Sample 45 had a 6 bp tandem duplication in CBL leading to the insertion of the

amino acids valine (V) and aspartic acid (D) after position 390. **B:** One sample identified in a cohort screen for mutations in *CBL* exons 8 and 9 carried two mutations, one in exon 8 (W408C) and a second one in intron 8 at the splice acceptor site (G to A). PCR subcloning and analysis of colony DNA revealed that the two mutations are on different alleles. Depicted are two representative colonies. Colony 43 has the mutation in exon 8 but not in intron 8 whereas colony 17 shows the opposite case. **A,B:** Depicted are the genomic (letters) as well as the respective amino acid (box chains) sequences. Numbers indicate amino acid positions in the Cbl protein. Amino acids, which are substituted due to mutations are in red boxes. The splice site alteration is a red circle. Black arrows indicate the positions of the mutations below the Sanger sequencing traces. **C:** Overview of *CBL* mutagenesis in MPN. Different genetic mechanisms are involved in increasing mutant gene dosage of *CBL*. Each panel shows schematically the two parental copies of chromosomes 11 (blue and yellow) and the *CBL* gene (white rectangles). Mutations are indicated with asterisks. From left to right: heterozygous mutation in *CBL*; uniparental disomy introduces homozygous *CBL* mutations; gain of a part of chromosome 11q leads to a duplication of the *CBL* mutation while one wild type allele is still present; compound heterozygosity established by two different mutations on the different alleles of the *CBL* gene in one cell. In addition, the loss of a part of chromosome 11q deleting one *CBL* allele and leaving the other allele unaffected (wild type *CBL*) is likely to introduce phenotypes due to haploinsufficiency.

Figure 4: Mutations detected in *DDB1, MLL* and *LMO2*.

A: Sample 36 harbored an 11q UPD as indicated by the blue bar below the chromosome 11 ideogram. We found two somatic mutations in *DDB1* and *CBL*. As

can be seen in the Sanger sequencing traces, both mutations are homozygous due to amplification by the UPD. **B:** In sample 50 a tandem duplication in *MLL* exon 3 was detected. The top graph shows whole exome coverage data across *MLL* exon 3. The data is plotted as the log2 ratio of the normalized exome sequencing coverage in the patient sample divided by the median normalized coverage of 8 independent control samples at each genomic position (X-axis). The position of the duplication is indicated by the red bar. Sanger sequencing confirmed an in-frame tandem duplication of 171 amino acids as shown at the bottom. **C:** A common deleted region on chromosome 11p targets *LMO2*. All deletions in the analyzed cohort that span the *LMO2* locus are depicted next to the chromosome 11 ideogram. Red bars indicate deletions, green bars indicate gains. In sample 42, which harbored a deletion spanning the LMO2 locus, we also detected a point mutation in *LMO2*. The middle section shows a signal intensity plot measuring copy number from Affymetrix microarrays. The plot depicts signal intensity (log_2 scale) differences between the patient and a healthy control pool for each probe (as implemented in the Affymetrix Genotyping Console software). The deletion in sample 42 can be seen as the deviation from 0 for all probes in the deleted genomic region (X-axis). The point mutation in *LMO2* as identified by Sanger sequencing is depicted at the bottom of panel C. **A,B and C:** Depicted are the genomic (letters) as well as the respective amino acid (box chains) sequences. Numbers above the boxes indicate amino acid positions in the proteins. Amino acids substituted in the patient samples are indicated by red boxes. The red circle indicates a splice site mutation. Reference and mutant sequences are shown. The arrows indicate the site of mutations below the Sanger sequencing traces.

Table 1: Cohort descriptives.

disease entity	specific diagnosis	total n	n with chr 11 lesions
MPN	Polycythemia vera	177	3
	post-PV MF	48	3
	post-PV AML	19	3
	Essential thrombocythemia	91	2
	post-ET MF	18	1
	post-ET AML	9	1
	Primary Myelofibrosis	85	5
	post-PMF AP	7	0
	post-PMF AML	16	6
MDS	MDS (chronic phase)	61	3
	post-MDS AML	40	5
de novo AML	*de novo* AML	180	19
CML	CML	62	1
total		*813*	*52*

n, number of samples; MPN, myeloproliferative neoplasms; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; PV, polycythemia vera; ET, essential thrombocythemia; PMF, primary myelofibrosis; MF, myelofibrosis; AP, accelerated phase; chr, chromosome

Figure 1

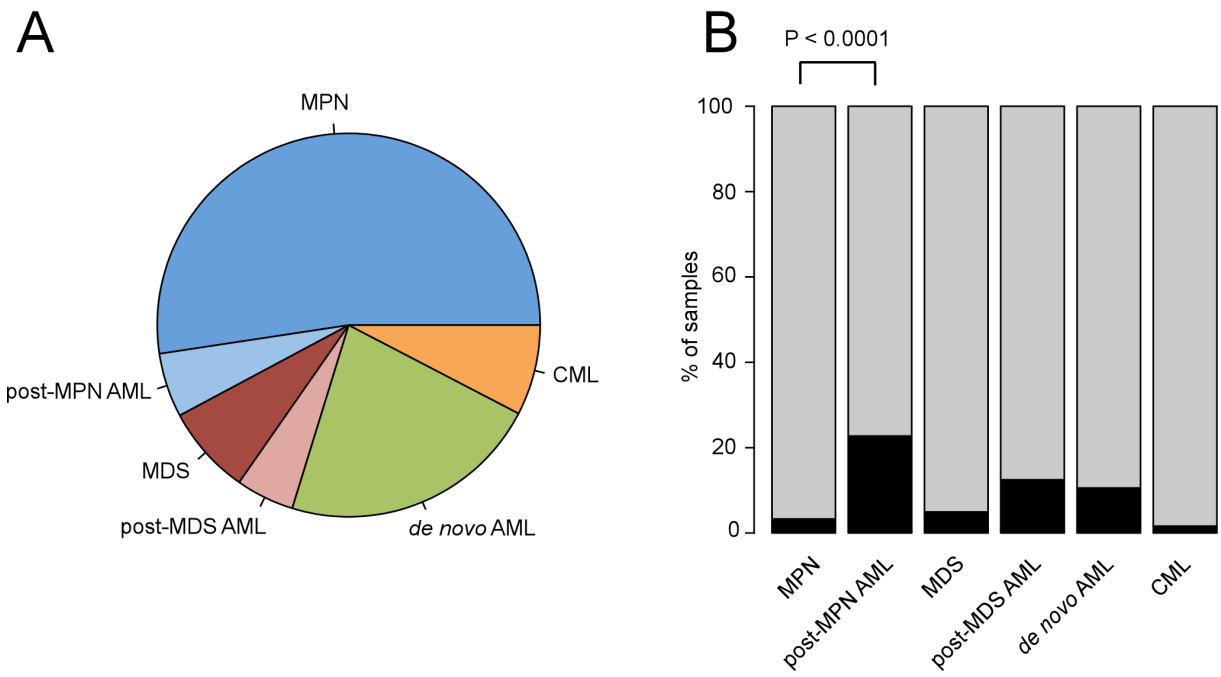


Figure 2

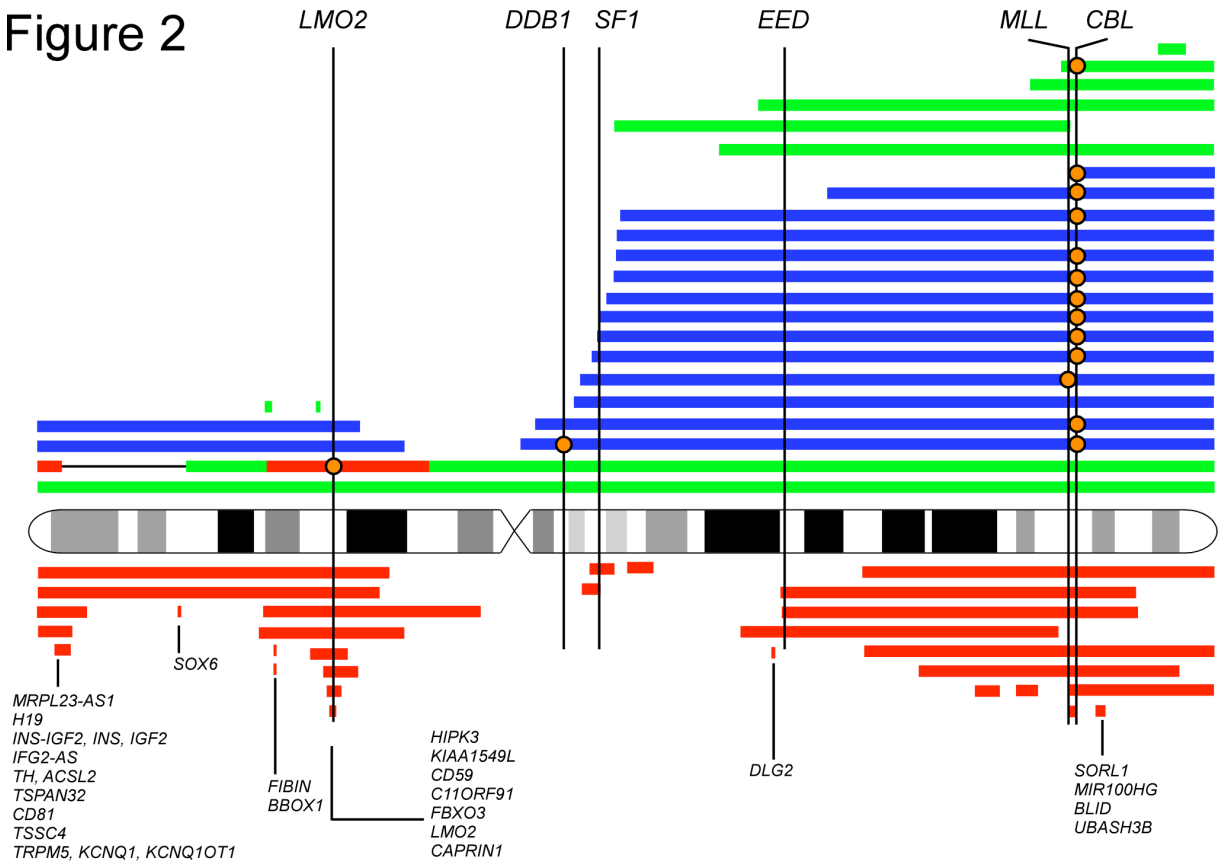
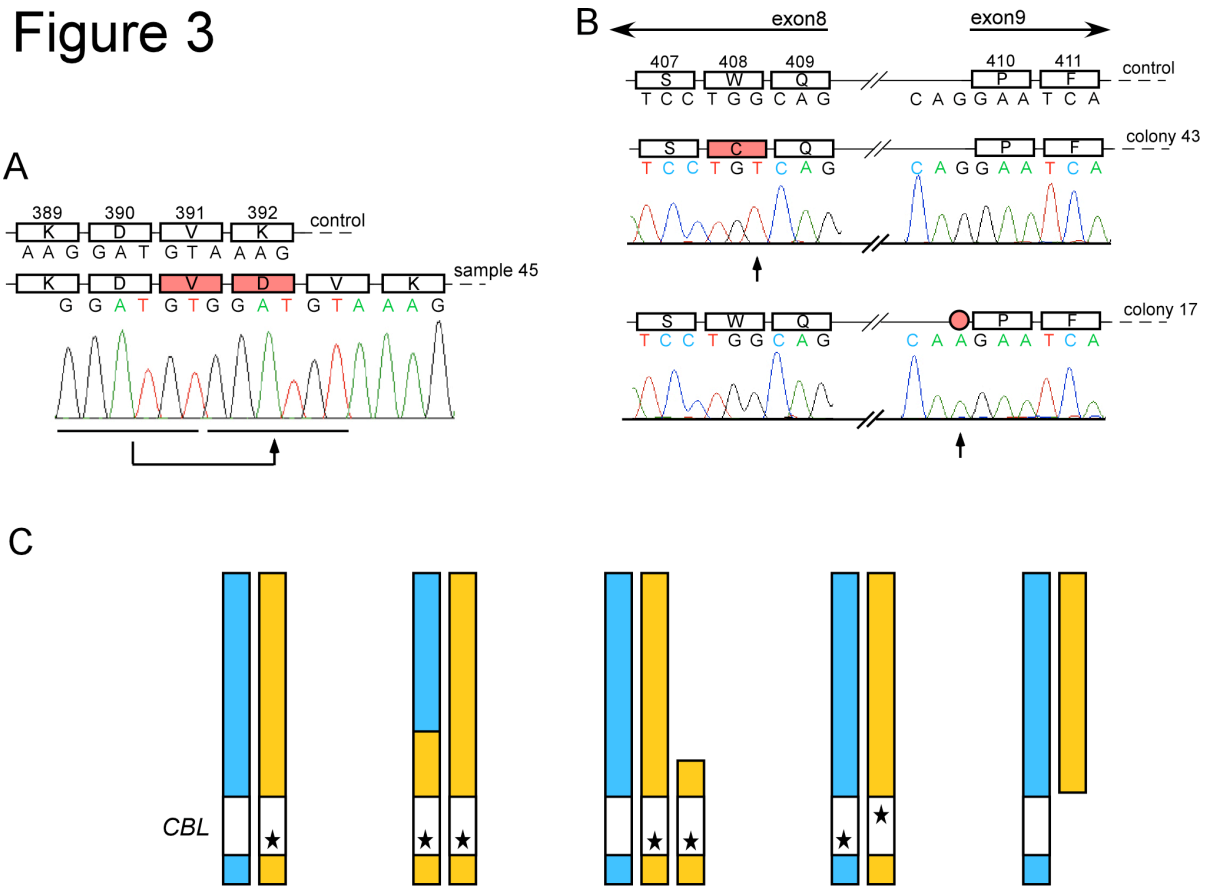


Figure 3


A
389 390 391 392
K D V K
A A G G A T G T A A A G
control
K D V D V K
G G A T G T G G A T G T A A A G
sample 45
B
exon8
exon9
407 408 409 410 411
S W Q P F
T C C T G G C A G C A G G A A T C A
control
S C Q P F
T C C T G T C A G C A G G A A T C A
colony 43
S W Q P F
T C C T G G C A G C A A G A A T C A
colony 17
C
CBL

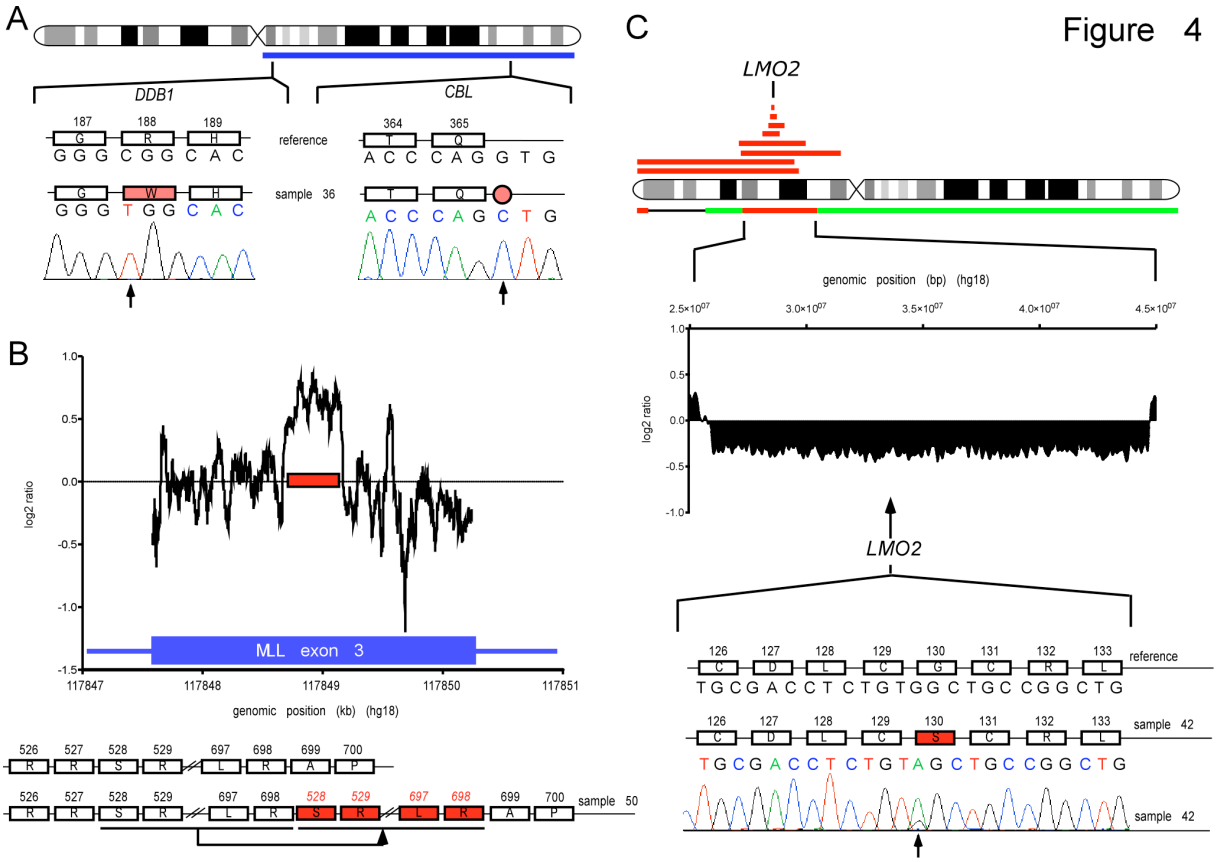

A
DDB1
CBL
187 188 189
G R H
G G G C G G C A C
reference
364 365
T Q
A C C C A G G T G
G W H
G G G T G G C A C
sample 36
T Q
A C C C A G C T G
B
log2 ratio
1.0
0.5
0.0
-0.5
-1.0
-1.5
MLL exon 3
117847 117848 117849 117850 117851
genomic position (kb) (hg18)
526 527 528 529 697 698 699 700
R R S R L R A P
526 527 528 529 697 698 528 529 697 698 699 700
R R S R L R S R L R A P
sample 50
C
LMO2
genomic position (bp) (hg18)
2.5×10^07 3.0×10^07 3.5×10^07 4.0×10^07 4.5×10^07
log2 ratio
1.0
0.5
0.0
-0.5
-1.0
LMO2
126 127 128 129 130 131 132 133
C D L C G C R L
T G C G A C C T C T G T G G C T G C C G G C T G
reference
126 127 128 129 130 131 132 133
C D L C S C R L
T G C G A C C T C T G T A G C T G C C G G C T G
sample 42
sample 42

Figure 4

4. GENERAL DISCUSSION

Since the results of the two studies presented in this thesis have been discussed in the respective manuscripts, I want to focus in this section on two additional aspects. First I will discuss the very recent literature that is related to what we published in manuscript #1 and that was published after this paper. Second, I will put our findings in a larger context discussing the overall genetic complexity that has been discovered in MPN. I will describe possible implications for our understanding of the disease biology and will give an outlook on the relevance of genetic studies for future treatment of MPN.

4.1 Recent literature in the context of the work presented in manuscript #1

The study published in *Blood* is still the largest study reported using high resolution microarray analysis in MPN (Milosevic & Kralovics, 2013). We were able to reproduce findings from classical cytogenetics such as the fact that deletions of chromosome 13 and 20, trisomies of chromosomes 8 and 9, duplications of chromosome 1q, as well as uniparental disomies of chromosome 9 are the most common aberrations in MPN (Amiel et al, 1995; Bacher et al, 2005; Kralovics et al, 2002; Reilly et al, 1997; Swolin et al, 1988; Tefferi et al, 2001). In addition we could confirm known and identify novel target genes of aberrations such as *FOXP1*, *TET2* (Delhommeau et al, 2009), *IKZF1* (Jager et al, 2010), *CUX1*, *ETV6* and *RUNX1* on chromosomes 3p, 4q, 7p, 7q, 12p and 21q, respectively. All of these genes except for *TET2* are transcription factors. As transcription factors are the key regulators of hematopoiesis (Orkin & Zon, 2008) (compare Figure 1) it is remarkable that a majority of chromosomal lesions appear to target transcription factors in MPN. Some of them, although they have not been mapped as targets of large chromosomal aberrations, have been studied intensively in myeloid malignancies before. *ETV6* has been associated with translocations involving over 40 chromosomal bands and 30 translocation partner genes. Five mechanisms by which *ETV6* translocations mediate leukemogenesis are discussed, among them, loss of function of *ETV6* (De Braekeleer et al, 2012). The latter is consistent with recurrent deletions of *ETV6* that we describe in manuscript #1. *RUNX1* mutations were already discussed in MPN and

have been associated with leukemic transformation (Ding et al, 2009). The functional association of other deletion targets like *FOXP1* or *IKZF1* in MPN is still not well understood. The transcription factor that we mapped as the target of chromosome 7q deletions, *CUX1*, was identified as the likely target of chromosome 7q deletions in neoplasms of the uterus (Schoenmakers et al, 2013). The gene was identified when Schoenmakers et al. analyzed primary cell lines of two specific patients that harbored no deletions, but inversions on chromosome 7. One breakpoint of the inversion was within the coding region of *CUX1* in both patients thereby disrupting the gene (Schoenmakers et al, 2013). In MPN translocations with a breakpoint in 7q22, the cytoband containing the *CUX1* gene, have been described (Tefferi et al, 2001). It remains to be seen if *CUX1* is the target of such translocations in MPN. A recent study in AML identified a translocation event between chromosomes 7 and 17 that led to the disruption of *CUX1* (McNerney et al, 2013). Also chromsome 7q deletions were detected in this study. RNA sequencing in 23 leukemia samples revealed down-regulation of CUX1 expression in patients with those deletions (McNerney et al, 2013). The group further identified a *CUX1* associated gene expression profile enriched for genes involved in cell cycle. Following a haploinsufficiency model, they analyzed shRNA mediated *CUX1* knockdown in drosophila as well as in a xenograft mouse model. In drosophila they reported overgrowth of hemocytes (myeloid cell equivalents) whereas in the mouse model they showed an engraftment advantage of human hematopoietic progenitors transduced with anti *CUX1* shRNA (McNerney et al, 2013). This work provides the first functional links between *CUX1* deletion and hematopoietic malignancies and underlines our finding that *CUX1* is an important target of chromosome 7q deletions.

We reported in manuscript #1 that chromosome 6p deletions in MPN significantly associated with disease progression to post-MPN AML. This result was followed up in our own lab by Puda et al. (Puda et al, 2012). The study focused on genetic lesions involved in post-MPN and post-MDS AML. A patient was identified that harbored a uniparental disomy of chromosome 6p leading to the duplication of a small chromosomal deletion to homozygosity. This deletion only contained the *JARID2* gene, which is therefore the likely target of chromsome 6p deletions (Puda et al, 2012). On chromosome 12p the authors reported a common deleted region that contained only 5 genes one of which is *AEBP2* and on chromosome 17q a

uniparental disomy amplified a somatic point mutation in *SUZ12*. Also several deletions affected *SUZ12* (Puda et al, 2012). All three genes, *JARID2*, *AEBP2* and *SUZ12* are members of the polycomb repressive complex 2 (PRC2), a multi-subunit protein complex involved in negative regulation of transcription. This is interesting as the core unit of PRC2 contains the *EZH2* gene for which we had reported UPD associated mutations in two patients in manuscript #1. It is therefore likely that the PRC2 complex plays an important role in MPN pathogenesis.

Furthermore the study by Puda et al. implicates that deletions can target multiple tumor-suppressors. In manuscript #1 we showed that *ETV6* is a target of chromosome 12p deletions. Puda et al report two CDRs on 12p, one targeting *ETV6*, another more centromeric one including *AEBP2,* the PRC2 member (Puda et al, 2012). Several large deletions covered both loci presumably conferring additional clonal advantage compared to single gene deletions (Puda et al, 2012). This does not revoke strategies to map common deleted regions and their target genes, it just points to the fact that large deletions although covering a previously identified target gene might also target other tumor-suppressors relevant for the disease. Other recent studies also reported mutations of *SUZ12* and other PRC2 members in MPN and other myeloid malignancies (Brecqueville et al, 2011; Score et al, 2012).

By mapping *TET2* as the commonly deleted target of chromosome 4q in manuscript #1 we confirmed a finding originally published by Delhommeau et al. (Delhommeau et al, 2009). The functional link between *TET2* loss and myeloid malignancies was presented short after our publication by Moran-Crusio and colleagues (Moran-Crusio et al, 2011). In a shRNA mediated knockdown of *TET2* in bone marrow cells they observed a genome wide reduction of 5-hydroxymethylcytosine – the primary product of TET2. This was associated with an increased replating capacity of cells with *TET2* knockdown compared to *TET2* wildtype cells. They used a conditional knockout model in the mouse, confirming that also in the mouse *TET2* knockout leads to increased replating capacity of hematopoietic progenitors in vitro and to an increased self-renewal capacity in vivo. By cell surface marker analysis of *TET2* knockout cells they identified up-regulation of the stem cell marker c-kit and through gene expression profiling they identified expression patterns commonly found in multipotent hematopoietic progenitor cells. In vivo mice showed an increase of the stem cell compartment in the bone marrow as well as marked splenomegaly after 20

weeks post *TET2* deletion. Overall the mice displayed a myeloid malignancy like phenotype (Moran-Crusio et al, 2011).

A study by Rumi and colleagues analyzed 29 MPN patients of whom serial samples for different disease stages were available in order to find chromosomal aberrations associated with disease progression (Rumi et al, 2011). The authors identified chromosome 5, 7 and 17p aberrations to be significantly associated with post-MPN AML. They furthermore showed that the acquisition of one or more of these aberrations, of which chromosome 5 and 7 were also identified in manuscript #1, is associated with inferior survival (Rumi et al, 2011).

4.2 Genetic complexity in MPN

4.2.1 Different affected genes – the same disease

Since the discovery of mutations affecting *JAK2* in virtually all patients with PV and around 50% of patients with ET or PMF, hopes were raised that the genetics of the three classical *BCR-ABL1* - negative MPNs would be as straight forward as the genetics of CML with similar therapeutic successes (one gene – one target – one drug). In recent years however, a number of other genes have been found mutated in MPN albeit with much lower frequencies, often below 10%, depending on the disease entity (Milosevic & Kralovics, 2013). In addition many of these mutations are not disease specific, but occur across several myeloid diseases (Tefferi, 2010). This raises the question in what ways mutations in a number of different genes can contribute to the same disease and if some of the genetic lesions are passengers with no impact on the disease pathogenesis. The fact that a gene is found mutated in multiple patients and across multiple disease entities, however, suggests an importance of that gene for the disease pathogenesis even if the overall mutational frequency is low. The genes discussed in this thesis are therefore unlikely to be passengers. In addition these genes are not random hits in the genome. Many of them have been shown to belong to similar classes of proteins or to be involved in similar pathways or protein complexes what will be discussed in detail in this chapter.

PRC2 complex. One example is the above-mentioned PCR2 complex. *JARID2*, *AEBP2*, *EZH2* or *SUZ12*, genes that have been found mutated or deleted in myeloid diseases, are members of this complex (Brecqueville et al, 2011; Puda et al, 2012; Score et al, 2012). Although the individual mutational frequency of these genes is low in MPN, the mutations account for the same mechanism, which is the disruption of PRC2 mediated transcriptional repression (Puda et al, 2012).

JAK/STAT pathway. Other mutations target similar pathways, most importantly the JAK/STAT pathway. Mutations of *JAK2* affect two thirds of all MPN patients and elicit constitutively activated signaling (Baxter et al, 2005; James et al, 2005; Kralovics et al, 2005; Levine et al, 2005). Activating mutations of the thrombopoietin receptor MPL, which signals via JAK2, are described in around 10% of PV and ET cases (Chaligne et al, 2008; Pardanani et al, 2006; Pikman et al, 2006). We (manuscript #2), and others (Dunbar et al, 2008; Grand et al, 2009; Sanada et al, 2009) reported mutations in the E3 ubiquitin ligase *CBL*. CBL is known to be a negative regulator of cytokine signaling by targeting a variety of signaling associated proteins – among them JAK2 and MPL - for degradation (Schmidt & Dikic, 2005; Thien & Langdon, 2001). Loss of the tumor suppressor function of CBL confers activated signaling through JAK2 (Sanada et al, 2009). *LNK* is a negative regulator of thrombopoietin signaling. Mutations of the gene are described in JAK2-wildtype MPN leading to increased phospho-STAT3/5 levels (Oh et al, 2010). Taken together mutations in *JAK2, MPL, CBL* and *LNK* independently account for activated JAK/STAT signaling.

Transcription factors. Several of the genes reported in manuscript #1 and manuscript #2 including *FOXP1, IKZF1, CUX1, ETV6, RUNX1* or *LMO2* belong to the functional group of transcription factors. Hematopoietic differentiation is regulated by transcription factors and for *ETV6, RUNX1,* o*r LMO2* the role in the hematopoietic system as well as in malignancies is well studied (see above).

Epigenetic modification. Genes involved in epigenetic regulation have recently gained a lot of attention in hematological malignancies, both on the level of DNA methylation as well as on the level of histone modification. *TET2* has been found to be involved in demethylation of DNA (He et al, 2011; Ito et al, 2011). Inactivating mutations or deletions of *TET2* were therefore expected to be associated with DNA hypermethylation. Interestingly, mutations of *IDH1/2*, enzymes involved in citric acid

cycle, have recently been associated with DNA hypermethylation in AML (Figueroa et al, 2010). Mutations in *IDH1* and *IDH2* have been found to be mutually exclusive and they showed comparable DNA hypermethylation profiles distinct from the profiles in normal bone marrow (Figueroa et al, 2010). Furthermore Figueroa et al. showed that *TET2* mutant AML samples displayed similar hypermethylation patterns as the *IDH1/2* mutant samples and that aberrations in *TET2* and the IDH enzymes are also mutually exclusive. They hypothesized, therefore, that both enzymes act by a similar mechanism. Wildtype IDH enzymes had been shown before to produce α-KG from isocitrate, whereas mutant IDH1 and 2 produce 2-HG (Dang et al, 2009). As TET2 is an α-KG dependant enzyme Figueroa et al reasoned, that the catalytic action of TET2 could be inhibited by the accumulation of 2-HG in IDH mutant cells (Figueroa et al, 2010). In their study TET2 transfection into 293T cells led to a decrease in 5-mC levels associated with an increase in 5-hmC levels, as expected. Coexpression of TET2 with mutant IDH1 (but not wildtype IDH1) was able to reverse that observation leading to increase in 5-mC and decrease in 5-hmC. With this finding the authors demonstrated that IDH1/2 mutations likely act via inhibition of TET2 on DNA methylation (Figueroa et al, 2010). The authors thereby functionally linked two frequently mutated genes in myeloid malignancies.

Similar to DNA methylation, histone methylation is another epigenetic mechanism that regulates gene expression. Similar to TET2, Jumonji C - domain containing histone demethylases have also been shown to require α-KG as cofactors (Tsukada et al, 2006). In line with this, Xu and colleagues showed that 2-HG, produced by mutant IDH1/2, is not only a TET inhibitor, but also an inhibitor of histone demethylases (Xu et al, 2011). In glioma, mutant IDH1 was associated with increased histone methylation levels compared to wildtype IDH1 (Xu et al, 2011). Focusing on AML, Sasaki and colleagues published a study on a knock-in mouse model of IDH1-R132H, the most frequently detected IDH1 mutation in AML (Sasaki et al, 2012). When IDH1-R132H was selectively expressed in hematopoietic cells, the mice developed AML like features. Studying the epigenetic regulation in these cells, the authors reported hypermethylated histones, suggesting that mutant IDH1 mediated histone hypermethylation is relevant in AML (Sasaki et al, 2012).

I have discussed the PRC2 complex as another frequently mutated player in histone modification. In manuscript #1 we reported mutations in one of the core component genes of PRC2, the histone methyltransferase *EZH2*. Interestingly, EZH2 has also been linked to DNA methylation via interactions with DNMT1, DNMT3A and DMNT3B and it has been shown that the binding of these DNMTs to their respective target sites on DNA is dependent on EZH2 (Vire et al, 2006). That is interesting, as it seems to be a direct link between histone modification and DNA methylation. DNMT3A is frequently found mutated in myeloid malignacies (Abdel-Wahab et al, 2011; Ley et al, 2010; Stegelmann et al, 2011; Yamashita et al, 2010).

JAK2 has recently been found involved in epigenetic regulation of transcription as well (Dawson et al, 2009). The protein has been detected in the nucleus of cells, where it is involved in histone phosphorylation and thereby regulation of gene expression. Both, wildtype JAK2 and JAK2-V617F mediated histone phosphorylation. One of the genes positively regulated by JAK2 mediated phosphorylation was *LMO2* (Dawson et al, 2009). We reported deletions of *LMO2* in several patients and one patient with a deletion and a point-mutation of *LMO2* in manuscript #2. This suggests a tumor suppressor function of *LMO2* which seems to be in contrast to the over-expression of *LMO2* mediated by JAK2. However, the majority of the patients in our study had either *de novo* or post-MPN AML. It is quite possible that elevated LMO2 expression confers an advantage in the chronic phase of MPN, which is associated with high proliferation and differentiation of hematopoietic progenitors. However at the switch to post-MPN AML *LMO2* expression might not be compatible with the associated differentiation arrest. Further research is needed to clarify the role of LMO2 at different stages of MPN disease development.

Taken together these examples show that the genes found mutated in MPN are linked, either by type (e.g. transcription factors) or more importantly by function. So far these “functional systems” like the JAK signaling pathway or the epigenetic regulators and the transcription factors seem “loosely” interconnected. One crosslink between JAK2 signaling and epigenetic regulation of gene expression is JAK2 itself. Additional studies are needed to clarify if there are further functional links between these systems and if there is something like a single MPN disease network that connects all the aberrantly expressed genes. It would have been hard to imagine that so many individual genes could have led to similar disease phenotypes without any

common biological mechanism. The above-mentioned examples are probably the first insight into a specific MPN network.

4.2.2 The same gene - different diseases

On the other hand many of the mutations reported in MPN also play a role in other myeloid malignancies (Tefferi, 2010). In addition to the question of how aberrations in multiple different genes can contribute to the same phenotype, researchers will also have to answer the question of how the same gene (and in some cases even the same point mutation) can contribute to different disease phenotypes. One explanation could be that the germline genetic background plays a role. Early hints in that direction came from JAK2-V617F mouse models that generated PV like phenotypes on a C57Bl/6 background and a phenotype with erythrocytosis, bone marrow fibrosis and elevated leukocyte counts on a Balb/c background (Wernig et al, 2006). The combination of germline and somatic genetic aberrations has been shown to influence phenotype as well. In a case report from our lab, Harutyunyan et al. presented a PV patient with a heterozygous germline variant in *FANCM* that introduced a premature stop-codon, thereby truncating the protein. The patient somatically acquired a uniparental disomy that duplicated the truncated *FANCM* allele to homozygosity (Harutyunyan et al, 2011). The *FANCM* gene has been associated with the hereditary *Fanconi anemia* disorder. During the course of the disease the affected MPN patient developed anemia most likely associated with the acquisition of the UPD and the full loss of FANCM function (Harutyunyan et al, 2011). As large aberrations are common cytogenetic events in MPN (manuscript #1, manurcript #2) it is plausible that other germline variants could contribute to myeloid malignancies in a similar fashion probably as determinants of the disease phenotype. To take that further, the genetic pathogenesis of different myeloid malignancies could indeed be molecularly similar and relatively minor determinants decide on the specific disease phenotype. From a systems biology perspective it could therefore be meaningful to integrate genetic aberrations from all myeloid malignancies into a “pan-myeloid” disease network that could explain and unite the several pieces of knowledge from the different disease entities.

4.2.3 MPN therapy

Such systems level approaches, be it in MPN or on a pan-myeloid level, could also help to identify novel targets for therapy. So far the only targeted therapy available in MPN is the recently approved JAK1 / JAK2 inhibitor ruxolitinib for therapy of primary and post-PV / post-ET myelofibrosis (LaFave & Levine, 2012). Although this inhibitor is not specific for the JAK2-V617F mutation, patients show a clear reduction of splenomegaly and several constitutional symptoms. The malignant clone however could not be reduced as measured by JAK2-V617F mutational burden. This is discussed as a consequence of the upper dosage limit of ruxolitinib. Side effects include thrombocytopenia and anemia which is why a higher dose of ruxolitinib cannot be administered (LaFave & Levine, 2012). Interestingly the drug shows similar effects in JAK2-V617F positive and JAK2 wildtype MPN. A proposed interpretation was that the reduced cytokine levels observed in patients under ruxolitinib treatment are responsible for the observed effects (LaFave & Levine, 2012). On the basis of an MPN malignancy network another explanation could be possible. Based on the hypothesis that a disease network could be disturbed at different nodes, for example by mutating different genes of that network, one could hypothesize that treatment could also act from different nodes not necessarily only at the node that is mutated. In a simple example, it would be expected that ruxolitinib is also efficient for patients with *MPL* mutations since MPL is upstream of and signaling through JAK2. Indeed such strategies have been shown to work in other settings. Inhibitors of DOT1L are developed as therapeutics against leukemias with MLL-translocations. DOT1L is a protein recruited by MLL-fusion proteins to create a complex for histone methylation (Fathi & Abdel-Wahab, 2012). In another example patients with *BRCA1* or *BRCA2* mutant malignancies were treated with an inhibitor against the DNA-repair enzyme PARP. The treatment successfully demonstrated antitumor activity (Fong et al, 2009). A biological link between BRCA1/2 and PARP was identified previously. Both enzymes are involved in DNA-repair; the BRCAs in DNA double-strand break repair, and PARP in base excision repair. After mutation of BRCA1 or 2 in cancer, the additional drug mediated down-regulation of PARP leads to accumulation of DNA damage driving cells into apoptosis (Farmer et al, 2005).

With the current knowledge on the importance of epigenetic modifiers in MPN pathogenesis, future research will show to what extent inhibitors of epigenetic modifiers are feasible therapeutics. The first inhibitors of the DNA methyltransferases are already in use in AML and MDS and nicely discussed by Fathi and Abdel-Wahab (Fathi & Abdel-Wahab, 2012).

4.3 Summary and concluding remarks

During this thesis I used novel high throughput technologies to contribute to a better understanding of the genetic basis of the classical myeloproliferative neoplasms, a subgroup of blood cancers. Affymetrix Genome Wide Human SNP microarrays 6.0 provide highest resolution genotyping by analyzing over 1.8 million markers across the whole genome. Using this technology, large cohorts of patients can be genotyped at a genome-wide level in reasonable amounts of time. In the two studies presented in this thesis, both, the possibility of high-resolution genotyping as well as the availability of large patient cohorts enabled us to characterize chromosomal lesions to a high degree. In addition the use of whole-exome sequencing in combination with SNP microarrays allowed the identification of possible target genes of large chromosomal aberrations where no target mapping based on microarray data alone was possible. We confirmed already reported genetic aberrations and found novel genes, for some of which independent research groups proved functional relevance. The apparent genetic complexity in MPN seems to resolve in the fact that different mutated genes act in similar pathways or protein complexes. Further studies will show if there is additional biological connectivity between these functional entities and whether a single MPN pathogenesis network exists. Already current knowledge has led to the first targeted therapy in MPN against JAK2 and future therapies will be developed most likely against epigenetic modifiers in the near future. Systems biology approaches will be meaningful to further identify novel drug targets in the myeloproliferative neoplasms and to guide treatment strategies based on mutation patterns of individual patients.

5. REFERENCES

Abdel-Wahab O, Manshouri T, Patel J, Harris K, Yao J, Hedvat C, Heguy A, Bueso-Ramos C, Kantarjian H, Levine RL, Verstovsek S (2010) Genetic analysis of transforming events that convert chronic myeloproliferative neoplasms to leukemias. *Cancer Res* **70**(2)**:** 447-452

Abdel-Wahab O, Pardanani A, Rampal R, Lasho TL, Levine RL, Tefferi A (2011) DNMT3A mutational analysis in primary myelofibrosis, chronic myelomonocytic leukemia and advanced phases of myeloproliferative neoplasms. *Leukemia* **25**(7)**:** 1219-1220

Amiel A, Gaber E, Manor Y, Fejgin M, Joseph-Lerner N, Ravid M, Lishner M (1995) Fluorescence in situ hybridization for the detection of trisomies 8 and 9 in polycythemia vera. *Cancer Genet Cytogenet* **79**(2)**:** 153-156

Andrieux J, Demory JL, Caulier MT, Agape P, Wetterwald M, Bauters F, Lai JL (2003) Karyotypic abnormalities in myelofibrosis following polycythemia vera. *Cancer Genet Cytogenet* **140**(2)**:** 118-123

Aziz A, Baxter EJ, Edwards C, Cheong CY, Ito M, Bench A, Kelley R, Silber Y, Beer PA, Chng K, Renfree MB, McEwen K, Gray D, Nangalia J, Mufti GJ, Hellstrom-Lindberg E, Kiladjian JJ, McMullin MF, Campbell PJ, Ferguson-Smith AC, Green AR (2013) Cooperativity of imprinted genes inactivated by acquired chromosome 20q deletions. *J Clin Invest* **123**(5)**:** 2169-2182

Bacher U, Haferlach T, Kern W, Hiddemann W, Schnittger S, Schoch C (2005) Conventional cytogenetics of myeloproliferative diseases other than CML contribute valid information. *Ann Hematol* **84**(4)**:** 250-257

Bandi SR, Brandts C, Rensinghoff M, Grundler R, Tickenbrock L, Kohler G, Duyster J, Berdel WE, Muller-Tidow C, Serve H, Sargin B (2009) E3 ligase-defective Cbl mutants lead to a generalized mastocytosis and myeloproliferative disease. *Blood* **114**(19)**:** 4197-4208

Barbui T, Thiele J, Passamonti F, Rumi E, Boveri E, Ruggeri M, Rodeghiero F, d'Amore ES, Randi ML, Bertozzi I, Marino F, Vannucchi AM, Antonioli E, Carrai V, Gisslinger H, Buxhofer-Ausch V, Mullauer L, Carobbio A, Gianatti A, Gangat N, Hanson CA, Tefferi A (2011) Survival and disease progression in essential thrombocythemia are significantly influenced by accurate morphologic diagnosis: an international study. *J Clin Oncol* **29**(23)**:** 3179-3184

Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S, Vassiliou GS, Bench AJ, Boyd EM, Curtin N, Scott MA, Erber WN, Green AR (2005) Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet* **365**(9464)**:** 1054-1061

Beer PA, Campbell PJ, Scott LM, Bench AJ, Erber WN, Bareford D, Wilkins BS, Reilly JT, Hasselbalch HC, Bowman R, Wheatley K, Buck G, Harrison CN, Green AR (2008) MPL mutations in myeloproliferative disorders: analysis of the PT-1 cohort. *Blood* **112**(1)**:** 141-149

Bench AJ, Nacheva EP, Hood TL, Holden JL, French L, Swanton S, Champion KM, Li J, Whittaker P, Stavrides G, Hunt AR, Huntly BJ, Campbell LJ, Bentley DR, Deloukas P, Green AR (2000) 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* **19**(34)**:** 3902-3913

Bersenev A, Wu C, Balcerek J, Tong W (2008) Lnk controls mouse hematopoietic stem cell self-renewal and quiescence through direct interactions with JAK2. *J Clin Invest* **118**(8)**:** 2832-2844

Blij-Philipsen MV, Stellink F, Vlasveld LT (1997) Partial trisomy 9 in a patient with polycythemia vera. *Cancer Genet Cytogenet* **97**(1)**:** 81-82

Borgstrom GH, Knuutila S, Ruutu T, Pakkala A, Lahtinen R, de la Chapelle A (1984) Abnormalities of chromosome 13 in myelofibrosis. *Scand J Haematol* **33**(1)**:** 15-21

Brecqueville M, Cervera N, Adelaide J, Rey J, Carbuccia N, Chaffanet M, Mozziconacci MJ, Vey N, Birnbaum D, Gelsi-Boyer V, Murati A (2011) Mutations and deletions of the SUZ12 polycomb gene in myeloproliferative neoplasms. *Blood Cancer J* **1**(8)**:** e33

Bumm TG, Elsea C, Corbin AS, Loriaux M, Sherbenou D, Wood L, Deininger J, Silver RT, Druker BJ, Deininger MW (2006) Characterization of murine JAK2V617F-positive myeloproliferative disease. *Cancer Res* **66**(23)**:** 11156-11165

Buxhofer-Ausch V, Gisslinger H, Berg T, Gisslinger B, Kralovics R (2009) Acquired resistance to interferon alpha therapy associated with homozygous MPL-W515L mutation and chromosome 20q deletion in primary myelofibrosis. *Eur J Haematol* **82**(2)**:** 161-163

Campbell PJ, Green AR (2006) The myeloproliferative disorders. *N Engl J Med* **355**(23)**:** 2452-2466

Capdeville R, Buchdunger E, Zimmermann J, Matter A (2002) Glivec (STI571, imatinib), a rationally developed, targeted anticancer drug. *Nat Rev Drug Discov* **1**(7)**:** 493-502

Chaligne R, Tonetti C, Besancenot R, Roy L, Marty C, Mossuz P, Kiladjian JJ, Socie G, Bordessoule D, Le Bousse-Kerdiles MC, Vainchenker W, Giraudier S (2008) New mutations of MPL in primitive myelofibrosis: only the MPL W515 mutations promote a G1/S-phase transition. *Leukemia* **22**(8)**:** 1557-1566

Chen Z, Notohamiprodjo M, Guan XY, Paietta E, Blackwell S, Stout K, Turner A, Richkind K, Trent JM, Lamb A, Sandberg AA (1998) Gain of 9p in the

pathogenesis of polycythemia vera. *Genes Chromosomes Cancer* **22**(4)**:** 321-324

Cho YS, Kim EJ, Park UH, Sin HS, Um SJ (2006) Additional sex comb-like 1 (ASXL1), in cooperation with SRC-1, acts as a ligand-dependent coactivator for retinoic acid receptor. *J Biol Chem* **281**(26)**:** 17588-17598

Cortes JE, Talpaz M, O'Brien S, Faderl S, Garcia-Manero G, Ferrajoli A, Verstovsek S, Rios MB, Shan J, Kantarjian HM (2006) Staging of chronic myeloid leukemia in the imatinib era: an evaluation of the World Health Organization proposal. *Cancer* **106**(6)**:** 1306-1315

Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, Fantin VR, Jang HG, Jin S, Keenan MC, Marks KM, Prins RM, Ward PS, Yen KE, Liau LM, Rabinowitz JD, Cantley LC, Thompson CB, Vander Heiden MG, Su SM (2009) Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature* **462**(7274)**:** 739-744

Dawson MA, Bannister AJ, Gottgens B, Foster SD, Bartke T, Green AR, Kouzarides T (2009) JAK2 phosphorylates histone H3Y41 and excludes HP1alpha from chromatin. *Nature* **461**(7265)**:** 819-822

De Braekeleer E, Douet-Guilbert N, Morel F, Le Bris MJ, Basinko A, De Braekeleer M (2012) ETV6 fusion genes in hematological malignancies: a review. *Leuk Res* **36**(8)**:** 945-961

Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Masse A, Kosmider O, Le Couedic JP, Robert F, Alberdi A, Lecluse Y, Plo I, Dreyfus FJ, Marzac C, Casadevall N, Lacombe C, Romana SP, Dessen P, Soulier J, Viguie F, Fontenay M, Vainchenker W, Bernard OA (2009) Mutation in TET2 in myeloid cancers. *N Engl J Med* **360**(22)**:** 2289-2301

Ding Y, Harada Y, Imagawa J, Kimura A, Harada H (2009) AML1/RUNX1 point mutation possibly promotes leukemic transformation in myeloproliferative neoplasms. *Blood* **114**(25)**:** 5201-5205

Dingli D, Schwager SM, Mesa RA, Li CY, Dewald GW, Tefferi A (2006) Presence of unfavorable cytogenetic abnormalities is the strongest predictor of poor survival in secondary myelofibrosis. *Cancer* **106**(9)**:** 1985-1989

Dunbar AJ, Gondek LP, O'Keefe CL, Makishima H, Rataul MS, Szpurka H, Sekeres MA, Wang XF, McDevitt MA, Maciejewski JP (2008) 250K single nucleotide polymorphism array karyotyping identifies acquired uniparental disomy and homozygous mutations, including novel missense substitutions of c-Cbl, in myeloid malignancies. *Cancer Res* **68**(24)**:** 10349-10357

Estey EH (2011) Acute myeloid leukemia: 2012 update on diagnosis, risk stratification, and management. *Am J Hematol* **87**(1)**:** 89-99

Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, Santarosa M, Dillon KJ, Hickson I, Knights C, Martin NM, Jackson SP, Smith GC, Ashworth A (2005) Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* **434**(7035)**:** 917-921

Fathi AT, Abdel-Wahab O (2012) Mutations in epigenetic modifiers in myeloid malignancies and the prospect of novel epigenetic-targeted therapy. *Adv Hematol* **2012:** 469592

Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, Li Y, Bhagwat N, Vasanthakumar A, Fernandez HF, Tallman MS, Sun Z, Wolniak K, Peeters JK, Liu W, Choe SE, Fantin VR, Paietta E, Lowenberg B, Licht JD, Godley LA, Delwel R, Valk PJ, Thompson CB, Levine RL, Melnick A (2010) Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell* **18**(6)**:** 553-567

Fisher CL, Pineault N, Brookes C, Helgason CD, Ohta H, Bodner C, Hess JL, Humphries RK, Brock HW (2010) Loss-of-function Additional sex combs like 1 mutations disrupt hematopoiesis but do not cause severe myelodysplasia or leukemia. *Blood* **115**(1)**:** 38-46

Fong PC, Boss DS, Yap TA, Tutt A, Wu P, Mergui-Roelvink M, Mortimer P, Swaisland H, Lau A, O'Connor MJ, Ashworth A, Carmichael J, Kaye SB, Schellens JH, de Bono JS (2009) Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N Engl J Med* **361**(2)**:** 123-134

Garzon R, Volinia S, Liu CG, Fernandez-Cymering C, Palumbo T, Pichiorri F, Fabbri M, Coombes K, Alder H, Nakamura T, Flomenberg N, Marcucci G, Calin GA, Kornblau SM, Kantarjian H, Bloomfield CD, Andreeff M, Croce CM (2008) MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. *Blood* **111**(6)**:** 3183-3189

Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, Jaenisch R (2003) Induction of tumors in mice by genomic hypomethylation. *Science* **300**(5618)**:** 489-492

Gelsi-Boyer V, Trouplin V, Adelaide J, Bonansea J, Cervera N, Carbuccia N, Lagarde A, Prebet T, Nezri M, Sainty D, Olschwang S, Xerri L, Chaffanet M, Mozziconacci MJ, Vey N, Birnbaum D (2009) Mutations of polycomb-associated gene ASXL1 in myelodysplastic syndromes and chronic myelomonocytic leukaemia. *Br J Haematol* **145**(6)**:** 788-800

Georgii A, Buhr T, Buesche G, Kreft A, Choritz H (1996) Classification and staging of Ph-negative myeloproliferative disorders by histopathology from bone marrow biopsies. *Leuk Lymphoma* **22 Suppl 1:** 15-29

Gery S, Gueller S, Chumakova K, Kawamata N, Liu L, Koeffler HP (2007) Adaptor protein Lnk negatively regulates the mutant MPL, MPLW515L associated with myeloproliferative disorders. *Blood* **110**(9)**:** 3360-3364

Grand FH, Hidalgo-Curtis CE, Ernst T, Zoi K, Zoi C, McGuire C, Kreil S, Jones A, Score J, Metzgeroth G, Oscier D, Hall A, Brandts C, Serve H, Reiter A, Chase AJ, Cross NC (2009) Frequent CBL mutations associated with 11q acquired uniparental disomy in myeloproliferative neoplasms. *Blood* **113**(24)**:** 6182-6192

Groffen J, Stephenson JR, Heisterkamp N, de Klein A, Bartram CR, Grosveld G (1984) Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. *Cell* **36**(1)**:** 93-99

Harutyunyan A, Gisslinger B, Klampfl T, Berg T, Bagienski K, Gisslinger H, Kralovics R (2011) Rare germline variants in regions of loss of heterozygosity may influence clinical course of hematological malignancies. *Leukemia* **25**(11)**:** 1782-1784

He YF, Li BZ, Li Z, Liu P, Wang Y, Tang Q, Ding J, Jia Y, Chen Z, Li L, Sun Y, Li X, Dai Q, Song CX, Zhang K, He C, Xu GL (2011) Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. *Science* **333**(6047)**:** 1303-1307

Hungerford DA, Nowell PC (1962) Chromosome studies in human leukemia. III. Acute granulocytic leukemia. *J Natl Cancer Inst* **29:** 545-565

Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y (2011) Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science* **333**(6047)**:** 1300-1303

Jabbour E, Kantarjian H (2012) Chronic myeloid leukemia: 2012 update on diagnosis, monitoring, and management. *Am J Hematol* **87**(11)**:** 1037-1045

Jager R, Gisslinger H, Passamonti F, Rumi E, Berg T, Gisslinger B, Pietra D, Harutyunyan A, Klampfl T, Olcaydu D, Cazzola M, Kralovics R (2010) Deletions of the transcription factor Ikaros in myeloproliferative neoplasms. *Leukemia* **24**(7)**:** 1290-1298

James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, Garcon L, Raslova H, Berger R, Bennaceur-Griscelli A, Villeval JL, Constantinescu SN, Casadevall N, Vainchenker W (2005) A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature* **434**(7037)**:** 1144-1148

Kim K, Choi J, Heo K, Kim H, Levens D, Kohno K, Johnson EM, Brock HW, An W (2008) Isolation and characterization of a novel H1.2 complex that acts as a repressor of p53-mediated transcription. *J Biol Chem* **283**(14)**:** 9113-9126

Kralovics R, Guan Y, Prchal JT (2002) Acquired uniparental disomy of chromosome 9p is a frequent stem cell defect in polycythemia vera. *Exp Hematol* **30**(3)**:** 229-236

Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, Tichelli A, Cazzola M, Skoda RC (2005) A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med* **352**(17)**:** 1779-1790

Kreft A, Buche G, Ghalibafian M, Buhr T, Fischer T, Kirkpatrick CJ (2005) The incidence of myelofibrosis in essential thrombocythaemia, polycythaemia vera and chronic idiopathic myelofibrosis: a retrospective evaluation of sequential bone marrow biopsies. *Acta Haematol* **113**(2)**:** 137-143

Kvasnicka HM, Thiele J (2010) Prodromal myeloproliferative neoplasms: the 2008 WHO classification. *Am J Hematol* **85**(1)**:** 62-69

La Starza R, Wlodarska I, Aventin A, Falzetti D, Crescenzi B, Martelli MF, Van den Berghe H, Mecucci C (1998) Molecular delineation of 13q deletion boundaries in 20 patients with myeloid malignancies. *Blood* **91**(1)**:** 231-237

Lacout C, Pisani DF, Tulliez M, Gachelin FM, Vainchenker W, Villeval JL (2006) JAK2V617F expression in murine hematopoietic cells leads to MPD mimicking human PV with secondary myelofibrosis. *Blood* **108**(5)**:** 1652-1660

LaFave LM, Levine RL (2012) JAK2 the future: therapeutic strategies for JAK-dependent malignancies. *Trends Pharmacol Sci* **33**(11)**:** 574-582

Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ, Boggon TJ, Wlodarska I, Clark JJ, Moore S, Adelsperger J, Koo S, Lee JC, Gabriel S, Mercher T, D'Andrea A, Frohling S, Dohner K, Marynen P, Vandenberghe P, Mesa RA, Tefferi A, Griffin JD, Eck MJ, Sellers WR, Meyerson M, Golub TR, Lee SJ, Gilliland DG (2005) Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell* **7**(4)**:** 387-397

Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, Kandoth C, Payton JE, Baty J, Welch J, Harris CC, Lichti CF, Townsend RR, Fulton RS, Dooling DJ, Koboldt DC, Schmidt H, Zhang Q, Osborne JR, Lin L, O'Laughlin M, McMichael JF, Delehaunty KD, McGrath SD, Fulton LA, Magrini VJ, Vickery TL, Hundal J, Cook LL, Conyers JJ, Swift GW, Reed JP, Alldredge PA, Wylie T, Walker J, Kalicki J, Watson MA, Heath S, Shannon WD, Varghese N, Nagarajan R, Westervelt P, Tomasson MH, Link DC, Graubert TA, DiPersio JF, Mardis ER, Wilson RK (2010) DNMT3A mutations in acute myeloid leukemia. *N Engl J Med* **363**(25)**:** 2424-2433

Li J, Kent DG, Chen E, Green AR (2011a) Mouse models of myeloproliferative neoplasms: JAK of all grades. *Dis Model Mech* **4**(3)**:** 311-317

Li Z, Cai X, Cai CL, Wang J, Zhang W, Petersen BE, Yang FC, Xu M (2011b) Deletion of Tet2 in mice leads to dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies. *Blood* **118**(17)**:** 4509-4518

Mardis ER, Ding L, Dooling DJ, Larson DE, McLellan MD, Chen K, Koboldt DC, Fulton RS, Delehaunty KD, McGrath SD, Fulton LA, Locke DP, Magrini VJ, Abbott RM, Vickery TL, Reed JS, Robinson JS, Wylie T, Smith SM, Carmichael L, Eldred JM, Harris CC, Walker J, Peck JB, Du F, Dukes AF, Sanderson GE, Brummett AM, Clark E, McMichael JF, Meyer RJ, Schindler

JK, Pohl CS, Wallis JW, Shi X, Lin L, Schmidt H, Tang Y, Haipek C, Wiechert ME, Ivy JV, Kalicki J, Elliott G, Ries RE, Payton JE, Westervelt P, Tomasson MH, Watson MA, Baty J, Heath S, Shannon WD, Nagarajan R, Link DC, Walter MJ, Graubert TA, DiPersio JF, Wilson RK, Ley TJ (2009) Recurring mutations found by sequencing an acute myeloid leukemia genome. *N Engl J Med* **361**(11)**:** 1058-1066

McNerney ME, Brown CD, Wang X, Bartom ET, Karmakar S, Bandlamudi C, Yu S, Ko J, Sandall BP, Stricker T, Anastasi J, Grossman RL, Cunningham JM, Le Beau MM, White KP (2013) CUX1 is a haploinsufficient tumor suppressor gene on chromosome 7 frequently inactivated in acute myeloid leukemia. *Blood* **121**(6)**:** 975-983

Milosevic JD, Kralovics R (2013) Genetic and epigenetic alterations of myeloproliferative disorders. *Int J Hematol* **97**(2):183-97

Moran-Crusio K, Reavie L, Shih A, Abdel-Wahab O, Ndiaye-Lobry D, Lobry C, Figueroa ME, Vasanthakumar A, Patel J, Zhao X, Perna F, Pandey S, Madzo J, Song C, Dai Q, He C, Ibrahim S, Beran M, Zavadil J, Nimer SD, Melnick A, Godley LA, Aifantis I, Levine RL (2011) Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. *Cancer Cell* **20**(1)**:** 11-24

Mossuz P, Girodon F, Donnard M, Latger-Cannard V, Dobo I, Boiret N, Lecron JC, Binquet C, Barro C, Hermouet S, Praloran V (2004) Diagnostic value of serum erythropoietin level in patients with absolute erythrocytosis. *Haematologica* **89**(10)**:** 1194-1198

Najfeld V, Montella L, Scalise A, Fruchtman S (2002) Exploring polycythaemia vera with fluorescence in situ hybridization: additional cryptic 9p is the most frequent abnormality detected. *Br J Haematol* **119**(2)**:** 558-566

Nowell PC, Hungerford DA (1960) Chromosome studies on normal and leukemic human leukocytes. *J Natl Cancer Inst* **25:** 85-109

Nowell PC, Hungerford DA (1961) Chromosome studies in human leukemia. II. Chronic granulocytic leukemia. *J Natl Cancer Inst* **27:** 1013-1035

Nowell PC, Hungerford DA (1962) Chromosome studies in human leukemia. IV. Myeloproliferative syndrome and other atypical myeloid disorders. *J Natl Cancer Inst* **29:** 911-931

Oh ST, Simonds EF, Jones C, Hale MB, Goltsev Y, Gibbs KD, Jr., Merker JD, Zehnder JL, Nolan GP, Gotlib J (2010) Novel mutations in the inhibitory adaptor protein LNK drive JAK-STAT signaling in patients with myeloproliferative neoplasms. *Blood* **116**(6)**:** 988-992

Orkin SH, Zon LI (2008) Hematopoiesis: an evolving paradigm for stem cell biology. *Cell* **132**(4)**:** 631-644

Pardanani A, Lasho T, Finke C, Oh ST, Gotlib J, Tefferi A (2010) LNK mutation studies in blast-phase myeloproliferative neoplasms, and in chronic-phase disease with TET2, IDH, JAK2 or MPL mutations. *Leukemia* **24**(10)**:** 1713-1718

Pardanani AD, Levine RL, Lasho T, Pikman Y, Mesa RA, Wadleigh M, Steensma DP, Elliott MA, Wolanskyj AP, Hogan WJ, McClure RF, Litzow MR, Gilliland DG, Tefferi A (2006) MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. *Blood* **108**(10)**:** 3472-3476

Passamonti F, Rumi E, Pungolino E, Malabarba L, Bertazzoni P, Valentini M, Orlandi E, Arcaini L, Brusamolino E, Pascutto C, Cazzola M, Morra E, Lazzarino M (2004) Life expectancy and prognostic factors for survival in patients with polycythemia vera and essential thrombocythemia. *Am J Med* **117**(10)**:** 755-761

Pastore C, Nomdedeu J, Volpe G, Guerrasio A, Cambrin GR, Parvis G, Pautasso M, Daglio C, Mazza U, Saglio G, et al. (1995) Genetic analysis of chromosome 13

deletions in BCR/ABL negative chronic myeloproliferative disorders. *Genes Chromosomes Cancer* **14**(2)**:** 106-111

Pikman Y, Lee BH, Mercher T, McDowell E, Ebert BL, Gozo M, Cuker A, Wernig G, Moore S, Galinsky I, DeAngelo DJ, Clark JJ, Lee SJ, Golub TR, Wadleigh M, Gilliland DG, Levine RL (2006) MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. *PLoS Med* **3**(7)**:** e270

Policitemia GIS (1995) Polycythemia vera: the natural history of 1213 patients followed for 20 years. Gruppo Italiano Studio Policitemia. *Ann Intern Med* **123**(9)**:** 656-664

Puda A, Milosevic JD, Berg T, Klampfl T, Harutyunyan AS, Gisslinger B, Rumi E, Pietra D, Malcovati L, Elena C, Doubek M, Steurer M, Tosic N, Pavlovic S, Guglielmelli P, Pieri L, Vannucchi AM, Gisslinger H, Cazzola M, Kralovics R (2012) Frequent deletions of JARID2 in leukemic transformation of chronic myeloid malignancies. *Am J Hematol* **87**(3)**:** 245-250

Reeves BR, Lobb DS, Lawler SD (1972) Identity of the abnormal F-group chromosome associated with polycythaemia vera. *Humangenetik* **14**(2)**:** 159-161

Reilly JT, Snowden JA, Spearing RL, Fitzgerald PM, Jones N, Watmore A, Potter A (1997) Cytogenetic abnormalities and their prognostic significance in idiopathic myelofibrosis: a study of 106 cases. *Br J Haematol* **98**(1)**:** 96-102

Rowley JD (1973) Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature* **243**(5405)**:** 290-293

Rumi E, Harutyunyan A, Elena C, Pietra D, Klampfl T, Bagienski K, Berg T, Casetti I, Pascutto C, Passamonti F, Kralovics R, Cazzola M (2011) Identification of genomic aberrations associated with disease transformation by means of high-

resolution SNP array analysis in patients with myeloproliferative neoplasm. *Am J Hematol* **86**(12)**:** 974-979

Sanada M, Suzuki T, Shih LY, Otsu M, Kato M, Yamazaki S, Tamura A, Honda H, Sakata-Yanagimoto M, Kumano K, Oda H, Yamagata T, Takita J, Gotoh N, Nakazaki K, Kawamata N, Onodera M, Nobuyoshi M, Hayashi Y, Harada H, Kurokawa M, Chiba S, Mori H, Ozawa K, Omine M, Hirai H, Nakauchi H, Koeffler HP, Ogawa S (2009) Gain-of-function of mutated C-CBL tumour suppressor in myeloid neoplasms. *Nature* **460**(7257)**:** 904-908

Sasaki M, Knobbe CB, Munger JC, Lind EF, Brenner D, Brustle A, Harris IS, Holmes R, Wakeham A, Haight J, You-Ten A, Li WY, Schalm S, Su SM, Virtanen C, Reifenberger G, Ohashi PS, Barber DL, Figueroa ME, Melnick A, Zuniga-Pflucker JC, Mak TW (2012) IDH1(R132H) mutation increases murine haematopoietic progenitors and alters epigenetics. *Nature* **488**(7413)**:** 656-659

Schmidt MH, Dikic I (2005) The Cbl interactome and its functions. *Nat Rev Mol Cell Biol* **6**(12)**:** 907-918

Schoenmakers EF, Bunt J, Hermers L, Schepens M, Merkx G, Janssen B, Kersten M, Huys E, Pauwels P, Debiec-Rychter M, van Kessel AG (2013) Identification of CUX1 as the recurrent chromosomal band 7q22 target gene in human uterine leiomyoma. *Genes Chromosomes Cancer* **52**(1)**:** 11-23

Score J, Hidalgo-Curtis C, Jones AV, Winkelmann N, Skinner A, Ward D, Zoi K, Ernst T, Stegelmann F, Dohner K, Chase A, Cross NC (2012) Inactivation of polycomb repressive complex 2 components in myeloproliferative and myelodysplastic/myeloproliferative neoplasms. *Blood* **119**(5)**:** 1208-1213

Scott LM, Scott MA, Campbell PJ, Green AR (2006) Progenitors homozygous for the V617F mutation occur in most patients with polycythemia vera, but not essential thrombocythemia. *Blood* **108**(7)**:** 2435-2437

Scott LM, Tong W, Levine RL, Scott MA, Beer PA, Stratton MR, Futreal PA, Erber WN, McMullin MF, Harrison CN, Warren AJ, Gilliland DG, Lodish HF, Green AR (2007) JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. *N Engl J Med* **356**(5)**:** 459-468

Shide K, Shimoda HK, Kumano T, Karube K, Kameda T, Takenaka K, Oku S, Abe H, Katayose KS, Kubuki Y, Kusumoto K, Hasuike S, Tahara Y, Nagata K, Matsuda T, Ohshima K, Harada M, Shimoda K (2008) Development of ET, primary myelofibrosis and PV in mice expressing JAK2 V617F. *Leukemia* **22**(1)**:** 87-95

Shtivelman E, Lifshitz B, Gale RP, Canaani E (1985) Fused transcript of abl and bcr genes in chronic myelogenous leukaemia. *Nature* **315**(6020)**:** 550-554

Smith BR (1990) Regulation of hematopoiesis. *Yale J Biol Med* **63**(5)**:** 371-380

Spiers AS, Bain BJ, Turner JE (1977) The peripheral blood in chronic granulocytic leukaemia. Study of 50 untreated Philadelphia-positive cases. *Scand J Haematol* **18**(1)**:** 25-38

Spivak JL (2002) Polycythemia vera: myths, mechanisms, and management. *Blood* **100**(13)**:** 4272-4290

Stegelmann F, Bullinger L, Schlenk RF, Paschka P, Griesshammer M, Blersch C, Kuhn S, Schauer S, Dohner H, Dohner K (2011) DNMT3A mutations in myeloproliferative neoplasms. *Leukemia* **25**(7)**:** 1217-1219

Stein BL, Williams DM, O'Keefe C, Rogers O, Ingersoll RG, Spivak JL, Verma A, Maciejewski JP, McDevitt MA, Moliterno AR (2011) Disruption of the ASXL1 gene is frequent in primary, post-essential thrombocytosis and post-polycythemia vera myelofibrosis, but not essential thrombocytosis or polycythemia vera: analysis of molecular genetics and clinical phenotypes. *Haematologica* **96**(10)**:** 1462-1469

Sverdlow S, Campl E, Harris N, Jaffe E, Pileri S, Stein H, Thiele J, Vardiman J (2008) *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*, Lyon: International Agency for Research on Cancer.

Swolin B, Weinfeld A, Westin J (1988) A prospective long-term cytogenetic study in polycythemia vera in relation to treatment and clinical course. *Blood* **72**(2)**:** 386-395

Szpurka H, Gondek LP, Mohan SR, Hsi ED, Theil KS, Maciejewski JP (2009) UPD1p indicates the presence of MPL W515L mutation in RARS-T, a mechanism analogous to UPD9p and JAK2 V617F mutation. *Leukemia* **23**(3)**:** 610-614

Tefferi A (2000) Myelofibrosis with myeloid metaplasia. *N Engl J Med* **342**(17)**:** 1255-1265

Tefferi A (2010) Novel mutations and their functional and clinical relevance in myeloproliferative neoplasms: JAK2, MPL, TET2, ASXL1, CBL, IDH and IKZF1. *Leukemia* **24**(6)**:** 1128-1138

Tefferi A, Lasho TL, Abdel-Wahab O, Guglielmelli P, Patel J, Caramazza D, Pieri L, Finke CM, Kilpivaara O, Wadleigh M, Mai M, McClure RF, Gilliland DG, Levine RL, Pardanani A, Vannucchi AM (2010) IDH1 and IDH2 mutation studies in 1473 patients with chronic-, fibrotic- or blast-phase essential thrombocythemia, polycythemia vera or myelofibrosis. *Leukemia* **24**(7)**:** 1302-1309

Tefferi A, Mesa RA, Schroeder G, Hanson CA, Li CY, Dewald GW (2001) Cytogenetic findings and their clinical relevance in myelofibrosis with myeloid metaplasia. *Br J Haematol* **113**(3)**:** 763-771

Tefferi A, Vardiman JW (2008) Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. *Leukemia* **22**(1)**:** 14-22

Thiele J, Kvasnicka HM (2005) Diagnostic impact of bone marrow histopathology in polycythemia vera (PV). *Histol Histopathol* **20**(1)**:** 317-328

Thiele J, Kvasnicka HM, Boeltken B, Zankovich R, Diehl V, Fischer R (1999) Initial (prefibrotic) stages of idiopathic (primary) myelofibrosis (IMF) - a clinicopathological study. *Leukemia* **13**(11)**:** 1741-1748

Thiele J, Kvasnicka HM, Diehl V (2005) Initial (latent) polycythemia vera with thrombocytosis mimicking essential thrombocythemia. *Acta Haematol* **113**(4)**:** 213-219

Thiele J, Rompcik V, Wagner S, Fischer R (1992) Vascular architecture and collagen type IV in primary myelofibrosis and polycythaemia vera: an immunomorphometric study on trephine biopsies of the bone marrow. *Br J Haematol* **80**(2)**:** 227-234

Thien CB, Langdon WY (2001) Cbl: many adaptations to regulate protein tyrosine kinases. *Nat Rev Mol Cell Biol* **2**(4)**:** 294-307

Tiedt R, Hao-Shen H, Sobas MA, Looser R, Dirnhofer S, Schwaller J, Skoda RC (2008) Ratio of mutant JAK2-V617F to wild-type Jak2 determines the MPD phenotypes in transgenic mice. *Blood* **111**(8)**:** 3931-3940

Tsukada Y, Fang J, Erdjument-Bromage H, Warren ME, Borchers CH, Tempst P, Zhang Y (2006) Histone demethylation by a family of JmjC domain-containing proteins. *Nature* **439**(7078)**:** 811-816

Velazquez L, Cheng AM, Fleming HE, Furlonger C, Vesely S, Bernstein A, Paige CJ, Pawson T (2002) Cytokine signaling and hematopoietic homeostasis are disrupted in Lnk-deficient mice. *J Exp Med* **195**(12)**:** 1599-1611

Vire E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C, Morey L, Van Eynde A, Bernard D, Vanderwinden JM, Bollen M, Esteller M, Di Croce L, de Launoit

Y, Fuks F (2006) The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* **439**(7078)**:** 871-874

Ward PS, Patel J, Wise DR, Abdel-Wahab O, Bennett BD, Coller HA, Cross JR, Fantin VR, Hedvat CV, Perl AE, Rabinowitz JD, Carroll M, Su SM, Sharp KA, Levine RL, Thompson CB (2010) The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. *Cancer Cell* **17**(3)**:** 225-234

Wernig G, Mercher T, Okabe R, Levine RL, Lee BH, Gilliland DG (2006) Expression of Jak2V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model. *Blood* **107**(11)**:** 4274-4281

Xing S, Wanting TH, Zhao W, Ma J, Wang S, Xu X, Li Q, Fu X, Xu M, Zhao ZJ (2008) Transgenic expression of JAK2V617F causes myeloproliferative disorders in mice. *Blood* **111**(10)**:** 5109-5117

Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim SH, Ito S, Yang C, Xiao MT, Liu LX, Jiang WQ, Liu J, Zhang JY, Wang B, Frye S, Zhang Y, Xu YH, Lei QY, Guan KL, Zhao SM, Xiong Y (2011) Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. *Cancer Cell* **19**(1)**:** 17-30

Yamashita Y, Yuan J, Suetake I, Suzuki H, Ishikawa Y, Choi YL, Ueno T, Soda M, Hamada T, Haruta H, Takada S, Miyazaki Y, Kiyoi H, Ito E, Naoe T, Tomonaga M, Toyota M, Tajima S, Iwama A, Mano H (2010) Array-based genomic resequencing of human leukemia. *Oncogene* **29**(25)**:** 3723-3731

Zaleskas VM, Krause DS, Lazarides K, Patel N, Hu Y, Li S, Van Etten RA (2006) Molecular pathogenesis and therapy of polycythemia induced in mice by JAK2 V617F. *PLoS One* **1:** e18

6 ABBREVIATIONS

2-HG	2-hydroxyglutarate
293T	a human kidney cell line
5-hmC	5 hydroxymethyl cytosine
5-mC	5 methyl cytosine
A	alanine (aminoacid)
α-KG	alpha ketoglutarate
ABL1	c-abl oncogene 1 (gene)
AEBP2	AE binding protein 2 (gene)
AKT	v-akt murine thymoma viral oncogene homolog (gene)
AML	acute myeloid leukemia
BaF/3	a murine pro-B cell line
BALB/c	a mouse strain
BCR	breakpoint cluster region (gene)
BRCA1, BRCA2	breast cancer 1 or 2 (genes)
C57Bl/6	a mouse strain
CBL	Casitas B-lineage lymphoma proto-oncogene (gene)
CDR	common deleted region
CML	chronic myeloid leukemia
CUX1	cut-like homeobox 1
DDB1	damage-specific DNA binding protein 1 (gene)
DNMT1, 2, or 3A	DNA (cytosine-5-)-methyltransferase 1, 2, or 3 alpha (genes)
DOT1L	DOT1-like, histone H3 methyltransferase (S. cerevisiae)
ERK	extracellular-signal-regulated kinase (genes or pathway)
ET	essential thrombocythemia
ETV6	ets variant 6 (gene)
EZH2	enhancer of zeste homolog 2 (Drosophila) (gene)
F	phenylalanine (aminoacid)
FANCM	Fanconi anemia, complementation group M (gene)
FDCP	cell line
FGFR1	fibroblast growth factor receptor 1 (gene)
FISH	fluorescence in situ hybridization
FOXP1	forkhead box P1 (gene)

HSC	hematopoietic stem cell
IDH1, IDH2	isocitrate dehydrogenase 1 or 2 (genes)
IKZF1	IKAROS family zinc finger 1 (Ikaros) (gene)
JAK1, JAK2, JAK3	Janus kinase 1, 2 or 3 (genes)
JARID2	jumonji, AT rich interactive domain 2
K	lysine (aminoacid)
L	leucine (aminoacid)
LMO2	LIM domain only 2 (gene)
LNK	see SH2B3
Mb	Megabases
MDS	myelodysplastic syndrome
MF	myelofibrosis
MLL	mixed lineage leukemia (gene)
MPL	myeloproliferative leukemia virus oncogene (thrombopoietin receptor) (gene)
MPN	myeloproliferative neoplasm
MYC	v-myc myelocytomatosis viral oncogene homolog (avian) (gene)
N	asparagine (aminoacid)
p	short arm of a chromosome
P	proline (aminoacid)
PARP	poly (Adenosine diphosphate-ribose) polymerase (gene)
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide (gene)
PDGFRB	platelet-derived growth factor receptor, beta polypeptide (gene)
PI3K	phosphatidylinositol-3-kinase
PMF	primary myelofibrosis
PRC2	Polycomb Repressive Complex 2
PV	polycythemia vera
q	long arm of a chromosome
RB1	retinoblastoma 1 (gene)
RUNX1	runt-related transcription factor 1 (gene)
S	serine (aminoacid)

SH2B3	SH2B adaptor protein 3 (gene), alias LNK
STAT	signal transducer and activator of transcription (genes)
SUZ12	suppressor of zeste 12 homolog (Drosophila) (gene)
T	threonine (aminoacid)
TET1, TET2	tet methylcytosine dioxygenase 1 or 2 (genes)
TYK2	Tyrosine kinase 2 (gene)
UPD	uniparental disomy
V	valine (aminoacid)
W	tryptophan (aminoacid)
WHO	World Health Organisation

CURRICULUM VITAE

Thorsten Klampfl
born 23 August 1980 in St. Pölten, Austria
Austrian citizenship
married, 2 children

Karl-Heinz-Straße 67/6/11
1230 Vienna, Austria
+43-650-6240246
thorsten.klampfl@gmail.com

Research Interests

- Human genetics and genomics; novel genomic technologies
- Hematopoiesis and hematological (myeloid) malignancies
- Translational research
- Combination of wet-lab and computational biology / systems biology

Current position

2008 – present	PhD student at the CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Lab of Robert Kralovics **„Oncogene and tumor suppressor network in myeloproliferative neoplasms**“
2011/2012	**Consultant for Haplogen GmbH (Viennese biotech company)** support in setting up a custom next-generation sequencing protocol using Illumina technology

Research experience

April – June 2008	Internship at Boehringer Ingelheim Austria GmbH Dept. of Medicinal Chemistry / Structural Research (Supervisor Dr. D. Kuhn) **Computational approaches to mine and analyze high throughput data**
2006 – 2008	Technical Assistant at the Institute of Cancer Research, Medical University of Vienna, Lab of Prof. Brigitte Marian **Project: The effects of 12S-Lipoxygenase on colorectal cancer growth**
2005 - 2007	Master's thesis at the Institute of Cancer Research Medical University of Vienna, Lab of Prof. Brigitte Marian **"The Influence of 12S-Lipoxygenase on Genes Involved in Metastasis in the Colorectal Cancer Cell Line Caco-2"**

Education

2000 – 2007	Studies of **biology** with specialisation in **human genetics** **Master's degree** (Mag. rer.nat.) with distinction, University of Vienna, Austria
1998-1999	Two terms studies of **informatics**, Technical University of Vienna, Austria
1998	**Final high school exam** ("Matura") at the BG/BRG St. Pölten, Austria

PUBLICATIONS

Genome integrity of myeloproliferative neoplasms in chronic phase and during disease progression.
Klampfl T*, Harutyunyan A*, Berg T, Gisslinger B, Schalling M, Bagienski K, Olcaydu D, Passamonti F, Rumi E, Pietra D, Jäger R, Pieri L, Guglielmelli P, Iacobucci I, Martinelli G, Cazzola M, Vannucchi AM, Gisslinger H, Kralovics R.*
Blood. 2011 Jul 7;118(1):167-76.

p53 lesions in leukemic transformation.
*Harutyunyan A, **Klampfl T**, Cazzola M, Kralovics R.*
N Engl J Med. 2011 Feb 3;364(5):488-90.

Rare germline variants in regions of loss of heterozygosity may influence clinical course of hematological malignancies.
*Harutyunyan A, Gisslinger B, **Klampfl T**, Berg T, Bagienski K, Gisslinger H, Kralovics R.*
Leukemia. 2011 Nov;25(11):1782-4.

Frequent deletions of JARID2 in leukemic transformation of chronic myeloid malignancies.
*Puda A, Milosevic JD, Berg T, **Klampfl T**, Harutyunyan AS, Gisslinger B, Rumi E, Pietra D, Malcovati L, Elena C, Doubek M, Steurer M, Tosic N, Pavlovic S, Guglielmelli P, Pieri L, Vannucchi AM, Gisslinger H, Cazzola M, Kralovics R.*
Am J Hematol. 2012 Mar;87(3):245-50.

Clinical significance of genetic aberrations in secondary acute myeloid leukemia
*Milosevic JD, Puda A, Malcovati L, Berg T, Hofbauer M, Stukalov A, **Klampfl T**, Harutyunyan AS, Gisslinger H, Gisslinger B, Burjanivova T, Rumi E, Pietra D, Elena C, Vannucchi AM, Doubek M, Dvorakova D, Robesova B, Wieser R, Koller E, Suvajdzic N,Tomin D, Tosic N, Colinge J, Racil Z, Steurer M, Pavlovic S, Cazzola M, Kralovics R*
Am J Hematol. 2012, Nov;87(11):1010-6.

An RNA-Seq strategy to detect the complete coding and non-coding transcriptome including full-length imprinted macro ncRNAs.
*Huang R, Jaritz M, Guenzl P, Vlatkovic I, Sommer A, Tamir IM, Marks H, **Klampfl T**, Kralovics R, Stunnenberg HG, Barlow DP, Pauler FM.*
PLoS One. 2011; 6(11):e27288

Identification of genomic aberrations associated with disease transformation by means of high-resolution SNP array analysis in patients with myeloproliferative neoplasm.
*Rumi E, Harutyunyan A, Elena C, Pietra D, **Klampfl T**, Bagienski K, Berg T, Casetti I, Pascutto C, Passamonti F, Kralovics R, Cazzola M.*
Am J Hematol. 2011 Dec;86(12):974-9.

Deletions of the transcription factor Ikaros in myeloproliferative neoplasms.
*Jäger R, Gisslinger H, Passamonti F, Rumi E, Berg T, Gisslinger B, Pietra D, Harutyunyan A, **Klampfl T**, Olcaydu D, Cazzola M, Kralovics R.*
Leukemia. 2010 Jul;24(7):1290-8.

*equal contribution