MEDICAL UNIVERSITY OF VIENNA

Frequent chromosomal aberrations of chromosome 6 and deletions of genes encoding polycomb repressive complex 2 subunits in the leukemic transformation of chronic myeloid malignancies

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

Doctor of Philosophy

Submitted by

Ana Puda

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

Vienna, 2019

DECLARATION

This thesis is compiled in a cumulative format, with the author of the included manuscript being the author of the thesis. The prologue preceeding the manuscript describes the exact contribution of Ana Pudja and other co-authors to the published manuscript. In addition, seven chapters in the Results section are included in the thesis, containing not yet published data. Some of the whole genome SNP arrays, from which data was used in section 3.2, were performed by Ashot Harutyunyan, Jelena D. Milosevic Feenstra, Thorsten Klampfl and Tiina Berg. Some experiments in section 3.5, 3.6. and 3.7. were performed with the help of Klaudia Nägele and Roland Jäger. The plots for Figure 12 were kindly provided by Fiorella Schischlik. Initial parts of the bioinformatic data analysis in section 3.7. were performed by Michael Schuster and Doris Chen. All other experiments were performed by Ana Pudja. Figures 5-8 and Tables 1-5 were taken from other publications and are partially or fully reprinted, with permission from the publishers. The thesis was entirely written by Ana Pudja.

The work presented in this thesis was performed by the author at the CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences in Vienna, Austria, in the laboratory of Dr. Robert Kralovics.

The Manuscript presented in the thesis was published in the American Journal of Hematology: “Frequent deletions of *JARID2* in leukemic transformation of chronic myeloid malignancies” by Puda A. et al, *Am J Hematol* 2012; 87(3): 245-250. Reprinted with permission.

Table of Contents

ACKNOWLEDGMENTS

I would like to thank Dr. Robert Kralovics for the opportunity he gave me to do my PhD in his lab under his supervision, for the scientific expertise he has shared with me and for the support he has given me all along my PhD, both professionally and personally.

I would like to thank the whole team of the Kralovics lab for the scientific discussions, knowledge exchange, fun, positive atmosphere and support throughout the years. I am thankful to Ashot Harutyunyan, Thorsten Klampfl and Roland Jäger for all the advice and shared knowledge, Jelena Milosevic Feenstra for the shared projects and publications and for all the things we have experienced together during our PhD days, and for being an amazing friend ever since, Tiina Berg for her help and for keeping the lab and team organized and efficient, Klaudia Bagienski, Martin Schalling, Doris Chen, Fiorella Schischlik, Harini Nivarthi and Nicole Them for their kind assistance, support, advice and cheerful days in the lab. The whole group contributed to making the working environment a great place for doing a PhD, developing scientific and personal skills and collecting valuable experiences.

I would like to thank my PhD Committee members Prof. Dr. Sylvia Knapp and Prof. Dr. Heinz Gisslinger for their support and constructive feedback they gave me for my PhD project, as well as internal and external collaborators for their contributions to my PhD project and publication.

I would like to thank Giulio Superti Furga, as the director of CeMM, for giving me the chance to become part of the community of the institute, for leading us in such a unique way and for teaching me skills beyond the scientific ones.

An enormous contribution to the success of my PhD was given by some very special people in my life, who have been with me all along the way and have given me tremendous support, encouragement, understanding, love and motivation to get to where I am now. Thank you, my very special friends Nataša, Željka, Irena, Tamara, Ivan, Natašica and Maka! You are such a big support, no matter what and no matter where from! Thank you, Anel and Milana, for beeing the best friends to me and my family, allowing me to find time and motivation to finish my PhD. Thank you, my dearest friend Tatjana, for your amazing support at all times! Thank you for our precious friendship, for your creativity in finding the best words and ways for keeping me motivated and thank you for continuously believing in me!

Words are not enough to express my gratefulness to my family, who has given me courage, faith, strength, love, motivation and much more, who is always there for me and who is my endless source of support. Thank you, Mom, for being a role model, for teaching me what really matters in life, for putting my wishes in front of your own, for loving me, for being with me at all times! Thank you, Filip, for being the best brother! Thank you, Baka, for believing in me and for teaching me so many valuable life lessons. Thank you, Bilja and Bora for being there for me my whole life! Thank you, my parents in law Jasmina and Dragan and my sister in law Dragana, for loving me, believing in me and supporting me!

My loving husband Miloš, thank you for beeing just the way you are, for your endless love, support, understanding, patients and encouragement! Thank you for the wonderful family we have built together! I would not have been who I am without you!

My dearest Igor, Nina and Mia, thank you for giving me the privilege of enjoying my role as a Mom every single moment! Thank you for your smiles, love, sparkling eyes, big souls and for being my biggest motivation!

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Myeloproliferative neoplasms (MPN) lacking the BCR-ABL1 fusion protein (Ph-negative MPN), comprise a group of clonal hematological malignancies affecting the myeloid lineage, and are in most cases driven by somatic mutations in three driver genes – *JAK2*, *CALR* and *MPL*. Patients frequently harbor additional mutations in a variety of non-driver genes. Based on clinical features, Ph-negative MPN are classified into three disease entities – polycythemia vera (PV), essential throbocythemia (ET) and primary myelofibrosis (PMF), all with a risk of progression to acute myeloid leukemia (AML). Myelodysplastic syndrome (MDS) is a myeloid malignancy, clinically different from MPN but with an overlapping mutational landscape and risk of progression to AML. In addition to somatic mutations chromosomal aberrations occur in a subset of chronic phase MPN and MDS patients.

With the use of genome wide high resolution SNP arrays we have investigated the aberrational profile of 77 AML patients with history of MPN (n=46) or MDS (n=31), identifying at least one chromosomal aberration in 64/77 (83.1%) patients. Detailed examination of the deletion profile identified focal homozygous deletions on chromosomes 6 and 12, affecting genes *JARID2* and *AEBP2*, respectively, both encoding co-factors of the polycomb repressive complex 2 (PRC2). We have investigated the deletion profile of genes encoding PRC2 subunits (*EZH2*, *SUZ12*, *EED*, *EZH1*, *JARID2*, *AEBP2*) in an expanded pan-myeloid cohort of 905 patients (including MPN, MDS, sAML and dnAML), and detected at least one PRC2 member (range 1-4) to be deleted in 6.6% of the entire cohort. Out of the 60 detected deletions 11 were focal (<2Mb). PRC2 deletions are strongly enriched in post-MPN AML (40%), post-MDS AML (22.5%) and dnAML (16.2%), compared to very low deletion rates in chronic phase MPN (1%) and no deletion detected in MDS. We were also able to detect frequent co-deletions of PRC2 members and *NF1* or *ETV6* in individual patients, associating these double deletions with leukemic transformation as well. Our data implicates the role of PRC2 loss in disease progression from chronic MPN or MDS to AML, as well as in *de novo* AML, and a likely cooperation of the loss of PRC2 function with other mutations or deletions in the progressive disease evolution.

In addition, we have observed a significant enrichment of chromosome 6p and 12p aberrations (covering *JARID2* and *AEBP2*, respectively) in sAML compared to chronic myeloid disorders. Sanger sequencing of DNA from patients harboring chromosome 6p lesions revealed no mutations of the *JARID2* gene. We therefore hypothesize that *JARID2* is preferentially targeted by deletions and not mutations and that it through haploinsufficiency acts as a tumor suppressor involved in the progression of myeloid neoplasms.

Finally, we have explored the mutational profile of chromosome 6p in patients with aberrations of this genomic region and identified no recurrent mutation and common target in the four selected patients. We have detected a somatic mutation in the *FAM65B* gene, and by intersecting exome sequencing data with the aberrational profile of chromosome 6 we have defined several commonly affected regions covering a number of genes with potential roles in the development of myeloid malignancies.

The findings of the research performed in this thesis has contributed to a better understanding of the genetics of disease progression of MPN and MDS by identifying deletions of PRC2 subunits in a subset of patients. It has identified *JARID2* as a novel tumor suppressor affected by deletions in leukemic progression of MPN. Finally, this study has provided a solid basis for further research in the field, with the aim of delineating the prognostic value and clinical impact of PRC2 loss in myeloid malignancies, which might serve as a valuable biomarker for future classification and management of affected patients.

ZUSAMMENFASSUNG

Der Begriff Myeloproliferative Neoplasien (MPN) umfasst eine Gruppe maligner klonaler Erkrankungen der hämatopoetischen Stammzelle und ist in den meisten Fällen auf somatische Mutationen dreier Gene – *JAK2*, *CALR* und *MPL,* zurückzuführen. Darüber hinaus weisen Patienten jedoch häufig Mutationen in weiteren Genen auf. In Abhängigkeit der klinischen Merkmale werden Ph- MPN unterteilt in Polycythämia vera (PV), Essentielle Thrombozythämie (ET) sowie Primäre Myelofibrose (PMF), die das Risiko zur Entstehung Akuter Myeloischer Leukämie (AML) gemein haben.
Das Myelodysplastische Syndrom (MDS) bildet eine weitere Klasse myeloider Erkrankungen, die zwar klinisch von MPN abgegrenzt ist, sich jedoch hinsichtlich des Mutationsspektrums und des Risikos zur Entstehung von AML überschneidet. Neben somatischen Mutationen weist ein Teil der Patienten mit chronischer Phase der MPN und MDS auch chromosomale Abberrationen auf. Mittels genomweiter hochauflösender SNP Microarray Technologie wurde das veränderte genetische Profil von 77 AML Patienten mit vorangegangener MPN (n=46) oder MDS (n= 31) untersucht und hierbei chromosomale Aberrationen in 64/77 (83,1%) der Patienten identifiziert. Eine detaillierte Analyse jener Regionen führte zur Identifizierung homozygoter Deletionen auf Chromosom 6 und 12, respektive die Gene *JARID2* und *AEBP2* betreffend, welche beide für Kofaktoren des Polycomb Repressive Complex 2 (PRC2) kodieren. Das Deletionsprofil jener Gene, welche die PRC2 Untereinheit kodieren (*EZH2, SUZ12, EED, EZH1, JARID2, AEBP2*) in einer erweiterten pan-myeloiden Kohorte von 905 Patienten wurde untersucht. In 6,6% der Untersuchten wurde die Deletion von zumindest einer PRC2 Untereinheit detektiert.

Im Vergleich zur geringen Deletionsrate in Patienten mit chronischer MPN (1%), sind Deletionen in PRC2 in post-MPN AML (40%), post MDS-AML (22,5%) und dnAML (16,2%) stark angereichert. Auch wurden häufig Kodeletionen von PRC2 Untereinheiten sowie in *NF1* oder *ETV6* nachgewiesen. Unsere Daten deuten darauf hin, dass der Verlust von PRC2 am Fortschreiten der chronischen MPN oder MDS zu AML, sowie der *de novo* Entsteheung von AML beteiligt ist und implizieren auch einen möglichen Zusammenhang zwischen dem funktionalen Verlust von PRC2 und dem Auftreten weiterer Mutationen oder Deletionen in der progressiven Kranheitsphase.

Zusätzlich wurde in sAML eine signifikante Anreicherung von chromosomalen Aberrationen innerhalb der Abschnitte 6p und 12p (welche Respektive *JARID2* und *AEBP2* abdecken) gegenüber chronisch myeloischen Erkrankungen festgestellt. Die Sangersequenzierung von Patienten mit Läsionen auf Chromosom 6p ergab jedoch keine Mutationen in *JARID2*. Es ist daher davon

auszugehen, dass *JARID2* vorzugsweise ein Angriffspunkt für Deletionenist und dass es durch Haploinsuffizienz als Tumorsuppressor in der Entwicklung myeloider Neoplasien fungiert.

Abschließend wurde das Mutationsprofil von Chromosom 6p in Patienten mit Aberrationen innerhalb dieses genomischen Abschnitts untersucht, allerdings keine wiederkehrende Mutation innerhalb der vier selektierten Patienten detektiert. Es wurde eine somatische Mutation in *FAM65B* aufgedeckt und beim Abgleich der Exomdaten mit dem Aberrationsprofil von Chromosom 6 einige Regionen gefunden, welche gemeinsam betroffen sind und Gene beinhalten, die potenziell an der Entstehung myeloider Malignitäten beteiligt sind.

Die Ergebnisse dieser Forschungsarbeit tragen durch die Identifizierung von Deletionen in PRC2 Untereinheiten in einer Untergruppe von Patienten zu einem besseren Verständnis der Genetik des Krankheitsverlaufs von MPN und MDS bei. Hierin wurde *JARID2* als neuer Tumorsuppresor identifiziert, welcher im Zuge der Entwicklung von MPN häufig von Deletionen betroffen ist. Abschließend stellt diese Arbeit eine fundierte Grundlage für weiterführende Tätigkeiten in diesem Forschungsbereich dar .

1. INTRODUCTION

Hematopoiesis is a tightly regulated process, in adults predominantly taking place in the bone marrow, producing around 10^{12} mature blood cells daily (Doulatov, Notta et al., 2012). It initiates from multipotent hematopoietic stem cells (HSC) with self-renewal and differentiation potential, and is organized as a hierarchy, giving rise to multipotent- and lineage-specific progenitors in a step-wise process, resulting in the production of all types of fully differentiated blood cells of the myeloid (erythrocytes, platelets, monocytes, dendritic cells and granulocytes – neutrophils, basophils, eosinophils, mast cells) and lymphoid (T-lymphocytes, B-lymphocytes, NK cells and dendritic cells) lineages (Doulatov et al., 2012). In a highly precise manner, the expression and silencing of specific genes and signaling pathways is synchronized to maintain the self-renewal capacity and to allow maturation of HSC for the production of sufficient numbers of blood cells (Antoniani, Romano et al., 2017). Dysregulation of such a balanced process leads to disordered hematopoiesis, and dependent on the affected gene/pathway and cell type may result in the accumulation of expanding mutated cells (immature or mature) and the development of different hematologic disorders, involving the lymphoid or the myeloid lineage. Intensive research of AML as a cancer model and characterization of leukemic stem cells has broadened the understanding of cancer evolution in general, with particular insight into the leukemic evolution (Ferrando & Lopez-Otin, 2017, Welch, Ley et al., 2012). Disorders of the myeloid hematopoietic lineage, including myeloproliferative neoplasms (MPN), myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML), originate in the hematopoietic stem cell compartment and are initiated by somatic mutations which supply the affected HSC with fitness advantage and result in a clonal outgrowth of mutated cells, named clonal hematopoiesis (Bonnet & Dick, 1997, Jamieson, Gotlib et al., 2006, Klampfl, Gisslinger et al., 2013a, Lapidot, Sirard et al., 1994, Woll, Kjallquist et al., 2014). Further cooperating mutations acquired at later steps of disease evolution may lead to disease progression. Clonal evolution in myeloid neoplasms can follow a linear or a parallel model, in which subsequent mutations occur on the background of the initial mutations (linear) or in separate clones (parallel) (Engle, Fisher et al., 2015, Martignoles, Delhommeau et al., 2018, Milosevic Feenstra, Nivarthi et al., 2016, Potter, Miraki-Moud et al., 2019, Walter, Shen et al., 2012). Hematopoietic stem cells accumulate somatic mutations throughout the process of ageing as well, which may lead to clonal hematopoiesis. Many of such mutations remain benign for years, but may serve as a background for additional cooperating mutations contributing to the development of hematologic malignancies, or may per se form a pool of pre-leukemic

cells which over time transform (Genovese, Kahler et al., 2014, Jaiswal, Fontanillas et al., 2014, Xie, Lu et al., 2014).

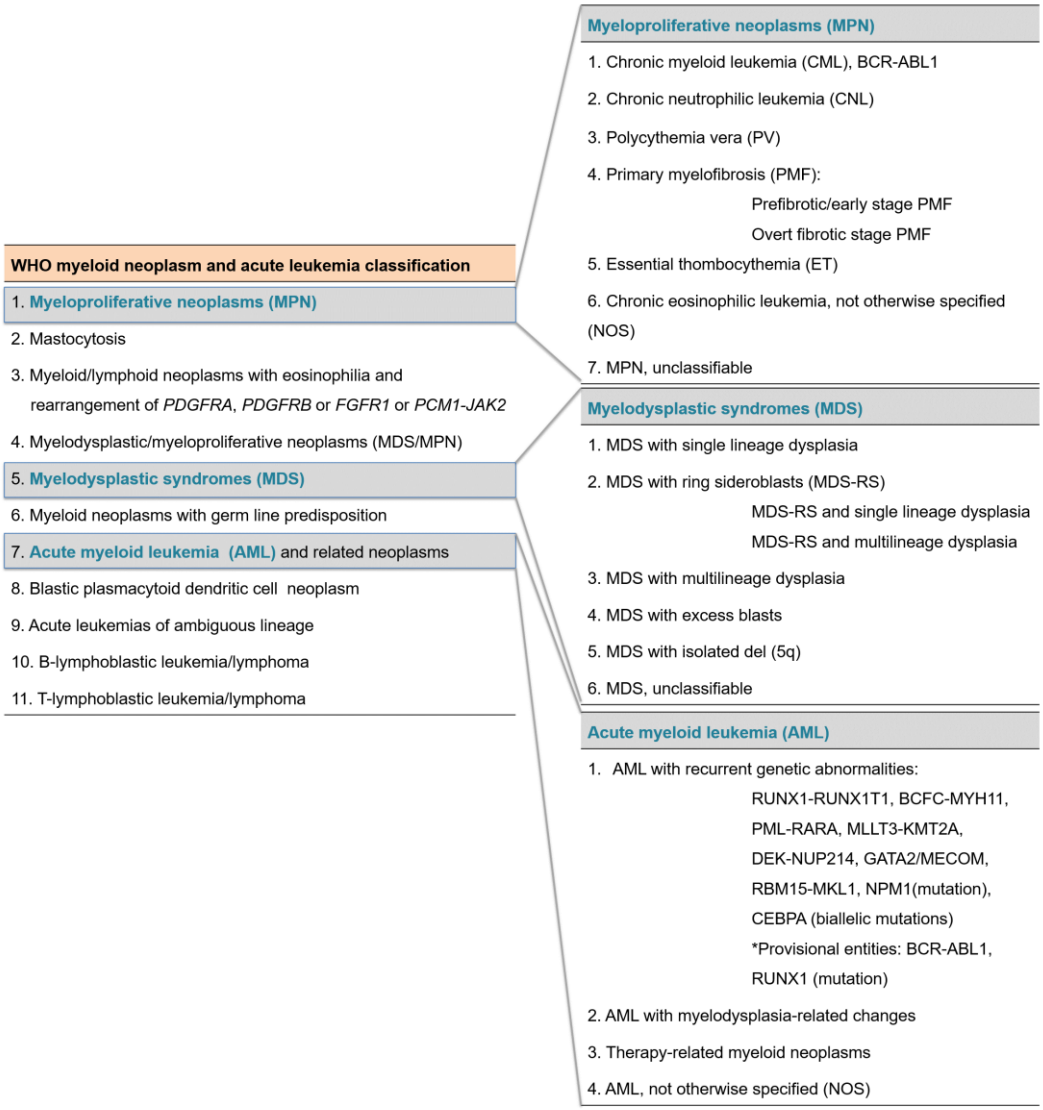
Despite the distinct morphological features in MDS and MPN a number of common somatic mutations and cytogenetic abnormalities involved in disease pathology are described, some of which are also detected in apparently healthy individuals with clonal hematopoiesis. Apart from occurring as a complication and disease progression of MPN or MDS, acute myeloid leukemia can also arise *de novo* (dnAML), without a previous history of chronic myeloid neoplasm. The research presented in this thesis is focused on specific genetic and cytogenetic defects detected in a pan-myeloid pool of patients (MPN, MDS and AML), with a focus on MPN and its comparison to MDS and AML, therefore clinical and genetic properties of these different diseases will be described in the introduction. The developing technology and huge efforts in understanding the genetics, epigenetics and underlying mechanisms of the development of myeloid neoplasm is providing solid evidence for the need of changing the guidelines for diagnosis and management of patients, by incorporating molecular genetics and various biomarkers in the classification. With this in mind, the latest recommendations for classification and diagnosis will be described in the introduction, as well as novel genetic findings in the field, expecting that new classifications of myeloid neoplasms will take many of these biomarkers into account in the near future.

1.1. Myeloproliferative neoplasms – clinical properties, diagnosis and management

First speculations about the common nature of phenotypically different myeloproliferative neoplasms were described by William Dameshek in 1951, when he grouped chronic myeloid leukemia, polycythemia vera, essential thrombocythemia and primary myelofibrosis as closely related diseases and named them “myeloproliferative disorders” (Dameshek, 1951). These disease entities are now named classical myeloproliferative neoplasms (MPNs), and are characterized by clonal hematopoiesis and increased production of fully functional terminally differentiated blood cells of the myeloid lineage. Patients with distinct MPNs may show common clinical manifestations and therefore require precision diagnosis, accurate risk stratification and adequate treatment. The latest World Health Organization (WHO) Classification of Tumors of Haematopoietic and Lymphoid Tissues from 2016 incorporates hematologic, morphologic and genetic properties in the classification of myeloid neoplasms, listing chronic myeloid leukemia (CML), chronic neutrophilic leukemia

(CNL), polycythemia vera (PV), primary myelofibrosis (PMF), essential thrombocythemia (ET), not otherwise specified chronic eosinophilic leukemia and unclassifiable MPN (MPN-U) as myeloproliferative neoplasms (Table 1) (Arber, Orazi et al., 2016).

Table 1. WHO 2016 classification of myeloid neoplasms and acute leukemia



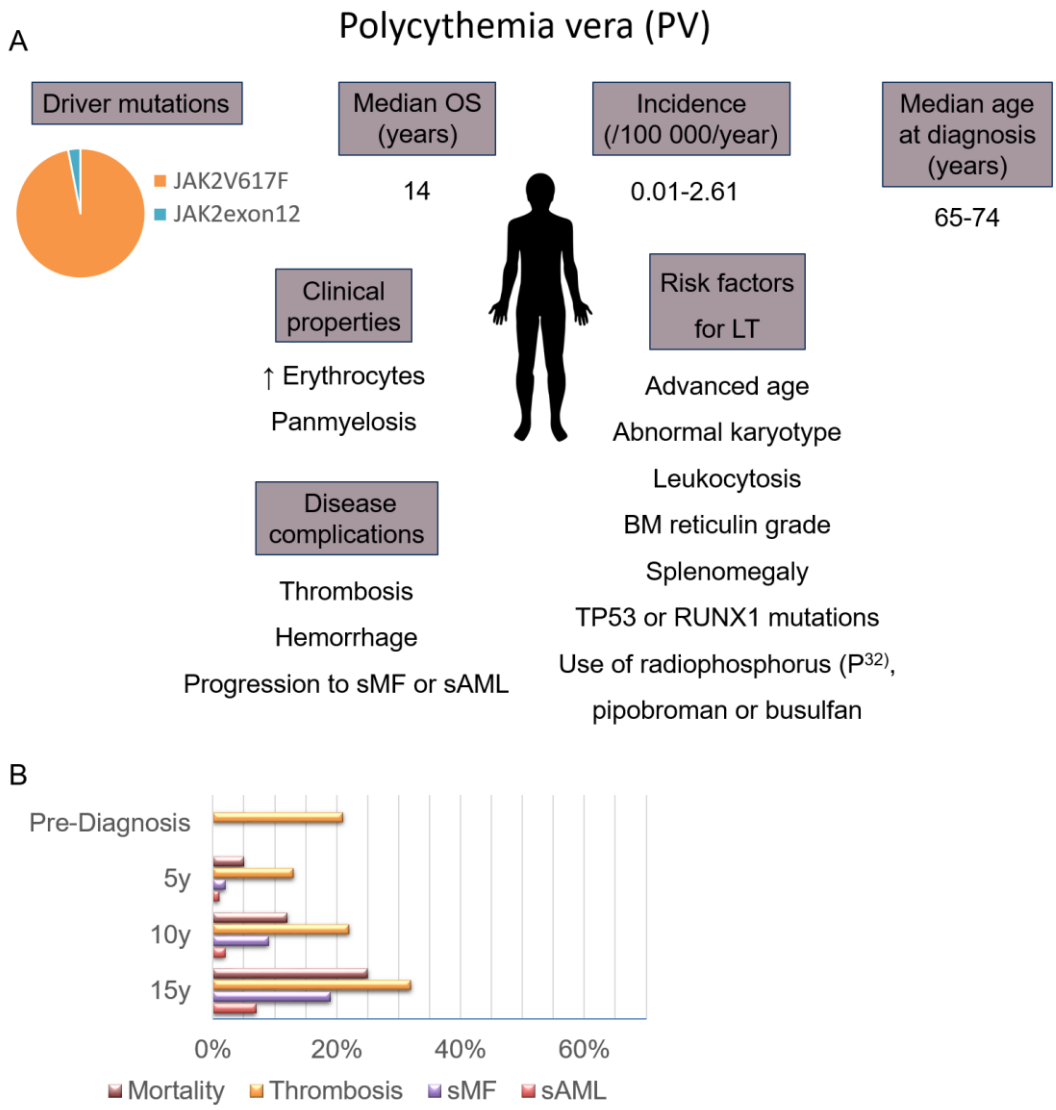
(The table was adapted and republished with permission of the American Society of Hematology, from The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia, Arber D. et al, Blood 2016 127:2391-2405; permission conveyed through Copyright Clearance Center, Inc.)

Chronic myeloid leukemia is characterized by the presence of a translocation of chromosomes 9 and 22, resulting in a well-studied *BCR-ABL1* fusion gene, which is the driver of clonal hematopoiesis in CML. The development of modern tyrosine-kinase inhibitor therapy has allowed efficient treatment of CML, leading to complete molecular remission and nearly normal or normal life expectancy in most treated patients (Jabbour & Kantarjian, 2018). The other three classical MPNs – PV, ET and PMF, lack the *BCR-ABL* fusion oncogene and are therefore named *BCR-ABL*$^-$ classical MPNs (also known as Philadelphia chromosome-negative classical MPNs; Ph$^-$ MPN). The identification of mutually exclusive somatic driver mutations in *JAK2*, *MPL* and *CALR* in MPN led to a more accurate classification of patients and has prospective clinical impact. A subgroup of MPN patients remain classified as triple negative, characterized by the lack of all three driver mutations (Klampfl et al., 2013a, Milosevic Feenstra et al., 2016). Thrombosis, hemorrhages and transformation to acute myeloid leukemia (AML) are frequent clinical complications of MPN (Swerdlow SH, 2016). Transitions between different disease stages or disease entities inside the *BCR-ABL*$^-$ MPNs have also been described, implicating that a complex multifactorial process is responsible for the disease initiation and progression.

1.1.1. Polycythemia vera

Polycythemia vera (PV) is represented by an increased number of erythrocytes, as well as pan-myelosis with an overproduction of granulocytes and megakaryocytes. The reported median age at diagnosis is in range from 65 to 74 years, with a median survival of 14 years and an incidence of 0.01 to 2.61 per 100 000 per year (Moulard, Mehta et al., 2014, Tefferi, Rumi et al., 2013, Titmarsh, Duncombe et al., 2014). The actual incidence of PV might be higher due to underdiagnosis of PV before the change of diagnostic criteria in 2016, when “masked PV” (mPV) was introduced and its resemblance to *JAK2*-mutant ET was recognized (Barbui, Thiele et al., 2014, Barbui, Thiele et al., 2018b). First diagnosis of PV is often made after increased hemoglobin levels, hematocrit or red blood cell mass are detected in a systematic blood test. In around 20% of cases arterial or venous thrombosis is documented in PV patients. Splenomegaly and hepatomegaly occur in 70% and 40% of patients, respectively. Symptoms most often reported by patients are headaches and dizziness, as well as visual disturbancies, itching and paraesthesias (Swerdlow SH, 2016). Thrombosis and hemorrhage represent the most common complications and cause of death in patients, and disease progression to myelodysplasia or acute myeloid leukemia occurs in up to 20% of patients (Figure 1) (Barbui et al., 2018b, Swerdlow SH, 2016).

Figure 1. Clinical and epidemiological properties of polycythemia vera



A. Representation of the most important clinical and epidemiological properties of PV, including the distribution of driver mutations, represented with a pie chart. B. Rates of Mortality, thrombosis, myelofibrotic and leukemic transformation in PV at different time points. Values are taken from previously published data (Barbui et al., 2018b), regrouped, and presented by a bar chart. OS, overall survival; ↑, increased; LT, leukemic transformation; sMF, secondary myelofibrosis; sAML, secondary AML; P^{32}, radiophosphorus; y, years.

Almost all PV patients are carriers of a somatic *JAK2* gain-of-function mutation. The most frequent is the *JAK2*V617F mutation, found in 95% of patients. However, 3% of PV patients have a mutation in exon 12 of *JAK2* instead (Kralovics, Passamonti et

al., 2005, Scott, Tong et al., 2007, Vainchenker & Kralovics, 2017). Properties of PV often include decreased serum erythropoietin (EPO) levels, increased bone marrow cellularity for the individual's age (mainly in the subcortical space which is hypocellular in healthy adults) and increased proliferation of erythrocytes, granulocytes and megakaryocytes (Swerdlow SH, 2016). The disease course is described through a continuum of three phases: 1. a pre-polycythaemic stage, where erythrocythosis is still moderate; 2. an overt polycythaemic stage, characterized by a strong increase in red cell mass; and 3. a post-polycythaemic stage (also known as post-PV myelofibrosis/post-PV MF), which is associated with increased BM fibrosis, a decrease in erythropoiesis and the presence of extramedullary haematopoiesis with consequential splenomegaly (Swerdlow SH, 2016). A further complication of the disease evolution is transformation to acute myeloid leukemia, which occurs in 7% of patients at 15 years after initial diagnosis, and is diagnosed with the presence of at least 20% of blasts in the bone marrow (Figure 1) (Barbui et al., 2018b, Swerdlow SH, 2016). According to the WHO 2016 classification, an integration of morphological, clinical and molecular genetic properties is the most effective and precise approach for the diagnosis and assessment of prognosis of MPN. The criteria for PV diagnosis, according to the latest classification, are represented in Table 2, where all major criteria must be met for diagnosis, or alternatively the minor criterion in addition to the first two major ones (Arber et al., 2016).

Progression to myelofibrosis occurs in 9% and 19% of PV patients 10 and 15 years after diagnosis, respectively (Figure 1B) (Barbui et al., 2018b). The median time to fibrotic transformation is reported to be between 8.5 and 20 years from the time of the initial diagnosis (Cerquozzi & Tefferi, 2015). Progression occurs more often in patients who had bone marrow fibrosis identified at initial diagnosis and in patients who have an allele burden of *JAK2*V617F mutation higher than 50% (Barraco, Cerquozzi et al., 2017, Passamonti, Rumi et al., 2010). Other risk factors for fibrotic transformation are older age, leukocytosis, thrombocytosis, splenomegaly and identification of the "masked PV" phenotype (Cerquozzi & Tefferi, 2015).

Table 2. The 2016 World Health Organization criteria for the diagnosis of PV

MAJOR CRITERIA		
1	2	3
↑ hemoglobin * or ↑ hematocrit ** or ↑ red cell mass ***	Hypercellular BM for age, with panmyelosis ****	Presence of *JAK2*V617F or *JAK2* exon 12 mutation
MINOR CRITERION		
↓ serum erythropoietin		

*hemoglobin > 16.5 g/dl in men, > 16 g/dl in women; ** hematocrit > 49% in men, >48% in women; *** >25% above mean normal predicted value; **** trilineage growth including prominent erythroid, granulocytic and megakaryocytic proliferation with pleomorphic mature megakaryocytes. In case of the presence of hemoglobin >18.5 g/dl (men)/>16.5 g/dl (women) or hematocrit >55.5% (men)/ >49.5% (women), and a *JAK2* mutation, and lower serum erythropoietin the major criterion number 2 is not required. ↑, increase; ↓, decrease. *(The table was adapted and republished with permission of the American Society of Hematology, from The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia, Arber D. et al, Blood 2016 127:2391-2405; permission conveyed through Copyright Clearance Center, Inc.)*

Leukemic transformation is a severe complication of MPN with poor prognosis, as the median survival after entering the blast phase is 3.6 months (Tefferi, Mudireddy et al., 2018c). The transformation is seen in 2% of PV patients after 10 years and in 7% after 15 years from initial diagnosis (Figure 1B) with the time to leukemic transformation being between 4.6 and 19 years from first diagnosis (Barbui et al., 2018b, Cerquozzi & Tefferi, 2015). Advanced age, abnormal karyotype, leukocytosis, BM reticulin grade and splenomegaly, as well as mutations of *TP53* or *RUNX1* are identified as risk factors for transformation, as well as the use of radiophosphorus (P^{32}), pipobroman and busulfan (Cerquozzi & Tefferi, 2015).

Polycythemia vera patients have an increased risk of thrombotic events. They occur in 22% and 32% of PV cases 10 and 15 years after diagnosis, respectively (Figure 1B) (Barbui et al., 2018b). Based on this risk for thrombosis and increased age as a risk factor for disease progression two risk stratification categories are established in PV: low-risk (age<60 years with no thrombosis history) and high-risk (age >60 or previous thrombosis). A newer prognostic model also integrates leukocytosis in the risk stratification, classifying PV patients in 3 risk categories based on a scoring system: low-risk, intermediate risk and high-risk (Rumi & Cazzola, 2017b).

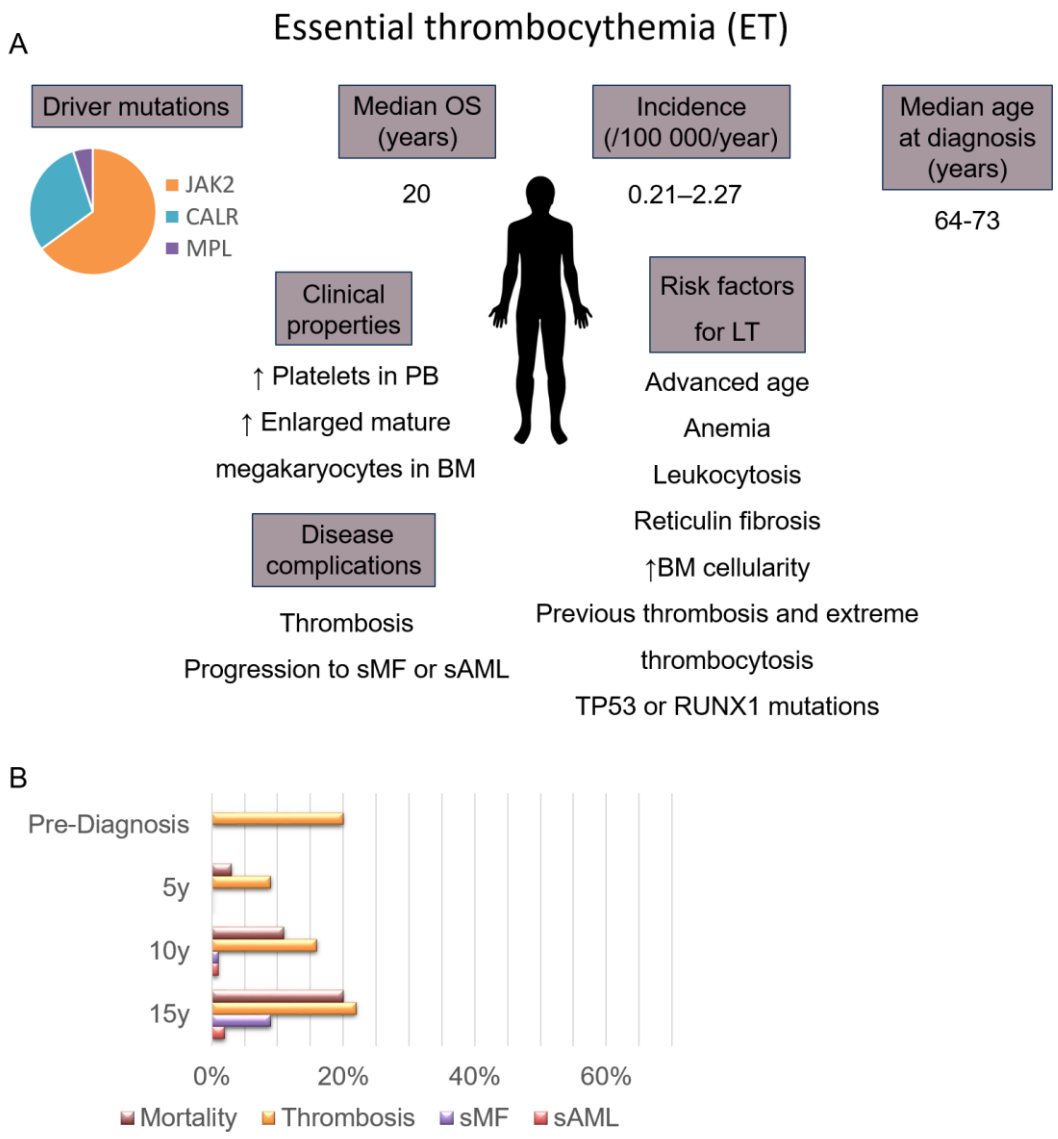
Management of PV includes phlebotomy, with the aim of keeping hematocrit levels below 45%, and low-dose acetyl salicylic acid. In high-risk patients cytoreductive therapy is recommended, with the choice of hydroxyurea (HU) or recombinant interferon alpha (rIFNα) as first-line therapy and ruxolitinib (a Jak1/Jak2 inhibitor) as second-line therapy or switch to rIFNα in patients who do not respond adequately to hydroxyurea (Barbui, Tefferi et al., 2018a, Vannucchi, Barbui et al., 2015). Ongoing studies show promising results for the use of pegylated interpheron alpha 2a in PV and ET patients, as a choice of first line therapy alternative to HU or second-line therapy in HU-nonresponders, as its use shows marked molecular response and it seems to be associated with beneficial outcome in patients (Ianotto, Chauveau et al., 2018, Masarova, Patel et al., 2017).

In summary, PV is a chronic phase MPN characterized by mutations in the *JAK2* gene in almost all patients and an overrepresentation of erythrocytes, as well as granulocytes and megakaryocytes in the bone marrow and peripheral blood. Disease complications include high risk of thrombosis and risks of disease progression to secondary myelofibrosis or acute myeloid leukemia. When leukemic transformation occurs the prognosis is poor with short overall survival.

1.1.2. Essential thrombocythaemia

Essential thrombocythaemia (ET) is characterized by an increased number of platelets in the peripheral blood, as well as an increase of the number of mature enlarged megakaryocytes in the bone marrow (Swerdlow SH, 2016). The incidence of ET is reported to be between 0.21 and 2.27 per 100 000 per year with a median age at diagnosis of 64 to 73 years and a median survival of 20 years (Figure 2) (Moulard et al., 2014, Tefferi et al., 2013, Titmarsh et al., 2014, Vannucchi et al., 2015).

Figure 2. Clinical and epidemiological properties of essential thrombocythemia



A. Representation of the most important clinical and epidemiological properties of ET, including the distribution of driver mutations, represented with a pie chart. B. Rates of Mortality, thrombosis, myelofibrotic and leukemic transformation in PV at different time points. Values are taken from previously published data (Barbui et al., 2018b), regrouped and represented by a bar chart. OS, overall survival; ↑, increased; PB, peripheral blood; BM, bone marrow; LT, leukemic transformation; sML, secondary myelofibrosis; sAML, secondary AML; y, years.

The majority of patients are diagnosed after a routine blood test, manifesting no symptoms at the time of diagnosis. In others, vascular occlusion or haemorrhage precede the initial diagnosis (Swerdlow SH, 2016). As thrombocythosis is not specific to ET only, the diagnosis of ET must be precise, excluding other types of neoplasms.

Therefore, a biopsy of the bone marrow is necessary for diagnosis. A typical biopsy shows a normal or moderately increased number of cells, with a significant proliferation of enlarged megakaryocytes with hyperlobulated nuclei. Highly increased granulopoiesis or a higher number of myeloblasts, as well as increased reticulin fibrosis, are not seen in ET (Swerdlow SH, 2016). Special care should be given to the distinction of ET from masked PV (mPV) and from prefibrotic primary myelofibrosis (pre-PMF) (Guglielmelli, Pacilli et al., 2017, Tiong, Casolari et al., 2016). The WHO 2016 classification of myeloid neoplasms has introduced changes in the diagnosis criteria in order to help differentiate these disease entities. According to the updated classification, for establishing a ET diagnosis all four major criteria or the first three in addition to the minor criterion need to be met, as presented in Table 3 (Arber et al., 2016).

Table 3. The 2016 World Health Organization criteria for the diagnosis of ET

MAJOR CRITERIA			
1	2	3	4
Platelet count ≥ 450 x 10^9/L	↑ BM proliferation with ↑enlarged, mature megakaryocytes with hyperlobulated nuclei and no ↑ or left shift in neutrophil granulopoiesis or erythropoiesis. Very rarely minor increase in reticulin fibrers (grade 1)	Not meeting WHO criteria for other myeloid neoplasms	Presence of *JAK2*, *CALR* or *MPL* mutation
MINOR CRITERION			
Presence of a clonal marker or Absence of evidence for reactive thrombocytosis			

↑, increased; BM, bone marrow; WHO, world health organization *(The table was adapted and republished with permission of the American Society of Hematology, from The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia, Arber D. et al, Blood 2016 127:2391-2405; permission conveyed through Copyright Clearance Center, Inc.)*

All three identified driver mutations of MPN (*JAK2, MPL* and *CALR*) are found in ET. *JAK2*V617F mutations are found in 50-60%, *CALR* in 20-25% and *MPL* in 2-3% of patients, and are mutually exclusive (Klampfl et al., 2013a, Vainchenker & Kralovics, 2017). The presence of these mutations is listed as a major criterion for MPN diagnosis, pointing out the importance of mutational screening for modern diagnostics (Arber et al., 2016). However, up to 20% of ET patients are triple negative for the driver mutations (Klampfl et al., 2013a, Milosevic Feenstra et al., 2016). In such patients

mutations in other genes or chromosomal abnormalities might be identified and can serve as markers of clonality.

Myelofibrotic transformation occurs in 1% and 9% of patients 10 and 15 years after the diagnosis of ET, respectively (Figure 2B) (Barbui et al., 2018b). The reported median time to progression to post-ET MF is 7 to 16 years from initial diagnosis with associated risk factors being advanced age, anemia, leukocytosis, increased reticulin formation in the BM, BM hypercellularity, use of anagrelide and mutations of *ASXL1* (Cerquozzi & Tefferi, 2015). On the contrary, patients with *JAK2*V617F mutations are associated with a lower risk of fibrotic transformation (Barbui, Thiele et al., 2011).

Blast transformation is seen less frequently in ET than in PV and occurs in 1% after 10 years and 2% of patients after 15 years from ET diagnosis (Figure 2B), with a median time to progression from 6 to 14 years (Barbui et al., 2018b, Cerquozzi & Tefferi, 2015). Age, anemia, leukocytosis, reticulin fibrosis, increased bone marrow cellularity, as well as previous thrombosis and extreme thrombocytosis are recognized as risk factors for leukemic transformation. Regarding molecular genetics, mutations of *TP53* or *RUNX1* are identified as factors increasing the risk for leukemic outbreak (Cerquozzi & Tefferi, 2015).

As in PV, ET patients are stratified in different risk categories. The IPSET-thrombosis system for risk stratification is recommended for use at diagnosis in all ET patients. In addition to age and history of thrombosis, it takes cardiovascular risk factors and *JAK2*V617F mutational status into account, and classifies patients into 4 risk categories: very low risk (age ≤60 years, no history of thrombosis, wt *JAK2*), low risk (age ≤60 years, no history of thrombosis, *JAK2*V617F mutation), intermediate risk (age >60 years, no history of thrombosis, wt *JAK2*) and high risk (age >60 years or history of thrombosis, in addition to a *JAK2*V617F mutation) (Passamonti, Thiele et al., 2012). The stratification of patients serves as a basis for therapy recommendation. A combination of low-dose acetyl salicylic acid and cytoreductive therapy is used, depending on the risk category of the patient. In high risk patients hydroxyurea or rINFα are recommended as a first-line choice for cytoreductive therapy, and a switch to rINFα or busulfan as second-line therapy in patients who inadequately respond to hydroxyurea (Tefferi, Vannucchi et al., 2018d).

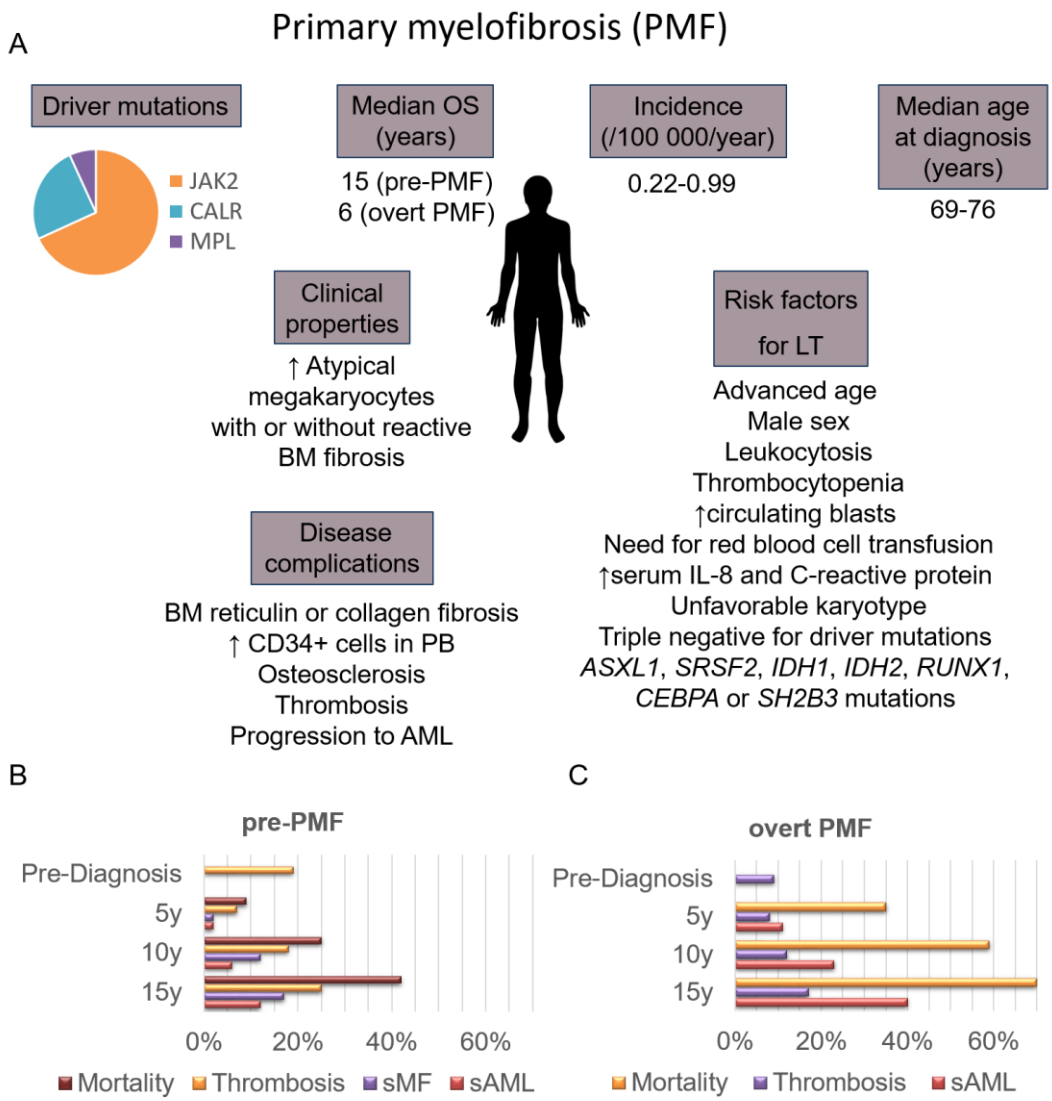
Summarized, increased platelet counts in the peripheral blood and megakaryocytes in the bone marrow define ET clinically, whereas mutations in one of the three MPN driver genes (*JAK2, CALR* and *MPL*) are defined as genetic causes in more than 80% of cases. Strict adhesion to diagnostic guidelines is necessary for avoiding misdiagnosis of ET-like disorders as ET. With a more benign clinical course than PV, ET associates with longer overall survival and lower risks of thrombotic events and fibrotic and leukemic disease progression.

1.1.3. Primary Myelofibrosis

Primary myelofibrosis is characterized by an expansion of morphologically atypical megakaryocytes as well as granulocytes in the bone marrow, with or without the presence of reactive bone marrow fibrosis, dependent on the disease stage (Swerdlow SH, 2016). The latest classification of myeloid neoplasms lists two subcategories of PMF – prefibrotic/early stage (pre-PMF) and overt fibrotic stage PMF (Table 1), and accordingly different diagnostic criteria for the two stages (Tables 4 and 5) (Arber et al., 2016). These are different evolution stages of the same disease and not different disease entities. In the prefibrotic stage reticulin fibrosis in the BM is minimal or absent and BM biopsy shows hypercellularity with increased numbers of neutrophils and atypical megakaryocytes. Due to thrombocythosis early stages of PMF often resemble ET and require careful examination of the bone marrow at diagnosis, with focus on the identification of abnormal megakaryocytes as the hallmark of PMF.

Clinical presentation, in terms of disease progression and mortality, differ in ET and pre-PMF, with worse outcome and prognosis for pre-PMF patients (Figure 3) (Barbui et al., 2018b). In most patients in which early PMF is diagnosed the disease progresses with time to overt PMF. First diagnosis is often made in the overt fibrotic phase. It associates with the presence of reticulin or collagen fibrosis in the bone marrow, extramedullary haematopoiesis (EMH) and an increase of CD34+ cells in peripheral blood. Osteosclerosis is detected in the bone marrow in advanced stages. As a consequence of EMH splenomegaly and hepatomegaly are detected in 90% and 50% of patients, respectively (Arber et al., 2016, Swerdlow SH, 2016).

Figure 3. Clinical and epidemiological properties of primary myelofibrosis



A. Representation of the most important clinical and epidemiological properties of PMF, including the distribution of driver mutations, represented with a pie chart. B. and C. Rates of mortality, thrombosis, myelofibrotic and leukemic transformation in pre-PMF and overt PMF, respectively, at different time points. Values are taken from previously published data (Barbui et al., 2018b), regrouped, and presented by a bar chart. OS, overall survival; ↑, increased; BM, bone marrow; PB, peripheral blood; AML, acute myeloid leukemia; LT, leukemic transformation; IL-8, interleukin-8; sML, secondary myelofibrosis; sAML, secondary AML; y, years.

The median age at diagnosis is in range from 69 to 76 years, with a median survival of 7.2 and 14.7 years for patients diagnosed in the fibrotic and prefibrotic stage, respectively (Guglielmelli et al., 2017, Moulard et al., 2014, Vannucchi et al.,

2015). The annual incidence of PMF is reported to be from 0.22 to 0.99 per 100.000 (Figure 3) (Titmarsh et al., 2014). Compared to all other Ph-negative MPN entities, overt PMF has the highest leukemic transformation rate, occurring in 23% and 40% of patients at a 10-year and 15-year follow up, respectively, as well as mortality rates in 35%, 59% and 70% at 5, 10 and 15 years post-diagnosis. These clinical manifestations of overt-PMF are markedly distinct from those of pre-PMF, where blast transformation and mortality occur at notably lower rates: 6% and 11% after 10 years and 12% and 20% after 15 years from initial diagnosis, respectively (Figure 3B) (Barbui et al., 2018b).

Table 4. The 2016 World Health Organization criteria for the diagnosis of pre-PMF

MAJOR CRITERIA		
1	2	3
Megakaryocytic proliferation and atypia, max. reticulin fibrosis – grade 1, ↑ BM cellularity (age-adjusted), ↑ granulopoiesis, often ↓ erythropoiesis	Not meeting WHO criteria for other myeloid neoplasms	Presence of *JAK2*, *CALR* or *MPL* mutation or Presence of another clonal marker or Absence of minor reactive BM reticulin fibrosis
MINOR CRITERION		
Anemia not atributed to a cormobid condition or Leukocytosis ≥ 11 x 10^9/L or Palpable splenomegaly or ↑LDH		

↑, increased; BM, bone marrow; ↓, decreased; WHO, world health organization; LDH, lactate dehydrogenase *(The table was adapted and republished with permission of the American Society of Hematology, from The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia, Arber D. et al, Blood 2016 127:2391-2405; permission conveyed through Copyright Clearance Center, Inc.)*

Table 5. The 2016 World Health Organization criteria for the diagnosis of overt PMF

MAJOR CRITERIA		
1	2	3
Megakaryocytic proliferation and atypia, reticulin and/or colagen fibrosis – grade 2 or 3	Not meeting WHO criteria for other myeloid neoplasms	Presence of *JAK2*, *CALR* or *MPL* mutation or Presence of another clonal marker or Absence of minor reactive BM reticulin fibrosis
MINOR CRITERION		
Anemia not atributed to a cormobid condition or Leukocytosis ≥ 11 x 10^9/L or Palpable splenomegaly or ↑LDH		

WHO, world health organization; BM, bone marrow; LDH, lactate dehydrogenase*(The table was adapted and republished with permission of the American Society of Hematology, from The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia, Arber D. et al, Blood 2016 127:2391-2405; permission conveyed through Copyright Clearance Center, Inc.)*

As in PV and ET, somatic mutations in driver genes are identified in PMF, leading to clonal expansion of haematopoietic stem cells. *JAK2*V617F mutations are found in 50-60%, mutations of *MPL* in around 5%, and *CALR* mutations in 25-30% of PMF cases (Vainchenker & Kralovics, 2017). However, up to 15% of PMF patients remain triple negative for the three mutations (Klampfl et al., 2013a, Milosevic Feenstra et al., 2016). In these patients search for other clonal markers at the time of diagnosis

(such as frequently identified mutations in other genes – *ASXL1, EZH2, TET2, IDH1/IDH2, SRSF2, SF3B1*) is recommended (Arber et al., 2016).

The triple negative group of PMF patients has a worse overall survival and higher risk of leukemic transformation compared to patients who are carriers of one of the driver mutations, among which *CALR* type 1 and type 1-like mutants have the most favorable prognosis in terms of blast transformation and longest median survival (Klampfl et al., 2013a, Tefferi, Guglielmelli et al., 2014a). Other risk factors for leukemic transformation in PMF are advanced age, male sex, leukocytosis, thrombocytopenia, increased number of circulating blasts, need for red blood cell transfusion, increase of serum interleukin-8 and C-reactive protein levels, unfavorable karyotype and the presence of *ASXL1*, *SRSF2*, *IDH1*, *IDH2*, *RUNX1*, *CEBPA* or *SH2B3* mutations (Tefferi, Lasho et al., 2016a, Vallapureddy, Mudireddy et al., 2019).

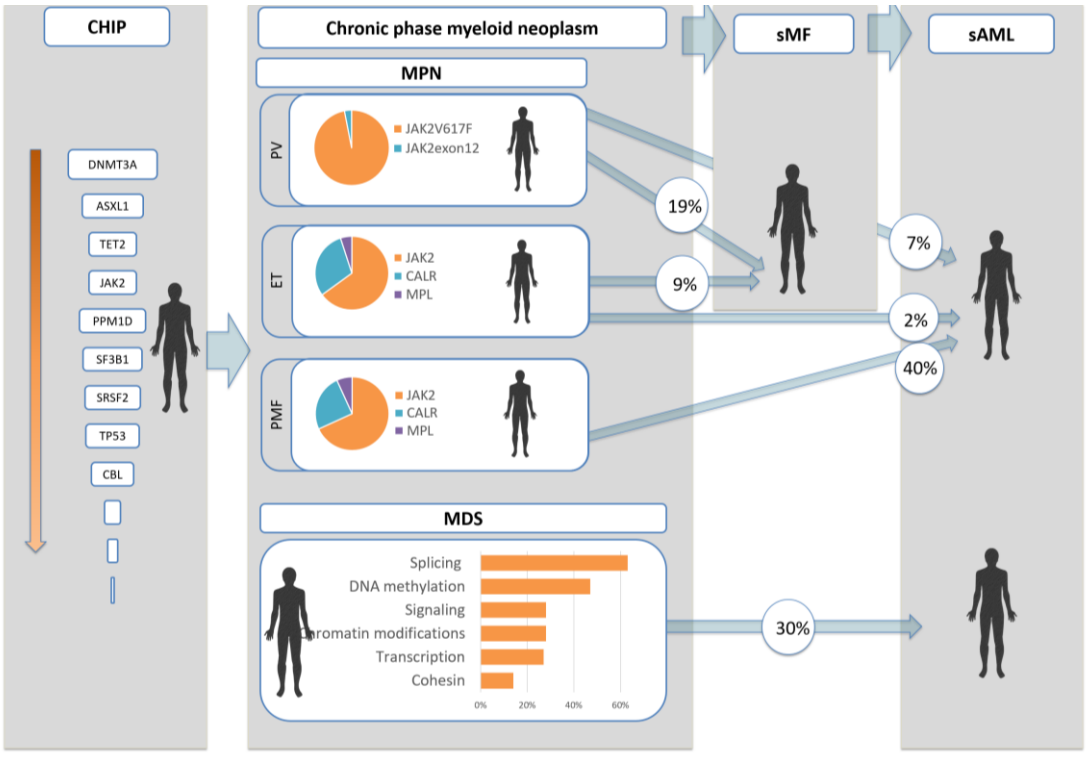
Management of PMF patients and treatment decisions require precise initial diagnosis, as well as risk stratification applying the most recent prognostic systems developed for PMF. Risk factors scored for the stratification are grouped in genetic (mutational status of *CALR*, *ASXL1*, *SRSF2*, *EZH2*, *IDH1*, *IDH2* and the *U2AF1Q157* mutation, and the identification of very high risk or unfavorable karyotype) and clinical (anemia, circulating blasts and constitutional symptoms). The newest mutation-enhanced international prognostic scoring system for transplant age patients (MIPSS70+ version 2.0) considers both clinical and genetic risk parameters and results in a 5-tiered (very low, low, intermediate, high and very high) risk stratification (Tefferi, Guglielmelli et al., 2018a). As no disease-modifying therapy is available for PMF, observation alone is recommended for low risk patients. In high and very high risk patients allogenic stem cell transplantation is recommended. Intermediate risk patients are treated with palliative therapy for occurring symptoms (erythropoiesis-stimulating agents for anemia, radiotherapy for localized bone pain and symptomatic non-hepatosplenic EMH and ruxolitinib or splenectomy for splenomegaly) (Tefferi, Guglielmelli et al., 2018b, Vannucchi et al., 2015). In pre-PMF patients with previous arterial thrombosis low dose aspirin is recommended, as are oral anticoagulants in cases with previous venous thrombosis. If thrombocytosis or leukocytosis occur hydroxyurea is considered as a first-line choice of cytoreduction therapy and a switch to rIFNα if recommended in patients resistant or intolerant to hydroxyurea (Finazzi, Vannucchi et al., 2018).

Taken together, PMF can be viewed as a continuum of two evolutional stages, with pre-PMF having a milder clinical presentation, including overall survival and risks of leukemic transformation, and overt PMF being associated with adverse prognosis. Generally, overt PMF has highest mortality rates and leukemic risks among all chronic phase classical Ph-negative MPNs. Atypical megakaryocytes in the bone marrow are

a hallmark of both pre-PMF and overt PMF, whereas BM fibrosis is usually present only in overt PMF, but can be detected to a minimal degree in pre-PMF in some cases. Mutations in one of the three driver mutations (*JAK2, MPL* and *CALR*) are present in almost 90% of PMF, with triple negative patients carrying the most adverse prognosis, whereas *CALR* (type 1 and type 1-like) mutants correlate with favorable prognosis.

1.2. Genetics of myeloproliferative neoplasms

Figure 4. From CHIP to sAML: driver mutations and disease progression



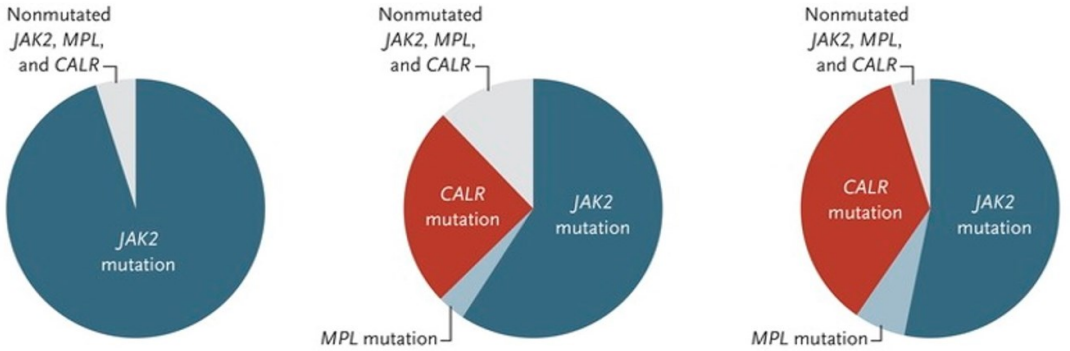
Disease progression from CHIP via chronic phase myeloid neoplasm to sAML is presented by arrows, with rates of transformation in 15 years represented in circles on the corresponding arrows. The most frequently mutated genes in CHIP are presented in declining order(from top to bottom). Driver mutations for MPN are presented with pie charts. The main classes of driver mutations in MDS are grouped by function and are presented with a bar chart. CHIP, clonal hematopoiesis of indeterminate potential; MPN, myeloproliferative neoplasm; MDS, myelodysplastic syndrome; sMF, secondary myelofibrosis; sAML, secondary acute myeloid leukemia

The cause and molecular pathways involved in the onset and evolution of myeloproliferative neoplasms were entirely unknown until the discovery of the first driver mutation in 2005, a mutation in the tyrosine kinase *JAK2* (Janus Kinase 2) (Baxter, Scott et al., 2005, James, Ugo et al., 2005, Kralovics et al., 2005, Levine,

Wadleigh et al., 2005). Since then the understanding of the molecular mechanisms causing myeloproliferative neoplasms has gone a long way, proving the significant role of activated JAK-STAT signaling in MPN pathogenesis (Vainchenker & Constantinescu, 2013). In addition to *JAK2*, mutations in another two genes were identified as MPN drivers - *CALR* (calreticulin) and *MPL* (myeloproliferative leukemia virus) (Klampfl et al., 2013a, Nangalia, Massie et al., 2013, Pikman, Lee et al., 2006). Somatic mutations in these three genes are mutually exclusive and are seen in the majority of MPN patients, leaving around 10% of ET and PMF cases with no detectable driver mutation (termed triple negative patients) (Klampfl et al., 2013a). Triple negative ET patients are associated with a benign disease course and lower thrombotic risk, whereas triple negative PMF is associated with higher leukemic transformation risk and shorter overall survival, compared to *JAK2* and *CALR* mutated MPN (O'Sullivan & Mead, 2019, Rumi, Pietra et al., 2014b). In addition to the three driver mutations patients often have other genes co-mutated (frequently involved in DNA methylation, histone methylation, RNA splicing, signaling pathways), which shape the unique patient-specific mutational profile, and together with other factors, such as inherited genetic predisposition, bone marrow microenvironment, age, gender and environmental exposures, contribute to the clinical disease course (Anderson, Duncombe et al., 2012, Geyer, Kosiorek et al., 2017, Godfrey, Chen et al., 2013). These mutations are not specific for MPN and are not mutually exclusive (Vainchenker & Kralovics, 2017). More than 50% of PV and ET and more than 80% of PMF patients have at least 1 mutated gene other than *JAK2*, *MPL* and *CALR* (Tefferi et al., 2016a, Tefferi, Lasho et al., 2016b). Not only is the type of mutation important for the disease evolution, but also is its variant allele frequency, the number of mutations, the order of mutation acquisition and the type of cell the initiating mutations occur in (Guglielmelli, Lasho et al., 2014, Mead & Mullally, 2017, Ortmann, Kent et al., 2015, Vannucchi, Antonioli et al., 2007). For instance, the occurrence of 2 or more somatic mutations in a single MPN patient is a poor prognostic factor, carrying an elevated risk for leukemic transformation and reduced overall survival (Guglielmelli et al., 2014, Lundberg, Karow et al., 2014). Molecular diagnostics and the identification of mutations in patients has become a valuable stratification, prognosis and management tool, as some mutations affect overall survival or therapy response. The following paragraphs give a closer description of the MPN driver mutations and their functions, together with an overview of the most frequent non-driver somatic mutations found in MPN.

1.2.1. Driver mutations of MPN – *JAK2*, *MPL* and *CALR*

Figure 5. Driver mutations in Ph-negative MPN



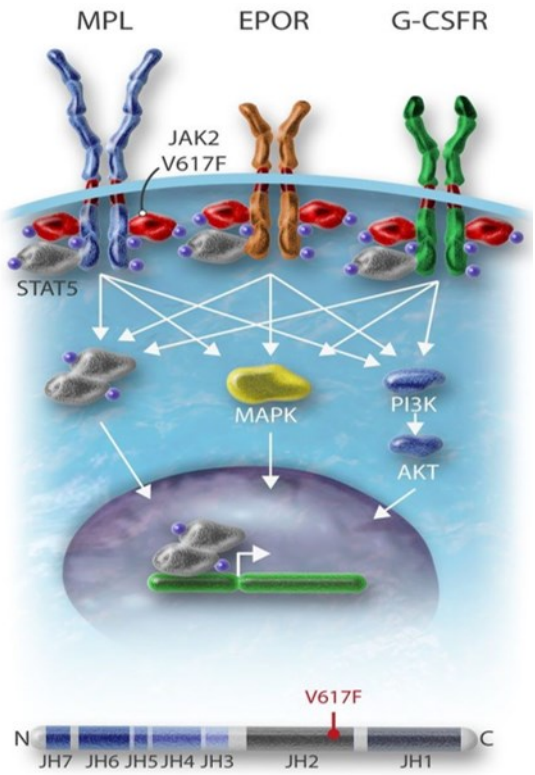
Polycythemia Vera | Essential thrombocythemia | Primary myelofibrosis

The three main driver mutation in MPN – in *JAK2*, *CALR* and *MPL*, are not uniquely represented in PV, ET and PMF. The frequencies of the mutations in the three disease entities are represented by pie charts. *(The Figure was reproduced and the figure legend adapted, with permission from Somatic Mutations of Calreticulin in Myeloproliferative Neoplasms, Klampfl T. et al. N Engl J Med 2013; 369:2379-2390; Copyright Massachusetts Medical Society)*

JAK2 mutations

The *JAK2* (Janus Kinase 2) gene is located on chromosome 9p and encodes a cytoplasmatic tyrosine kinase, which binds to type I cytokine receptors involved in myelopoiesis (erythropoietin receptor - EPOR, thrombopoietin receptor – MPL and granulocyte colony stimulating factor receptor – G-CSFR) (Vainchenker & Kralovics, 2017). Upon binding of cytokines to the receptor Jak2 is activated through transphosphorylation and acts by activating the downstream signaling pathways, mainly through STATs (signal transducer and activator of transcription), but also via MAPK (mitogen-activated protein kinase) and PI3K (phosphoinositide 3-kinase), resulting in transcriptional regulation of target genes (Figure 6). Jak2 binds to the cytoplasmatic domain of the cytokine receptor through its FERM (four-point-one ezrin, radixin, moesin) domain, located at the N terminus of the protein. In addition to a C-terminal catalytically active kinase domain (JH1) the JAK2 kinase contains a catalytically inactive pseudokinase domain (JH2) which inhibits the kinase domain when no cytokine is bound to the receptor. A SH2 (Src homology 2) domain is located between the FERT and the JH2 domain (Vainchenker & Constantinescu, 2013).

Figure 6. Cytokine receptors and signaling of JAK2V617F



JAK2V617F binds to cytokine receptors - MPL (thrombopoietin receptor), EPOR (erythropoietin receptor) and G-CSFR (granulocyte colony-stimulating factor receptor) and activates downstream signaling cascades. In the bottom panel the protein domains of the JAK2 kinase are presented, displaying the position of the JAK2V617F mutation in the pseudokinase JH2 domain. *(Republished with permission of the American Society of Hematology, from Genetic basis and molecular pathophysiology of classical myeloproliferative neoplasms, Vainchenker W & Kralovics R., Blood 2017 129:667-679 permission conveyed through Copyright Clearance Center, Inc.)*

The most common somatic mutation of *JAK2,* which is present in around 95% of PV, and 50-60% of ET and PMF patients (Figure 5), is a G to T gain of function mutation resulting in a valine to phenylalanine change at position 617 (*JAK2*V617F) (Baxter et al., 2005, James et al., 2005, Kralovics et al., 2005, Levine et al., 2005). The affected amino acid is positioned in the pseudokinase domain of the protein, inducing a conformational change which disrupts the inhibitory function of the JH2 domain and results in constitutional activation of Jak2 and the subsequent signaling cascade. As a consequence, increased myeloid cell proliferation and differentiation occur (Vainchenker & Constantinescu, 2013). Furthermore, the *JAK2*V617F mutated kinase is capable of overcoming negative regulation by SOCS3 (suppressor of cytokine signaling 3) (Hookham, Elliott et al., 2007). *JAK2*V617F mutations happen early in the

haematopoietic hierarchy, in the haematopoietic stem cells and haematopietic progenitors of all myeloid lineages (Anand, Stedham et al., 2011, Kralovics et al., 2005). Expression of *JAK2*V617F *in vitro* allows cytokine-independent survival and proliferation of interleukin-3 dependent BaF3 murine cells, but only in the presence of a type I cytokine receptor and a functional FERM domain of the kinase (Kralovics et al., 2005, Lu, Levine et al., 2005). The same *JAK2*V617F mutation causes different phenotypes in patients (ET, PV and PMF), through a selective mechanism which is not entirely understood. Part of the explanation can be found in the preferential binding of mutated Jak2 to different receptors (EPOR being involved in erythrocytosis, MPL in thrombocytosis and G-CSFR in granulopoiesis), and the activation of different STATs (activated STAT5 having a significant role in PV development, STAT1 and STAT3 in ET, and lower activation of STAT3 and STAT5 in MF) (Chen, Beer et al., 2010, Walz, Ahmed et al., 2012, Yan, Hutchison et al., 2012).

The dose dependency of *JAK2*V617F expression seems to also have a role in disease shaping and phenotype manifestation. Homozygous mutants provide stronger proliferative advantage to the affected cells *in vivo* compared to heterozygotes (Anand et al., 2011). Moreover, there is a correlation between the size of the homozygous clone and hemoglobin levels in PV and ET patients (Godfrey et al., 2013, Vannucchi et al., 2007). Mouse model studies of the functional effect of *JAK2*V617F demonstrate the myeloproliferative phenotype inducing potential of the mutation and show that low mutation expression results in a ET-like phenotype, compared to high expression which presents with PV-like phenotype, often progressing to secondary myelofibrosis (Akada, Yan et al., 2010, Lacout, Pisani et al., 2006, Li, Kent et al., 2014, Tiedt, Hao-Shen et al., 2008). A phenotypic switch from ET to PV was observed in knock in mice, associated with a transition from heterozygous to homozygous *JAK2*V617F expression (Li et al., 2014). A transition from heterozygous to homozygous *JAK2*V617F in mutation carriers arises through mitotic recombination resulting in an acquired uniparental disomy of chromosome 9p and loss of heterozygosity (LOH) for the affected region and is frequently seen in MPN, more often in PV and PMF than in ET (Kralovics, Guan et al., 2002, Wang, Wheeler et al., 2016). Homozygous *JAK2*V617F mutation seems to be associated with erythrocythosis (rather than thrombocytosis), which corresponds to higher frequencies of homozygous *JAK2*V617F seen in PV than ET and the hypothesis that homozygous *JAK2*V617F mutants preferentially bind to erythropoietin receptors (EPOR), unlike heterozygous *JAK2*V617 mutants which have higher binding affinity to the thrombopoietin receptor (MPL). The homozygous mutation is associated with higher risk of secondary myelofibrosis in both PV and ET patients (Cerquozzi & Tefferi, 2015).

In addition to the *JAK2*V617F mutation, mutations in exon 12 of *JAK2* are discovered in 3% of PV patients, which are specific for PV and not seen in ET and PMF (Scott et al., 2007). However, a progression of *JAK2* exon 12 mutant PV patients to secondary myelofibrosis may occur (Pietra, Li et al., 2008, Scott et al., 2007). The mutations in exon 12 are heterogeneous – from point mutations to small insertions or deletions. They are all positioned in the linker domain between the SH2 and JH2 domain, between residues 536 and 547, also resulting in constitutive activation of the kinase through a not yet understood mechanism which seems to differ from the one seen in *JAK2*V617F mutants. The close physical proximity of the pseudokinase domain and the linker loop (where exon 12 mutation changes occur) suggests that this region is important for the interaction of the pseudokinase and kinase domains of Jak2 (Albiero, Madeo et al., 2008, Lee, Ma et al., 2009). PV patients with exon 12 *JAK2* mutations present at a younger age with stronger erythrocythosis than seen in *JAK2*V617F patients, consistent with erythrocythosis seen in transgenic mouse models and the affinity of *JAK2* exon 12 mutants to bind EPOR (Scott et al., 2007, Yao, Ma et al., 2017). Exon 12 mutations result in stronger activation of the downstream signaling cascade, demonstrated by stronger activation of STAT5 compared to *JAK2*V617F, and also provide survival and proliferation capability to BaF3 cells in the absence of cytokines (Scott et al., 2007).

Jak2 can also be localized in the nucleus of progenitor CD34+ cells where it regulates transcription, by directly phosphorylating tyrosine 41 of histone 3 (H3Y41) and causing the release of the heterochromatin protein 1α (HP1α) (Dawson, Bannister et al., 2009, Rinaldi, Rinaldi et al., 2010). In the nucleus it also phosphorylates PRMT5, reducing its methyltransferase activity. Both of these actions of *JAK2*V617F affect the expression of target genes, some of which are involved in haematopoiesis and leukemogenesis (ex. *LMO2* and *NFE2*), through a mechanism different from the canonical JAK-STAT signaling pathway (Peeken, Jutzi et al., 2018). This might play a role in the pathogenesis of MPN but further research in this area is needed for better understanding of the functional effect of the observed chromatin modifications.

Recently, several variants of *JAK2* have been associated with progressing MPN, especially in combination with an existing *JAK2*V617F mutation, with a potential to serve as predictors of leukemic transformation (Benton, Boddu et al., 2019).

***MPL* mutations**

The *MPL* (Myeloproliferative Leukemia Virus; Thrombopoietin Receptor; TPOR) gene is positioned on chromosome 1p and encodes the thrombopoietin receptor, which is a homodimeric class I cytokine receptor involved in

megakaryopoiesis and hematopoietic stem cell quiecense. For its functional role in the regulation of haematopoiesis and platelet production it requires binding to its ligand, thrombopoietin. The presentation of MPL at the cell membrane is in a monomeric or unstable dimeric form, when inactive. Cytokine binding induces a conformational change of the receptor bringing it into an active dimeric conformation which further activates the JAK-STAT signaling pathway, through Jak2 bound to the intracellular domain of MPL (Vainchenker, Plo et al., 2019). The receptor contains distinct functional domains – the intracellular domain responsible for binding to JAK2, a juxtamembrane cytosolic domain which prevents ligand-independent dimerization of the receptor through a RWQFP motif, a transmembrane domain needed for membrane positioning and an extracellular domain which binds thrombopoietin. Missence *MPL* mutations in MPN were discovered in 2006 (Pikman et al., 2006). They occur in around 2% of ET and 5% of PMF patients, and are not identified as disease drivers in PV patients (Figure 5). The two hot spots of mutations of MPL are in exon 10 of the gene, resulting in changes of tryptophan at position 515 of the receptor (W515) located in the juxtamembrane domain, and serine at position 505 (S505) located in the transmembrane domain (Vainchenker et al., 2019). The following *MPL* mutations at position 515 are associated with MPN: W515L, W515K, W515R, W515A and W515G, with the first two being most frequently detected, predominantly as heterozygous mutations (Defour, Chachoua et al., 2016, Pikman et al., 2006). Only W515C and W515P do not activate MPL, whereas all other substitutions of W515 result in a TPO-independent dimerization and activation of MPL and subsequent activation of Jak2, increased STAT5 and STAT3 signaling and cytokine-independent growth. Retroviral mouse models expressing *MPL*W515L and *MPL*W515A induce an ET-like phenotype progressing to myelofibrosis (Pecquet, Staerk et al., 2010, Pikman et al., 2006). In ET patients a transition from heterozygous to homozygous MPL mutations, by acquired uniparental disomy of chromosome 1p, is associated with bone marrow fibrosis and disease progression (Rumi, Pietra et al., 2013). A less common mutation is the S505N mutation, also responsible for constitutive activation of MPL through stabilizing the active dimeric conformation, however with a weaker *in vivo* effect than the one observed in *MPL*W515 mutants (Vainchenker et al., 2019). Other non-canonical weak gain-of-function *MPL* mutations have been identified in ET and PMF patients at significantly lower rates, such as T119I, S204P, S204F and E230G in the extracellular domain and Y591N and Y591F in the intracellular domain (Cabagnols, Favale et al., 2016, Milosevic Feenstra et al., 2016). In addition, a novel MPL P222S mutation coupled with chr 1p UPD has recently been identified in a PMF patient (Poluben, Puligandla et al., 2019). The thrombopoietin receptor has a central role in MPN pathogenesis, not only in MPL mutant patients but also in JAK2 and CALR mutants

(Figures 6 and 7). Jak2 mutants require binding to either EPOR, MPL or G-CSFR for inducing MPN, whereas *CALR* and *MPL* mutations activate only MPL and upon receptor activation all three driver mutations manifest their effect through constitutively active JAK-STAT signaling (Lu, Huang et al., 2008, Sangkhae, Etheridge et al., 2014, Vainchenker & Kralovics, 2017). Germline *MPL* mutations have also been identified and are associated with hereditary thrombocytosis (Ding, Komatsu et al., 2009, Ding, Komatsu et al., 2004).

CALR mutations

The newest discovered of the three MPN driver mutations are mutations in the *CALR* (calreticulin) gene, located on the short arm of chromosome 19. They occur in around 30% of ET and PMF patients (Figure 5) (Klampfl et al., 2013a). More than 50 different types of frameshift mutations have been detected, all in exon 9 of the gene, and all resulting in a +1 frameshift (Klampfl et al., 2013a, Nangalia et al., 2013). Despite the large number of different mutations, they can be grouped into 4 subgroups based on their properties. The two most frequent mutations are named type 1 and type 2 mutations, representing 53% and 32% of *CALR* mutations in patients, and the ones resembling the induced structural changes are classified as type 1-like and type 2-like, occurring at much lower frequencies. The most prevalent *CALR* mutation, the type 1 mutation (also known as *CALR*del52), is a 52 bp deletion resulting in a L367fs*46 change of the protein. It is more frequent in PMF than in ET. The type 2 mutation is a 5 bp insertion (*CALR*ins5) which induces a K385fs*47 modification and is distributed at similar rates in ET and PMF. These changes introduce novel positively charged amino acid sequences at the C-terminal of the protein, with *CALR*ins5 preserving around half of the negative charges of the wt protein, unlike *CALR*del52 which replaces almost all of them. *CALR* mutations occur in the hematopoietic stem cells and early progenitors and are identified as early events in MPN clonal evolution (Klampfl et al., 2013a, Nangalia et al., 2013). They are most often heterozygous, with the allelic frequency at diagnosis usually ranging from 40% to 50%. Rarely, homozygous *CALR* mutations are identified in patients, usually resulting from an acquired uniparental disomy of chromosome 19, and are associated with disease progression and acquired myeloperoxidase deficiency in MF patients (Rumi et al., 2014b, Theocharides, Lundberg et al., 2016). *CALR*-mutated MPN patients have a younger age at diagnosis, higher platelet count and decreased number of leukocytes compared to *JAK2*-mutated MPN (Tefferi, Lasho et al., 2014b). In ET lower risk of thrombosis as well as a higher risk of fibrotic transformation is associated with *CALR* mutations, whereas in PMF

improved overall survival and lower red blood cell transfusion dependency is observed (Klampfl et al., 2013a, Nangalia et al., 2013, Pei, Wu et al., 2016, Rumi, Pietra et al., 2014a). The risk of myelofibrotic transformation in ET is associated with del52 mutants and not with ins5 mutants. Furthermore, *CALR*ins5 mutant patients have reduced risk of thrombosis, higher platelet counts and reduced overall survival (Cabagnols, Defour et al., 2015, Tefferi et al., 2014b, Tefferi, Wassie et al., 2014c). *CALR* mutations have also been identified in refractory anemia with ringed sideroblasts and thrombocytosis (RARS-T), chronic neutrophilic leukemia and atypical chronic myeloid leukemia (Klampfl et al., 2013a, Patnaik, Belachew et al., 2014).

Calreticulin is a Ca^{2+}-binding protein predominantly localized in the lumen of the endoplasmatic reticulum (ER) where it acts as a chaperone involved in quality control of newly produced glycoproteins, directing the inadequately folded proteins to the proteasome for degradation (Ellgaard & Frickel, 2003). The protein consist of three domains – a N-terminal domain holding the ER targeting signal sequence, a P-domain rich in proline with a role in Ca^{2+} binding, and a C-terminal domain holding a KDEL sequence (ER retention signal). The C-terminal domain in the wildtype protein is negatively charged and also serves as a low-affinity binding site for Ca^{2+} (Elf, Abdelfattah et al., 2016). The mutant protein lacks the KDEL sequence and contains positively charged residues replacing the negative ones of the wild type protein. In addition to its chaperone role, CALR is also involved in regulation of Ca^{2+} storage (Arnaudeau, Frieden et al., 2002). Scientific findings about the role of mutant *CALR* in MPN development are rapidly accumulating since the discovery of *CALR* mutations in 2013 and are building a model of their role in MPN pathogenesis. The cellular localization of the mutated form of the protein is mainly in the ER to Golgi intermediate (ERGIC) compartment. During MPL maturation in the ER, mutant CALR binds to the N-glycosylated residues of the extracellular receptor domain, activates the receptor prematurely, blocks its maturation and transports it to the cell surface (Figure 7B) (Chachoua, Pecquet et al., 2016, Elf et al., 2016). The activated receptor constitutively remains in this state, activates Jak2 bound to its intercellular domain, and furthermore the STAT5, MAPK/ERK and PI3K/AKT pathways (Araki, Yang et al., 2016, Chachoua et al., 2016, Klampfl et al., 2013a). This mimics the effect of *MPL* mutations. For this constitutive signaling activation mutant CALR requires its mutated C-terminal domain and its glycan binding site, as well as the N-glycosylation sites in the extracellular domain of MPL and tyrosine residues on the intracellular domain of MPL (Chachoua et al., 2016, Elf et al., 2016, Marty, Pecquet et al., 2016, Nivarthi, Chen et al., 2016). Interestingly, CALR binds to MPL through its N-domain but the mutant C-domain is required to allow this interaction by interfering with the P-domain/N-domain interaction which prevents binding to MPL (Araki et al., 2016). Another property of the CALR

mutant is to use its novel C-domain to form homomultimeric complexes, which happens prior to, and seems to be required for, MPL binding and activation (Araki, Yang et al., 2019). Mutant CALR can also bind to G-CSFR (Figure 7C), activating it to a much weaker extent than MPL, but this interaction is not sufficient for long-term cytokine-independent proliferation in hematopoietic cell lines (Chachoua et al., 2016). TPO-independent MPL activation and long-term cell proliferation is confirmed to be induced in *CALR* mutant cell lines. Recent findings discovered a role of wild type CALR in normal hematopoiesis, in self-renewal of hematopoietic stem cells and erythroid and megakaryocytic differentiation, by direct regulation of expression of genes involved in these processes (Salati, Prudente et al., 2017). The role of mutant CALR in transcription regulation of target genes involved in megakaryocytic differentiation has also been identified. It is involved in forming a chaperone complex with FLI1 and ERP57, which binds to the *MPL* promotor, promoting its transcription (Han, Schubert et al., 2016, Pronier, Cifani et al., 2018). Mutant CALR also regulates the expression of other factors important for megakaryocyte production – CD41 and NF-E2 and impairs normal functioning of hematopoietic stem cells (Han et al., 2016). Mouse models of *CALR* mutants have been developed, presenting an ET-like phenotype which progresses over time to myelofibrosis (mainly in *CALR*del52 mouse models), demonstrating the MPN-inducing property of mutant *CALR*, and confirming its driver mutation potential (Balligand, Achouri et al., 2016, Elf et al., 2016, Li, Prins et al., 2018, Marty et al., 2016). The observed thrombocytosis is milder and the rate of fibrotic transformation is lower in *CALR*ins5 mouse models than in *CALR*del52 (Marty et al., 2016). In transgenic *CALR*del52 mouse models homozygous mutations induced MF, whereas heterozygotes presented with an ET-like phenotype, implicating a role of expression dosage in differential phenotype manifestation (Shide, Kameda et al., 2017). Based on the knowledge about mutant *CALR*-induced MPN several strategies for treating *CALR*-mutant patients are being investigated, including application of JAK2 inhibitors alone or in combination MEK-ERK, mTOR or PARP inhibitors; inhibition of Golgi transport and N-glycosylation; competitive inhibition of mutant CALR by delivering wt CALR peptides to *CALR*-mutant cells; T-cell receptor-mediated immunotherapy (Merlinsky, Levine et al., 2019). This is still an open field of research and further pre-clinical and clinical studies are necessary for identifying the best approach for *CALR*-mutant MPN management.

Figure 7. Cytokine receptors and signaling of wt and mutant CALR

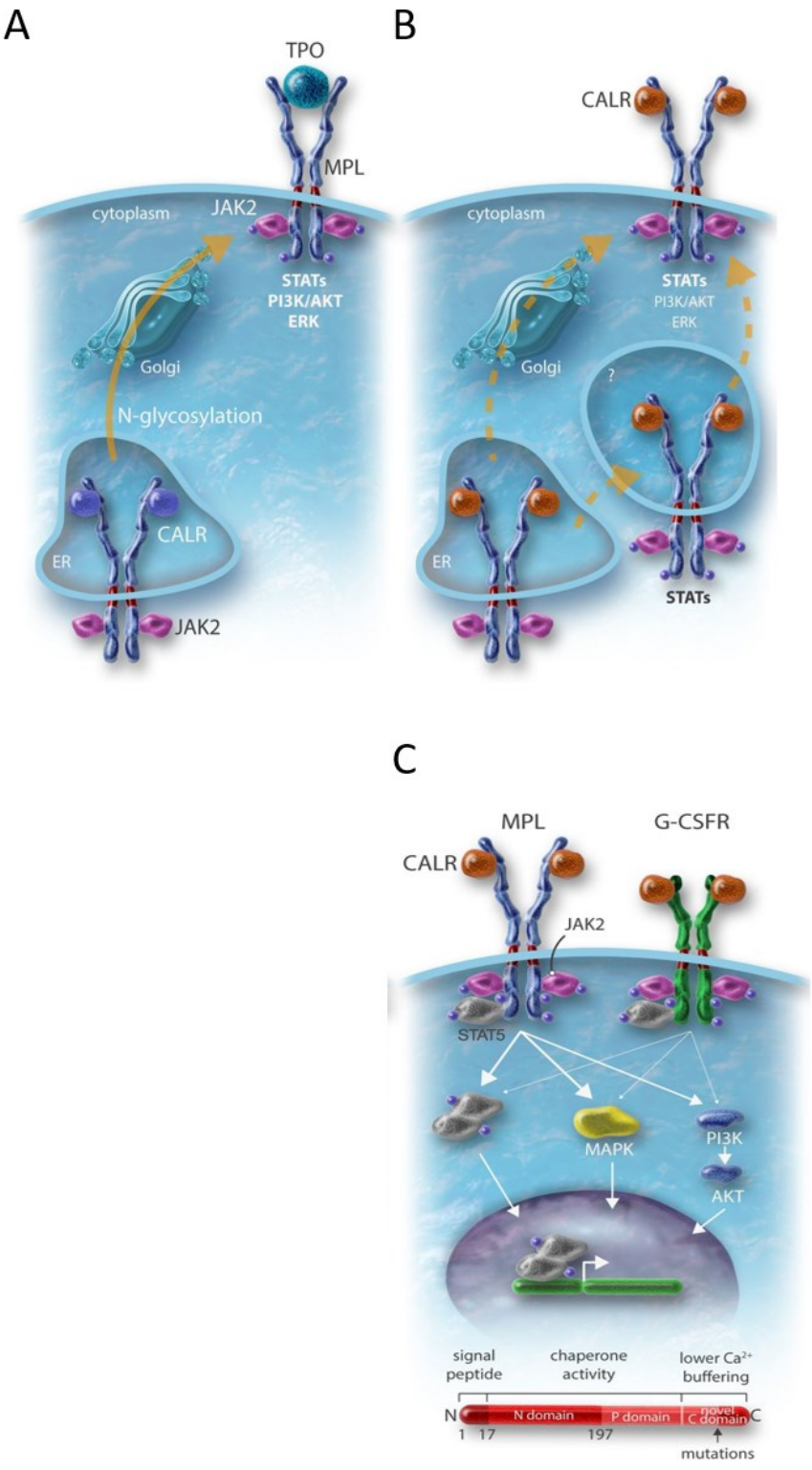


Figure 7. Cytokine receptors and signaling of wt and mutant CALR
A. WT CALR binds to the MPL receptor in the ER, disassociates afterwards, allowing trafficking and expression of the mature form of the MPL receptor at the cytoplasmatic membrane, where it binds TPO, and in a cytokine-dependent way activates signaling. B. Mutant CALR binds to the MPL receptor in the ER and is transported with it to the cell surface, expressing a immature receptor form with bound CALR on the cytoplasmatic membrane, constitutively activating the JAK-STAT pathway. C. Mutant CALR binds to cytokine receptors preferentially to MPL (thrombopoietin receptor), but also to G-CSFR (granulocyte colony-stimulating factor receptor), activating the downstream signaling. The protein domains of CALR are presented in the bottom panel, displaying the C domain where mutations occur. *(Republished with permission of the American Society of Hematology, from Genetic basis and molecular pathophysiology of classical myeloproliferative neoplasms, Vainchenker W & Kralovics R., Blood 2017 129:667-679 permission conveyed through Copyright Clearance Center, Inc.)*

1.2.2. Non-driver somatic mutations in MPN

TET2

TET2 (ten-eleven translocation oncogene family member 2) gene is located on chromosome 4q and encodes a protein of the TET family of proteins, with a role in gene regulation in hematopoietic stem cells through demethylation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) at CpG sites (Cimmino, Abdel-Wahab et al., 2011). Mutations in this epigenetic regulator occur in 10-15% of MPN, in all three classical MPN subtypes (PV, ET, PMF), as well as with all three driver mutations (*JAK2*, *CALR*, *MPL*) (Delhommeau, Dupont et al., 2009). All of the mutations result in a loss-of-function of the protein and accordingly, a reduction of 5hmC levels is observed in *TET2* mutant patients. *TET2* loss increases the self-renewal capacity of hematopoietic stem cells and skews differentiation to the myeloid lineage (Moran-Crusio, Reavie et al., 2011). It is also shown to induce hypermutagenicity of the affected hematopoietic stem and progenitor cells (Pan, Wingo et al., 2017). The order in which mutations arise relative to other mutations may contribute to the phenotype determination. When *TET2* mutations precede *JAK2*V617F an ET-like phenotype is likely to develop, in contrast to *TET2* mutations on a *JAK2*V617F background which give rise to a PV phenotype (Ortmann et al., 2015). In the case of co-mutated *CALR/TET2* cases *CALR* mutations seem to always occur first, potentially due to the fact that CALR mutations arise in younger individuals than other MPN-driving mutations (Nangalia et al., 2013). Acquired copy-neutral loss of heterozygosity of

chromosome 4q is seen in MPN, leading to a homozygous *TET2* mutation (Jankowska, Szpurka et al., 2009). In mouse models homozygous *TET2* mutations induce a more severe myeloid malignancy phenotype with a shorter latency period, compared to heterozygous *TET2* mutations (Li, Cai et al., 2011). *TET2* mutations are also detected in chronic myelomonocytic leukemia (CMML), MDS and AML at even higher frequencies than in MPN and are one of the most frequently identified mutations in clonal hematopoiesis associated with ageing (CHIP) (Genovese et al., 2014, Jaiswal et al., 2014, Smith, Mohamedali et al., 2010, Weissmann, Alpermann et al., 2012, Xie et al., 2014).

DNMT3A

The *DNMT3A* (DNA methyltransferase 3A) gene is located on chromosome 2p and is mutated in all MPN subtypes, in 5-10% of cases (Stegelmann, Bullinger et al., 2011). The mutational hot spot is at position 882, with R882H being the most frequently detected *DNMT3A* mutation but other mutations occur in different functional protein domains, all resulting in a loss of function. The encoded protein actively regulates DNA methylation by methylating the CpG dinucleotides, and therefore affecting the same biological processes as Tet2, most likely regulating the expression of genes involved in the development of hematopoietic stem cells. *DNMT3A* mutations provide self-renewal properties to hematopoietic stem cells and are shown to immortalize them, allowing for indefinitive propagation of the mutated cells (Jeong, Park et al., 2018). *DNMT3A* mutations are one of the most frequently seen in CHIP and are considered early events in myeloid neoplasm evolution (Genovese et al., 2014). They occur associated with *JAK2*, *MPL* and *CALR*, and are shown to most frequently precede *JAK2*V617F, inducing ET (Nangalia, Nice et al., 2015). However, when the *DNMT3A* mutation occurs as a secondary event to *JAK2*V617F a PV phenotype develops, confirming the importance of mutation order for MPN phenotype shaping. A DNA methylation-independent role of *DNMT3A* mutants has been revealed, showing that they silence differentiation-related genes in HSCs, by interacting with polycomb repressive complex 1 (PRC1), leading to HSC accumulation (Koya, Kataoka et al., 2016).

IDH1/IDH2

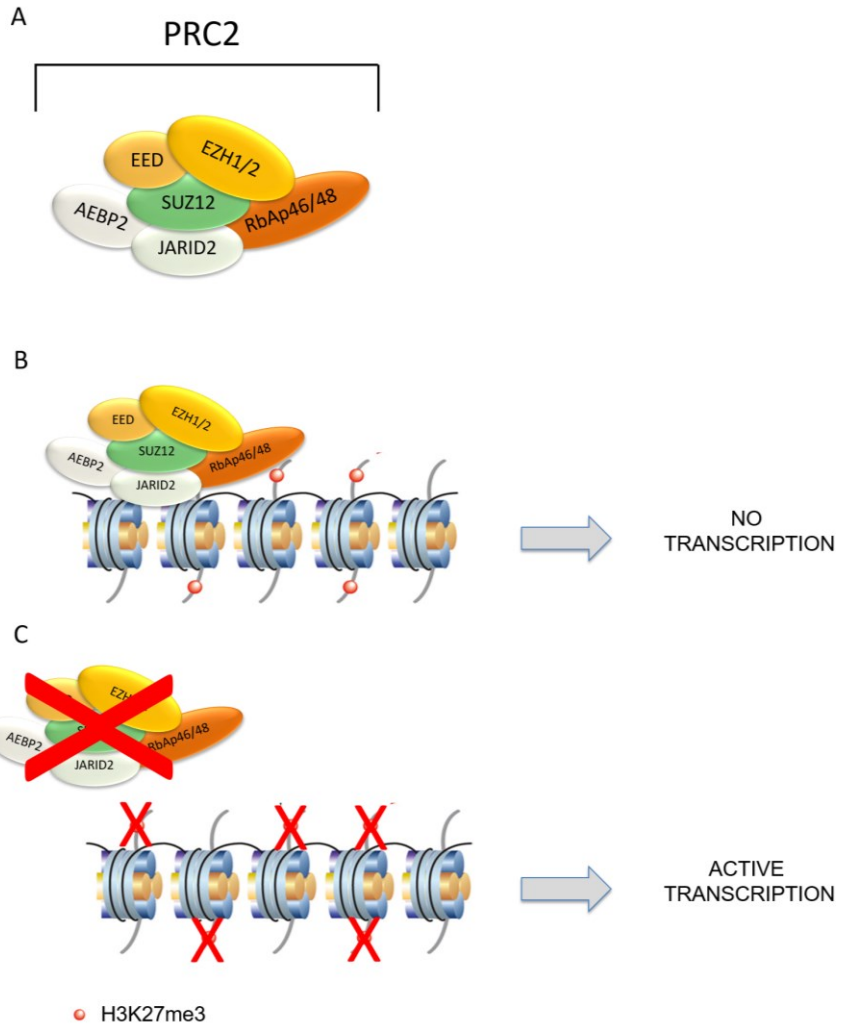
Isocitrate dehydrogenase 1 and 2 (Idh1 and Idh2) are enzymes which convert isocitrate to α-ketoglutarate, but the mutated proteins produce 2-hydroxyglutarate instead (Ye, Xiong et al., 2012). This indirectly affects DNA methylation, inhibits Tet2 activity and results in a block of differentiation. The *IDH1* and *IDH2* genes are located on chromosomes 2q and 15q, respectively, and are found mutated in 1-4% of MPN (Tefferi, Lasho et al., 2010). *IDH1* and *IDH2* mutations are associated with advanced phases of MPN and leukemic transformation (Green & Beer, 2010).

EZH2

Ezh2 (Enhancer of Zeste 2) is one of the core members of the polycomb repressive complex 2 (PRC2) which regulates chromatin structure and transcription of genes important for the regulation of self-renewal and differentiation of hematopoietic stem cells by thrimethylating lysine 27 of histone 3 (H3K27me3), a hallmark of repressed chromatin structures and epigenetic gene silencing (Figure 8B) (Margueron & Reinberg, 2011). Ezh2 is the catalytic subunit of the complex, requiring the presence of the other two core members (Suz12 and Eed) for its methyltransferase activity (Cao & Zhang, 2004). Eed binds to H3K27me3 marks allosterically inducing the activity of Ezh2, whereas Suz12 provides structural stability to the complex, necessary for its activity, by binding to all PRC2 subunits (Kasinath, Faini et al., 2018, Margueron & Reinberg, 2011). Other members of the PRC2 complex include RbAp46/RbAp48, which are also part of the complex core, co-factors Jarid2 and Aebp2, which moderate complex recruitment to specific loci and its activity, and alternatively Ezh1, which is a homolog of Ezh2 with reduced histone methyltransferase activity (Figure 8A).

Aebp2 and Jarid2 mimic histone H3 tails, bind to RbAp48 and Eed, respectively, and synergistically regulate its activity and recruitment. They also contribute to the stability of the complex by binding to Suz12 (Kasinath et al., 2018). The *EZH2* gene is located on chromosome 7q. *EZH2* loss, as well as chromosome 7 lesions are associated with poor prognosis and *EZH2* mutated PMF patients have increased risks of leukemic transformation (Guglielmelli, Biamonte et al., 2011, Tam, Abruzzo et al., 2009, Tefferi et al., 2018c). *EZH2* mutations detected in myeloid neoplasms are loss-of-function missence and frameshift mutations, contrary to gain-of-function mutations seen in lymphoid malignancies, occurring in 3% of MPN, with highest frequencies in PMF among the three disease entities (Lundberg et al., 2014).

Figure 8. The polycomb repressive complex 2 and its role in the transcription of target genes



A. The subunits of PRC2. Core members (EZH1, EZH2, SUZ12, EED and RbAp46/48) are associated with JARID2 and AEBP2. B. When recruited to target genes PRC2 thrimethylates H3K27 leaving H3K27me3 marks which leads to repression of the targeted gene. C. When PRC2 is inactivated the lack of H3K27me3 marks results in de-repression of the target gene and its active transcription. *(The figure was produced by the author of the thesis, using the previously published chromatin graphics from Jarid2 Methylation via the PRC2 Complex Regulates H3K27me3 Deposition during Cell Differentiation, Sanulli S. et al., Mol Cell. 2015 Mar 5;57(5):769-783, with permission under the terms of the Creative Commons Attribution Liscence (https://creativecommons.org/licenses/by/4.0/))*

A co-occurrence of *EZH2* and *JAK2*V617F mutations is often observed and their effects synergize in MPN phenotype initiation, as shown in mouse models, leading to a fast progressing disease with shortened overall survival (Sashida, Wang et al., 2016, Shimizu, Kubovcakova et al., 2016, Yang, Akada et al., 2016). *EZH2*

mutations also co-occur with *ASXL1* mutations (Guglielmelli et al., 2011). Both mutations are identified in long-term HSCs and have been described as early events in PMF evolution, preceding MPN driver mutations. These mutations can also occur secondary to a MPN driver mutation and contribute to disease progression (Lundberg et al., 2014). Double mutants present with lower hemoglobin levels, higher rates of additional mutations and worse prognosis (Triviai, Zeschke et al., 2019). Impaired erythrocytosis is observed in both *JAK2V617F/EZH2* and *ASXL1/EZH2* double mutants, with myeloid skewing of hematopoiesis (Triviai et al., 2019, Yang et al., 2016). *EZH2* loss abrogates the recruitment of the PRC2 complex to target genes, reduces H3K27me3 levels and results in their derepression (Figure 8C), some of which have important roles in self-renewal of HSC (ex. Hox genes) or regulation of erythropoiesis and fibrosis promotion (ex. Lin28b/Hmga2 axis) (Khan, Jankowska et al., 2013, Shimizu et al., 2016).

ASXL1

Asxl1 (Additional Sex Combs Like 1) is encoded by the *ASXL1* gene located on chromosome 20q. Mutations of the gene are frequently seen in MPN, preferentially in PMF, with an initially reported frequency of around 25% in PMF, and their presence is associated with shortened overall survival and increased risk of leukemic transformation, especially in *CALR* wt patients (Tefferi et al., 2014b, Vannucchi, Lasho et al., 2013, Yonal-Hindilerden, Daglar-Aday et al., 2015) A recent study detected *ASXL1* mutations by means of next generation sequencing in even 38% of PMF patients (Triviai et al., 2019). In PV and ET the reported incidences of *ASXL1* mutations are 12% and 11%, respectively. *ASXL1* mutations in MPN are mainly heterozygous missense or nonsense point mutations or frameshift mutations, located in exon 12. The mutations affect the C-terminal domain of the protein and result in a truncated form lacking the PHD (plant homeodomain) domain, important for histone mark recognition and recruitment of chromatin and transcription regulators (Sanchez & Zhou, 2011). Asxl1 directly interacts with Ezh2 and is involved in the recruitment of PRC2 to target loci, and therefore has an important role in epigenetic regulation of transcription (Abdel-Wahab, Adli et al., 2012). Loss of *ASXL1* in hematopoietic cells results in global loss of H2K27me3 marks and increased expression of target genes, including *HOXA* genes involved in leukemogenesis. Mutated Asxl1 also induces myeloid leukemogenesis through interactions with Bap1 (BRCA1-associated protein 1), which deubiquitinases Lysine 119 of Histone 2A (H2AK199Ub) and epigenetically regulates gene expression, by upregulating *HOXA* and *IRF8* expression (Asada, Goyama et al., 2018). As part of clonal hematopoiesis related to ageing (CHIP) *ASXL1*

is one of the most frequently mutated genes contributing to clonality of hematopoietic cells, and is also mutated in MDS, AML and CMML (Genovese et al., 2014, Jaiswal et al., 2014, Xie et al., 2014). The presence of *ASXL1* mutations in long-term haematopoietic stem cells indicates its role in early pathogenesis of myeloid malignancies (Triviai et al., 2019). *ASXL1* and *TET2* mutations co-occur in MPN. The cooperation of these mutations results in myeloid skewage of hematopoiesis through the activated Asxl1-Bap1 complex (Balasubramani, Larjo et al., 2015). As already described, the co-occurrence of *ASXL1* and *EZH2* mutations is known in MPN and is associated with adverse prognosis (Triviai et al., 2019).

Mutations affecting spliceosome proteins

The *SRSF2* (serine/arginine-rich splicing factor 2), *U2AF1* (U2 small nuclear RNA auxiliary factor 1), *SF3B1* (splicing factor 3B subunit 1), and *ZRSR2* (zinc finger RNA binding motif and serine/arginine rich 2) genes, located on chromosomes 17q, 21q, 2q and Xp, respectively, encode components of the spliceosome machinery and are seen mutated in MPN in a mutually exclusive manner, with highest frequencies observed in PMF patients (18%, 16%, 10% and 10% for the 4 genes respectively) (Tefferi et al., 2016a). *SF3B1* and *SRSF2* mutations are associated with poor prognosis and elevated rates of disease progression and leukemic transformation Aside mis-splicing of target genes, *SRSF2* and *U2AF1* mutations contribute to the MPN phenotype through a PRC2-dependant mechanism, as significant downregulation of *EZH2* and reduced H3K27me3 levels are observed in patients who are carriers of these mutations (Khan et al., 2013).

Mutations in signaling pathways

Having in mind the significance of the JAK-STAT signaling pathway in haematopoiesis and its causative role in the development of MPN it is not surprising that mutations in other members or regulators of the signaling pathway are found in MPN. *LNK* (lymphocyte specific adaptor protein), also known as *SH2B3* (SH2B adaptor protein 3), is found mutated in MPN, at very low frequencies, not mutually exclusive with MPN driver mutations and are associated with disease progression (Hurtado, Erquiaga et al., 2011, Oh, Simonds et al., 2010, Pardanani, Lasho et al., 2010). *LNK* mutations are also known to occur in *JAK2*-negative erythrocytosis (Lasho, Pardanani et al., 2010). The encoded protein negatively regulates JAK2 signaling initiated by activation of tyrosine kinases, EPOR or MPL, and when mutated induces cell proliferation and a MPN-like phenotype in *LNK* deficient mice (Oh et al.,

2010, Tong & Lodish, 2004, Tong, Zhang et al., 2005, Velazquez, Cheng et al., 2002). Both somatic and germline *LNK* mutations most likely synergize with MPN driver mutations in determining the disease phenotype (Rumi, Harutyunyan et al., 2016). *SOCS* (suppressor of cytokine signaling) genes, encoding a family of negative regulators of JAK-STAT signaling, are also found mutated and epigenetically inactivated in MPN (Jost, do et al., 2007, Suessmuth, Elliott et al., 2009). Inactivating mutations of the *CBL* (Casitas B-lineage lymphoma proto-oncogene) gene, located on chromosome 11q, are detected in MPN and are associated with acquired uniparental disomies of chromosome 11q (Grand, Hidalgo-Curtis et al., 2009, Klampfl, Milosevic et al., 2013b). Mutations occur more frequently in PMF (5%) than in PV and ET (1%) (Tefferi et al., 2016a, Tefferi et al., 2016b). Cbl negatively regulates tyrosine kinase signaling, through its E3 ubiquitine ligase function. In addition to acting on receptor tyrosine kinases as its targets (EPOR, MPL, PDGFR), Cbl also regulates the ubiquitination of other signaling molecules, including Jak2, Flt3 and c-Kit (Toffalini & Demoulin, 2010). Mutations in *NRAS*, a member of the Ras signaling cascade, and *NF1*, a negative regulator of Ras signaling, appear in MPN at very low frequencies. Microdeletions of chromosome 17q covering the *NF1* gene are described in MPN patients (Stegelmann, Bullinger et al., 2010). The role of *NF1* in MPN development is confirmed in *NF1* knockout mice which present with a MPN phenotype (Birnbaum, O'Marcaigh et al., 2000). The described mutations affecting the signaling pathways are associated with disease progression (Courtier, Carbuccia et al., 2017, Grand et al., 2009, Oh et al., 2010, Rampal, Ahn et al., 2014).

TP53

TP53 is a gene located on chromosome 17p, encoding the p53 transcription factor, which is one of the best studied proteins. *TP53* is found to be the most frequently mutated gene across different cancer types, with a frequency of 42% in a pan-cancer cohort, and shows association with poor outcome (Kandoth, McLellan et al., 2013, Lawrence, Stojanov et al., 2014). The protein acts as a tumor suppressor, regulating cell faith in response to DNA damage, either by inducing DNA damage repair or apoptosis. Dependent on the type and intensity of DNA damage, p53 directly activates and indirectly represses a large number of target genes, most of which are involved in cell cycle arrest, DNA repair and apoptosis (Hafner, Bulyk et al., 2019). Mdm4 is a E3 ligase ubiquitinase acting as an important negative regulator of p53 in basal cell conditions (Haupt, Maya et al., 1997, Honda, Tanaka et al., 1997). Upon stress, phosphorylation of p53 inhibits its interaction with Mdm4, resulting in accumulation of p53 (Shieh, Ikeda et al., 1997). In return, p53 regulates transcription

of *MDM4*, forming a negative regulation loop (Barak, Juven et al., 1993). *TP53* mutations are seen in chronic phase MNP at low frequencies (1-2%), co-occurring with any of the three driver mutations as well as in triple negative MPN, with a preference of co-occurrence with *JAK2*V617F. ET, but not PV and PMF, is associated with shorter overall survival in the presence of a *TP53* mutation and a strong enrichment of *TP53* mutations is seen in disease progression and leukemic transformation (16-35%) (Harutyunyan, Klampfl et al., 2011, Lasho, Mudireddy et al., 2018, Rampal et al., 2014, Tefferi et al., 2016a, Tefferi et al., 2016b, Venton, Courtier et al., 2018). A co-occurrence of *TP53* mutations with *JAK2*V617F synergize in the induction of clonal dominance and fully penetrant AML, as demonstrated in a mouse model (Rampal et al., 2014). A recent study detected low burden (VAF<12%) *TP53* mutations in 16% of the studied chronic MPN cohort, associated with increased age, with multiple *TP53* mutations occurring in one fourth of the *TP53*-mutated patients (Kubesova, Pavlova et al., 2018). Monoallelic *TP53* mutations evidently occur in chronic MPN more often than previously estimated, at low variant allele frequencies, and may remain stable over a long period of time. An onset of a secondary mutation or chromosomal lesion (17p del or 17p UPD) resulting in a loss of the wild type allele, induces a strong clonal expansion of the *TP53*-mutated clone and transformation to AML (Lundberg et al., 2014). Duplications of the chr1q region covering the *MDM4* gene are also observed in blast phase MPN, occurring in a mutually exclusive fashion with *TP53* mutations, leading to inactivation of the p53 pathway (Harutyunyan et al., 2011).

1.2.3. Germline variants as risk factors for MPN

In addition to the above described somatic mutations in MPN, several germline variants have been identified in MPN patients, some of which have shown to confer predisposition to developing MPN. The best studied is the *JAK2* 46/1 haplotype which occurs in one fourth of the healthy population and is a risk factor for developing MPN, preferentially caused by the JAK2V617F mutation (Olcaydu, Harutyunyan et al., 2009). Similarly, carriers of a germline SNP in the *TERT* gene (encoding a telomerase reverse transcriptase involved in telomerase length maintenance) have increased risks of developing different types of cancer, including MPN and AML, as well as carriers of polymorphisms at the *MECOM, TET2, SH2B3, ATM, CHEK, PINT, GFI1B and MYB* genes (Hinds, Barnholt et al., 2016, Rumi & Cazzola, 2017a, Tapper, Jones et al., 2015). Recently, the presence of a germline variant in the *TERT* gene has also been recognized as a predisposing factor for the development of clonal hematopoiesis (Zink, Stacey et al., 2017). A SNP in the genomic region between the *HBS1L* and *MYB* genes (rs9376092) is predisposing variant associated with ET (Tapper et al., 2015). In familial

MPN germline mutations of the *RBBP6* gene predispose to MPN in close relatives of affected individuals, as well as germline duplications of a 700kb region on chromosome 14q (Harutyunyan, Giambruno et al., 2016, Saliba, Saint-Martin et al., 2015).

1.2.4. Recurrent cytogenetic aberrations

Several recurrent cytogenetic aberrations have been described in MPN. With no significant difference in occurrence among the three subtypes of classical MPN – PV, ET and PMF, at least one chromosomal aberration is detected in over 60% of patients, as detected in a study of over 400 chronic phase samples investigated by high-resolution SNP arrays (Klampfl, Harutyunyan et al., 2011). The most frequently detected lesion is the loss of heterozygosity of chromosome 9p (9pUPD), associated with PV and *JAK2*V617F mutations. An acquired uniparental disomy occurs during mitotic recombination, amplifying a pre-existing heterozygous mutation to homozygosity, with no copy number change of the genomic region (Tuna, Knuutila et al., 2009). In the case of chromosome 9p the UPD gives rise to a homozygous *JAK2*V617F mutation, which as discussed before increases the proliferative potential of affected cells (Anand et al., 2011). Apart from chr9p UPD the most frequent chromosomal aberrations in MPN include deletions of chr13q, chr20q, chr4q, chr11q, chr3p and chr11p, gains of chr9, chr8, chr9p and uniparental disomy of chr1p and chr14q (Klampfl et al., 2011). An increase of the number of chromosomal aberrations is strongly associated with disease progression, significantly linking chr1q and chr9p aberrations with progression to secondary myelofibrosis or accelerated disease phase (<20% blasts), and aberrations of chromosomes 1q, 7q, 5q, 6p, 7p, 19q, 22q and 3q with leukemic transformation (Klampfl et al., 2011, Rumi, Harutyunyan et al., 2011, Thoennissen, Krug et al., 2010). Chromosome 1q UPD is strongly associated with mutations of the *MPL* gene, positioned in the affected region. Aberrations of chromosome 5, 7 and 17 are also identified as risk factors for leukemic transformation and are associated with decreased survival (Rumi et al., 2011). In addition, trisomy of chromosome 8 and gain of chr8q are associated with disease progression of *JAK2*V617F negative patients (Thoennissen et al., 2010). Not only important as risk factors for disease progression, the detection of chromosomal aberrations serves as a method of discovery of affected genes important for MPN pathogenesis. The minimal affected region of detected recurrent chromosomal aberrations in MPN pinpointed to and allowed the discovery of several genes contributing to the MPN phenotype or

disease progression, including *TET2* (chr4q), *IKZF1* (chr7p), *CUX1* (chr7q), *FOXP1* (chr3p), *ETV6* (chr12p), *RUNX1* (chr21q) and *NF1* (chr17q) (Stegelmann et al., 2010). Still, conventional karyotyping has a role in the detection of balanced chromosomal rearrangements, such as translocations and inversions. In the genetic risk assessment of chronic phase MPN the presence of trisomy 8, trisomy 9, loss of chromosome Y and del 20q in PV, and chromosome 3 inversion, chromosome 17q inversion, monosomy 7, del 7q, del 12p and del 11q in PMF are associated with adverse outcome (Tefferi & Vannucchi, 2017).

In summary, mutually exclusive mutations in *JAK2*, *CALR* and *MPL* are phenotypic drivers of MPN, all functioning through constitutive activation of the JAK-STAT signaling pathway, in a type 1 tyrosine kinase receptor dependent fashion. *JAK2* mutations (*JAK2*V617F but not exon 12 mutations) occur in PV, ET and PMF, whereas, *CALR* and *MPL* mutations occur only in ET and PMF. Expressed in cell lines all three genes, when mutated, induce cytokine-independent cell growth, and in mouse models induce an MPN phenotype, confirming their true disease-inducing potential. Mutations affecting DNA methylation, histone modifications, transcription factors, signaling pathways and the splicing machinery, as well as cytogenetic lesions often co-occur with mutations in the driver genes. The order of mutation acquisition as well as the synergetic potential of specific mutational combinations play a significant role in disease shaping. Mutations in some genes are considered early events, and others late events in the clonal evolution of MPN, some of which are established risk factors for leukemic transformation.

1.3. Myelodysplastic syndromes - clinical properties, diagnosis and management

Myelodysplastic syndromes (MDS) are another big group of chronic clonal myeloid neoplasms, characterized by one or more peripheral cytopenias and single or multilineage morphologic dysplasia of myeloid cells (Arber et al., 2016, Swerdlow SH, 2016). The dysplastic lineage does often not correspond to the identified cytopenia in MDS patients (Arber et al., 2016, Germing, Strupp et al., 2012, Verburgh, Achten et al., 2007). In some patients, an increase in the percentage of myeloblasts in the bone marrow and peripheral blood is seen. Ineffective haematopoiesis occurs and patients are at increased risk of developing AML (Swerdlow SH, 2016). The reported annual incidence of MDS globally ranges from 0.07 to 9.6 per 100 000, with the incidence significantly increasing with advanced age (Lubeck, Danese et al., 2016). MDS is most often diagnosed in older individuals, with a median age at diagnosis of 71-76 years (Sperling, Gibson et al., 2017). Cytopenia-related clinical symptoms are in most cases the first signs of MDS, however some patients are asymptomatic and cytopenias are identified after a routine blood test or tests performed for other intentions. The most common cytopenia in MDS is anemia, isolated or coexistent with thrombocythopenia or neutropenia. Anemic patients present with weakness and fatique, patients with thrombocytopenia with bleeding and petechiae, whereas recurrent infections and fever often occur as first symptoms of neutropenia (Swerdlow SH, 2016, Weinberg & Hasserjian, 2019). For precise diagnosis the bone marrow biopsy and aspirate are crucial, providing information about BM cellularity, cellular morphology and the percentage of blasts. The bone marrow is frequently found hyper- or normocellular, with the exception of hypocellular bone marrow of hypoplastic MDS patients (Swerdlow SH, 2016). The necessary requirement for MDS diagnosis is the detection of at least 1 cytopenia, in addition to at least one of the other relevant diagnostic properties – dysplasia in 1 or more lineages, increased blasts and/or the presence of MDS-defining cytogenetic lesions (Arber et al., 2016). The criterion for defining dysplasia is the presence of at least 10% of dysplastic cells in any lineage. Dependent on the number of dysplastic lineages identified in a patient, MDS is defined as single lineage or multilineage. The percentage of myeloblasts in the bone marrow and peripheral blood are evaluated at diagnosis and are necessary for defining MDS categories with or without excess blasts. Conventional karyotyping provides cytogenetical information and identifies chromosomal abnormalities important for diagnosis and assessment of prognosis. Recurrent chromosomal abnormalities detected in MDS, which are MDS-defining in cytopenic individuals, are del(5q), monosomy 7, del(7q), del(11q), del(12p), isochormosome 17 and complex karyotype (≥3 lesions) (Swerdlow SH, 2016). Trisomy

8, del(20q) and loss of chromosome Y are also recurrently seed in MDS, but are not MDS-defining diagnostic findings, due to their recurrence in other diseases. Morphologic categories of MDS are defined by the WHO classification of myeloid neoplasms, putting focus on the type of dysplasia and percentage of blasts and giving the identified cytopenia(s) a minor role for classification. Del(5q) is so far the only cytogenetic abnormality defining a separate subcategory of MDS (Table 1) (Arber et al., 2016).

The resemblance of clinical features of MDS and other causes of cytopenia or dysplasia challenges the diagnosis of MDS, especially in low grade MDS without excess blasts. Several pre-MDS conditions have been described which may but do not necessarily develop into MDS (Valent, Orazi et al., 2017). Idiopathic cytopenia of undetermined significance (ICUS) is characterized by single or multilineage cytopenia, without dysplasia and without MDS minimal diagnostic criteria. Interestingly, over 30% of ICUS patients have MDS-associated somatic mutations (Kwok, Hall et al., 2015). If clonal mutations with allele frequency ≥2% occur in individuals with ICUS, without other MDS-related criteria or signs of other clonal bone marrow neoplasia, the diagnosis of clonal cytopenia of unknown significance (CCUS) is made. In other individuals dysplasia is detected, without the presence of cytopenia, and if no cytogenetic or molecular marker of clonality is present this condition is named idiopathic dysplasia of undetermined significance (IDUS) (Valent et al., 2017). Recently, a condition named clonal haematopoiesis of undetermined potential (CHIP) has been described, characterized by the presence of somatic mutations in genes which are recurrently found mutated in myeloid malignancies, in peripheral blood of individuals with normal blood counts who lack evidence of any haematological malignancy or other clonal conditions (Genovese et al., 2014, Jaiswal et al., 2014, Xie et al., 2014). However, CHIP differs from MDS, lacking other MDS-defining features and should be diagnosed as a different disease entity, implicating the insufficiency of mutational testing and the importance of morphologic and cellular assessment of the bone marrow for MDS diagnosis. ICUS, IDUS, CCUS and CHIP are considered as potential pre-MDS conditions and can evolve over time into MDS. However, they can durably remain stable or progress into other myeloid or lymphoid neoplasms or even into other non-hematologic pathologies. Therefore, regular follow-up in such patients is recommended (Valent et al., 2017).

Upon diagnosis, MDS patients are stratified into risk categories, with the Revised International Prognostic Scoring System (IPSS-R) being the most widely used scoring system available (Greenberg, Tuechler et al., 2012). It incorporates information about blast percentage, the cytogenetic risk group and the level of cytopenia(s) into the scoring calculation and groups patients into 5 prognostic risk categories (very low,

low, intermediate, high and very high risk). The clinical course of the disease and outcome is highly variable depending on the risk category, with a median overall survival ranging from 9 months to around 9 years, (from very high risk IPSS-R to very low risk IPSS-R categories) (Greenberg et al., 2012).

Approximately one third of MDS patients transform to AML (Steensma, 2015). The prognosis for progression to AML also differs between risk categories, with increasing duration to leukemic transformation from very high risk to very low risk patients. As age is a risk factor for survival the calculated risk score requires age-adjustment. Other identified risk factors for survival in MDS are serum lactate dehydrogenase (LDH), ferritin and $ß_2$-microglobulin levels, as well as performance status and comorbidities (Gatto, Ball et al., 2003, Greenberg et al., 2012, Naqvi, Garcia-Manero et al., 2011, Wimazal, Sperr et al., 2001, Wimazal, Sperr et al., 2008).

Recommendations for management of MDS patients is based on personalized evaluation of patient's prognostic risk group and age, with a growing impact of genetics in therapy assessment. The aim of therapy for lower risk patients is transfusion independency and first line therapy frequently includes growth factor support, and is switched to lenalidomide or hypomethylating agents (5-azacitidine or decitabine) when no response to growth factors is observed (Montalban-Bravo & Garcia-Manero, 2018). Lenalidomide is shown to be particularly efficient in patients with 5q- MDS, leading to complete cytogenetic response in more than 50% of patients (Fenaux, Giagounidis et al., 2011, List, Dewald et al., 2006). However, *TP53* mutations in del5q patients predict resistance to lenalidomide, as domination of *TP53*-mutant subclones is detected after lenalidomide treatment (Jadersten, Saft et al., 2009, Mossner, Jann et al., 2016). Immunosuppressive therapy is recommended for some MDS patients, with hypocellular bone marrow and the presence of trisomy 8 reported to be predictors of positive response (Garg, Faderl et al., 2009, Sloand, Mainwaring et al., 2005, Sloand, Melenhorst et al., 2011, Tobiasson & Kittang, 2019). Other supportive care measures include prophylactic antibiotics for infection control and iron chelation. In lower risk patients not responding to first and second line therapies allogenic stem cell transplantation or clinical trials should be considered. In higher risk MDS the aim of therapy is survival prolongation, with allogenic haematopoietic stem cell transplantation (alloHSCT) being the only potential curative treatment to date. Standard first line therapy, either prior to alloHSCT or in patients who are not candidates for alloHSCT, involves the use of 5-azacitidine or decitabine (Montalban-Bravo & Garcia-Manero, 2018). Positive response to hypomethylating agents is associated with *TET2* mutations, without co-ocurring *ASXL1* mutations (Bejar, Lord et al., 2014, Itzykson, Kosmider et al., 2011). Chemotherapy used for treating *de novo* AML (anthracycline-araC) is occasionally used in higher risk MDS, however only in

younger patients with favorable karyotype, who are candidates for allogenic stem cell transplantation (Montalban-Bravo & Garcia-Manero, 2018). Mutations of *TP53* have been reported as strong molecular biomarkers for post-transplantation poor survival (Bejar, Stevenson et al., 2014).

1.4. Genetics of myelodysplastic syndromes

Unlike MPN where the majority of patients have one of the three driver genes mutated (*JAK2, CALR, MPL*), all affecting the same signaling pathway, in MDS a greater genetic heterogeneity of causative mutations exists. No single gene is mutated in the majority of cases and the identified mutations show no specificity for MDS. In addition, only few genes are found mutated at moderately high frequencies (up to 25%), whereas the others occur at low frequencies (≤5%). More than 30 driver mutations have been discovered in MDS, affecting different cellular processes, predominantly splicing factors and epigenetic regulators, but also the cohesion complex, transcription factors and signaling pathways (Figure 4) (Haferlach, Nagata et al., 2014, Papaemmanuil, Gerstung et al., 2013). A significant overlap of affected genes can be noticed in MDS and dnAML, however the frequencies at which they appear differ, for example with overrepresentation of *FLT3, NPM1, CEBPA, NRAS* and *PTPN11* in AML, and *SF3B1, ZRSR2, CUX1, ASXL1* and *U2AF1* in MDS (Ogawa, 2019). These findings implicate that the same mutations may be acquired early in one disease entity and late in the other, thereby shaping the outcome phenotype. The most frequently mutated genes in MDS, such as *SF3B1*, *TET2*, *ASXL1*, *SRSF2* and *DNMT3A*, are also the most commonly identified in clonal hematopoiesis of indeterminate potential (CHIP), therefore MDS may evolve from a preexisting CHIP phase in some cases (Genovese et al., 2014, Jaiswal et al., 2014, Xie et al., 2014). MDS patients harbor a median of 2-3 driver mutations. Increased numbers of mutations are often seen in high risk MDS and are associated with reduced leukemia-free and overall survival (Haferlach et al., 2014, Papaemmanuil et al., 2013). An increased risks of leukemic transformation is associated with mutations in *FLT3, NPM1, NRAS, IDH1, IDH2, WT1* and *PTPN11* (Makishima, Yoshizato et al., 2017). The most important cellular processes and affected genes in MDS will be described in the following paragraphs.

Mutations affecting spliceosome proteins

Mutations affecting the RNA splicing machinery are the most common group of mutations in MDS, affecting around 60% of cases, in a mutually exclusive manner (Inoue, Bradley et al., 2016, Yoshida, Sanada et al., 2011). *SF3B1* mutations occur in 23% of MDS cases and strongly correlate with the presence of ring sideroblasts (Bejar, Papaemmanuil et al., 2015, Papaemmanuil, Cazzola et al., 2011). Due to this association of a mutations with a clinical phenotype the WHO includes the mutation status into the diagnostic criteria for MDS with ring sideroblasts (MDS-RS) and reduces the obligatory blast count for diagnosis from 15% to 5% in the presence of a *SF3B1* mutation (Arber et al., 2016, Malcovati, Karimi et al., 2015). Carriers of a *SF3B1* mutation have a favorable prognosis in most cases, however, when present in combination with a complex karyotype it associates with adverse prognosis (Haase, Stevenson et al., 2019, Malcovati et al., 2015, Papaemmanuil et al., 2011). *SRSF2* mutations occur less frequently, in 14% of the patients, resulting in mis-splicing of several genes, including *EZH2, CASP8* and *CDK10* (Kim, Ilagan et al., 2015, Shiozawa, Malcovati et al., 2018). *SF3B1* and *SRSF2* genes are exclusively found as heterozygous and mutually exclusive mutations as homozygosity of single mutations or their co-ocurrence results in lethality and synthetic lethality, respectively. Both mutations are shown to also aberrantly affect haematopoiesis through the activation of MAPK and NF-κB signaling (Lee, North et al., 2018b). *U2AF1* mutations affect around 10% of MDS patients, and have also shown to target *EZH2* mRNA splicing (Shiozawa et al., 2018). Contrary to the favorable prognosis associated with *SF3B1* mutations, poor prognosis correlates with *SRSF2* and *U2AF1* mutations (Graubert, Shen et al., 2011, Thol, Kade et al., 2012). Other mutations affecting the splicing machinery in MDS include mutations of *ZRSR2* and *PRPF8*, observed in 6% and 3% of patients, respectively (Bejar et al., 2015).

Mutations of epigenetic regulators

Mutations of genes involved in DNA methylation (*DNMT3A, TET2, IDH1* and *IDH2*) and histone modifications (*EZH2, ASXL1* and *BCOR*) affect at least 60% of MDS patients and tend to occur early in MDS evolution, in the initiation or early progression phase (Bejar et al., 2015, Lindsley, 2017). With their roles in DNA methylation already described in Chapter 1.2.2., *TET2, DNMT3A, IDH2* and *IDH1* mutations are harbored by 26%, 13%, 3% and 2% of MDS patients, respectively, resulting in hypermethylation of genes involved in myeloid differentiation (Bejar et al., 2015, Delhommeau et al., 2009, Gelsi-Boyer, Trouplin et al., 2009, Ley, Ding et al., 2010, Rasmussen, Jia et al.,

2015, Walter, Ding et al., 2011). *DNMT3A* and *TET2* mutations frequently co-occur, synergistically contributing to disease progression (Haferlach et al., 2014, Zhang, Su et al., 2016). PRC2- and PRC1-associated genes *ASXL1, EZH2* and *BCOR* are mutated in 20%, 6% and 4% of MDS, respectively, and are associated with poor prognosis (Bejar et al., 2015, Damm, Chesnais et al., 2013).

Mutations of cohesin complex members

Cohesin is a multi-protein complex important for sister chromatide alignment allowing proper DNA repair during cell division, as well as for gene expression regulation by DNA loop stabilization and interactions with polycomb proteins. Members of the complex are Smc3, Smc1A, Rad21, Stag1 and Stag2 (Kagey, Newman et al., 2010, Michaelis, Ciosk et al., 1997, Schaaf, Kwak et al., 2013, Schaaf, Misulovin et al., 2013). Mutually exclusive mutations in cohesin complex member genes occur in 10% of low-risk MDS and in more than 15% of high-risk MDS, associate with disease progression and reduced overall survival (Thota, Viny et al., 2014). The *STAG2* gene has the highest frequency of mutations in MDS. An association of cohesion mutations with *RUNX1, ASXL1* and *BCOR* mutations is observed. Interestingly, no association of cohesion mutations and increased cytogenetic aberrations exists, implicating that the role cohesion mutations have in myelodysplasia is linked to gene expression regulation rather than to its chromatide-stabilization function in replication (Thota et al., 2014).

Mutations affecting signaling pathways

Mutations in members of the Ras signaling pathway are found in a total of MDS, with *NRAS, NF1, CBL* and *PTPN11* being the most frequent of them, but still at low rates of 6%, 5%, 4% and 1%, respectively (Haferlach et al., 2014). Mutations affecting other signaling pathways, such as *JAK2*, *FLT3* and *KIT*, are seen in MDS at much lower frequencies than in other myeloid neoplasms like AML and MPN. Signaling pathway mutations occur late in MDS evolution, often in disease progression to AML.

TP53

TP53 mutations, prevalently missense mutations, occur in 7.5%-18% of MDS cases (Bejar, Stevenson et al., 2011, Haferlach et al., 2014, Hou, Tsai et al., 2018, Walter, Shen et al., 2013). The presence of *TP53* mutations associates with complex karyotype, reduced number of additional mutations in driver genes, increased bone marrow blasts, reduced platelet counts and reduced overall survival (Bejar et al., 2011). A complex karyotype combined with *TP53* mutations associates with increased occurrence of chromosome 5q deletions and loss of heterozygosity of chromosome 17q (Yoshizato, Nannya et al., 2017). Even in the subgroup of MDS patients with a complex karyotype *TP53* mutations stratify patients in higher and lower risk groups with influenced median overall survival (0.6 vs. 1.5 years, respectively) (Haase et al., 2019). The p53 pathway can also be deregulated by *PPM1D* mutations, which encodes a phosphatase negatively regulating p53-dependant cellular responses. *PPM1D* mutations are not often seen in primary MDS (3%) but are enriched in therapy-related MDS (t-MDS), as well as *TP53* mutations, occurring in 20% and 37% of t-MDS cases, respectively (Hsu, Dayaram et al., 2018, Lindsley, 2017, Ok, Patel et al., 2015). In therapy related MDS and AML pre-existing *TP53* mutations are shown to provide clonal advantage to hematopoietic stem cells upon therapy (Wong, Ramsingh et al., 2015). Both *TP53* and *PPM1D* mutations are among the most frequently mutated genes identified in ageing-associated clonal hematopoiesis, providing the possibility for the prior existence of either of these mutations, which would outgrow upon cytotoxic therapy for treatment of other diseases (Busque, Mio et al., 1996, Champion, Gilbert et al., 1997).

Recurrent cytogenetic aberrations

At the time of diagnosis more than half of MDS patients present with large chromosomal aberrations detectable by conventional karyotyping, whereas in others only small copy number alterations or uniparental disomies are detected. In total, more than 80% of MDS patients carry chromosomal aberrations and the increase of karyotype complexity, which occurs during the course of the disease evolution, associates with poor prognosis (Gondek, Haddad et al., 2007, Haase, Germing et al., 2007, Wang, Wang et al., 2010, Zhang, Kim et al., 2015). Clonal cytogenetic evolution occurs in 27% of MDS in a 10-year follow up and strongly associates with reduced overall survival, increased risk of leukemic transformation and shortened time interval to leukemic onset (Neukirchen, Lauseker et al., 2017, Schanz, Sole et al., 2018). The most common recurrent aberrations include monosomy 7, del chr7q, del chr5q, trisomy

8, LOH chr17p, del chr20q, gain of chr 21q, del chr12p, del chr 11q and loss of chr Y, with 5% of MDS cases harboring a complex karyotype at diagnosis associated with very poor prognosis (Ogawa, 2019, Swerdlow SH, 2016). According to the IPSS-R scoring system used for MDS risk stratification a complex karyotype (> 3 aberrations), monosomy 7 and lesions of chromosome 3 are associated with high risk, whereas loss of chromosome Y and deletions of chr11q, chr5q, chr12p and chr20q predict favorable prognosis (Greenberg et al., 2012). Deletions of chromosome 5q occur in 10%-15% of MDS and are the single chromosomal aberration associated with a MDS subtype included in the WHO classification, also referred to as the 5q$^-$ syndrome (Arber et al., 2016, Boultwood, Fidler et al., 2002, Jerez, Gondek et al., 2012, Sole, Espinet et al., 2000). Haploinsufficiency of several genes located on chromosome 5q plays a role in the pathophysiology of the 5q$^-$ syndrome, characterized by macrocytosis, anemia and thrombocytosis, including *RPS14* (ribosomal protein S14), *CSNK1A1*(casein kinase 1 α1), miR-145 (micro RNA 145), miR-146a (micro RNA 146a), *DDX41* (DEAD-box helicase 41) and *G3BP1* (G3BP stress granule assembly factor 1) (Hosono, Makishima et al., 2017, Ribezzo, Snoeren et al., 2019). Patients with isolated deletions of chromosome 5q are treated with lenalidomide which induces ubiquitin-mediated degradation of CK1α (encoded by the *CSNK1A1* gene) and results in activation of the p53 pathway and apoptosis (Sperling et al., 2017). The presence of a *TP53* mutation in 5q$^-$ syndrome patients abolishes the effect of lenalidomide, as confirmed in *in vitro* studies of *TP53* knockdown cells, and is associated with poor prognosis (Jadersten et al., 2009, Jadersten, Saft et al., 2011, Kronke, Fink et al., 2015, Schneider, Adema et al., 2014). This is a relevant point for patient management as almost 20% of patients with 5q$^-$ syndrome harbor *TP53* mutations. Other mutations frequently co-ocurring with chr 5q deletions are those in *FLT3*, *NPM1* and *TET2* (Hosono et al., 2017). As in the case of chromosome 5q aberrations, deletions of part of chromosome 7 (7q) or the whole chromosome lead to haploinsufficiency of a number of genes, some of which are already known to have a role in hematological malignancies, including *CUX1*, *EZH2*, *KMT2C (MLL3)* and *SAMD9L* (Chen, Liu et al., 2014, McNerney, Brown et al., 2013, Nagamachi, Matsui et al., 2013, Nikoloski, Langemeijer et al., 2010).

In summary, MDS is a chronic myeloid neoplasms, with a risk of transformation to AML. Clinically and genetically it is a very heterogeneous group of diseases, characterized by morphologic dysplasia of myeloid cells, cytopenia(s) in peripheral blood and in some cases elevated blast counts. Several other conditions defined by cytopenias or dysplasia resemble the clinical properties of MDS but should not be misdiagnosed for MDS. Deletions of chromosome 5q and mutations in the *SF3B1* gene correlate with clinical phenotypes and are incorporated in the MDS classification

defining the MDS with isolated del(5q) and MDS with ring sideroblasts subclasses, respectively, both of which are associated with favorable prognosis without the occurrence of other cytogenetic events which affect it (ex. *TP53* mutations, complex karyotype). Other MDS patients harbor somatic mutations and/or cytogenetic lesions affecting different genes and pathways, with the most frequently affected being the spliceosome and cohesin complex members, epigenetic regulators and the p53 pathway.

1.5. Acute myeloid leukemia – clinical properties, classification and genetics

Acute myeloid leukemia (AML) is the most frequent leukemia in adults, with an incidence of 3.2 per 100000 per year for the general population. The incidence rises with age, from 0.2 to 20.1 per 100 000 per year in the US, for age groups <65 years and ≥65 years, respectively (Siegel, Miller et al., 2015). In the context of clinical ontogeny, AML can be classified as: 1. secondary AML (sAML) - subsequent to MPN or MDS, 2. therapy-related AML (tAML) - subsequent to therapy or 3. *de novo* AML (dnAML) - arising with no prior chronic phase or therapy and carrying better prognosis than sAML and tAML. The median age at diagnosis is 73 and 70 years for sAML and dnAML, respectively (Hulegardh, Nilsson et al., 2015). The median overall survival for dnAML patients decreases with age, from 13.1 years in patients younger than 55 years to 0.58 years in those older than 75. In sAML poor overall survival is observed in all age groups, with a median of 0.58 and 0.5 years in patients younger than 55 and older than 75 years, respectively (Hulegardh et al., 2015).

AML is a clonal disease blocking hematopoietic differentiation and is characterized by the accumulation of immature myeloid precursor cells (myeloblasts) in the bone marrow and peripheral blood, with a minimal presence of 20% myeloblasts required for diagnosis (Arber et al., 2016). The increase of blasts in the bone marrow leads to cytopenias and bone marrow failure. Patients present with unspecific symptoms related to cytopenias, such as weakness, fatigue, infections, fever and bleeding. Management of AML highly depends on the subtype and risk category, often including induction therapy (mainly a combination of cytarabine and anthracycline) followed by consolidation therapy (chemotherapy or allogeneic hematopoietic stem cell transplantation) in responders, with the aim of eradicating potential residual disease (De Kouchkovsky & Abdul-Hay, 2016). Despite the success of therapy and remission seen in patients, relapse often occurs with poor prognosis. The WHO classification of myeloid neoplasms incorporates genetic, morphologic and clinical factors in the categorization (Arber et al., 2016). Gene fusions are recurrently seen in AML, including *PML-RARA*, *MYH11-CBFB*, *RUNX1-RUNX1T1*, *BCR-ABL1*, *NUP98-*

NSD1 and several fusions involving the *MLL* gene (Cancer Genome Atlas ResearchLey et al., 2013). Favorable prognosis is associated with t(8,21) (*RUNX1-RUNX1T1*), inv(16) (*MYH11-CBFB*), t(15,17) (*PML-RARA*), *NPM1* mutations and biallelic *CEBPA* mutations, whereas inv(3) and t(6,9), *FLT3* internal tandem duplications (*FLT3*-ITD), *DNMT3A* mutations, chr5 and chr7 aberrations, as well as a complex karyotype confer adverse prognosis (De Kouchkovsky & Abdul-Hay, 2016). Over 40% of adult AML have no detectable large cytogenetic aberrations (Mrozek, Heerema et al., 2004). Recent studies have revealed that more than 96% of AML patients carry at least 1 somatic mutation, despite AML being very poor in the observed frequency of mutations compared to other cancer types (Cancer Genome Atlas Research et al., 2013, Kandoth et al., 2013, Lindsley, Mar et al., 2015, Papaemmanuil, Gerstung et al., 2016). Recurrently mutated genes in AML are grouped into functional categories and provide information about the complexity of causes and biological processes responsible for the development of *de novo* and secondary AML (Cancer Genome Atlas Research et al., 2013, Lindsley et al., 2015, Papaemmanuil et al., 2016). *NPM1* and *FLT3* mutations as well as *MLL*/11q23 and *CBF* rearrangements are overrepresented in dnAML compared to sAML (Lindsley et al., 2015, Milosevic, Puda et al., 2012). On the other hand, mutations affecting DNA methylation (*TET2, DNMT3A*), chromatin modifiers (*ASXL1, EZH2, BCOR*), RNA splicing (*SRSF2, SF3B1, U2AF1, ZRSR2*) and the cohesin complex (*STAG2*) are highly specific for sAML, tend to occur early in disease evolution and also correlate with poor outcome when detected in clinically diagnosed dnAML or tAML. In sAML mutations in members of the signaling pathways (*FLT3* and *RAS*) and transcription factors (*RUNX1*, *GATA2* and *CEBPA*) are acquired later in clonal evolution, during leukemic transformation. Copy number alterations and uniparental disomies are also enriched in sAML compared to dnAML, with chr9p UPD and chr7q del (targeting *CUX1*) significantly associated with sAML (Milosevic et al., 2012). Recurrent mutations in AML affecting DNA methylation (*IDH1, IDH2*), the transcription factor *RUNX1* and signaling pathways (*NRAS, KRAS, KIT, NF1, PTPN11, WT1*) are uniquely represented in dnAML and sAML. *TP53* mutations associate with complex karyotypes and reduced overall survival and seem to describe a distinct class of AML, with a specific clinical phenotype (Lindsley et al., 2015). Not only the type of mutation, but also their combinations contribute to disease shaping. For example, *DNMT3A* and *NPM1* mutations co-operate to induce AML, as shown in a mouse model. First *DNMT3A* induces clonal hematopoiesis, whereas *NPM1* as a secondary event is responsible for the leukemic transformation (Loberg, Bell et al., 2019). This is consistent with secondary *NPM1* mutations co-occurring with *DNMT3A* (as well as with *TET2* and *IDH1/2* mutations), seen in AML patients (Papaemmanuil et al., 2016). Based on gene expression data and DNA methylation patterns, another

separate AML subclass can be defined by triple mutations in *NPM1, DNMT3A* and *FLT3* (Cancer Genome Atlas Research et al., 2013). This data shows that *de novo* and secondary AML have different mechanisms of disease initiation and development, and should be treated as distinct diseases. However, some biological processes involved in the pathogenesis of the two are overlapping and understanding of this overlap may develop a deeper understanding of disease evolutions and dynamics.

Taken together, dnAML and sAML describe two different pathologies, with different molecular pathways responsible for their development. *NPM1* and *FLT3* mutations, together with rearrangements involving *MLL* or *CBP* are specific for dnAML, whereas mutations in components of the spliceosome, cohesin complex and epigenetic modifiers, as well as cytogenetic lesions targeting *JAK2* and *CUX1* are specific for sAML. Mutations in *IDH1, IDH2, RUNX1, NRAS, KRAS, NF1, WT1, KIT* and *PTPN11* are shared between dnAML and sAML. A distinct group of AML with poor prognosis appears to be defined by *TP53* mutations.

1.6. Clonal hematopoiesis

Myeloid neoplasms are characterized by clonal hematopoiesis caused by somatic mutations in the hematopoietic stem cells and clonality is often associated with cancer. However, recent findings have described clonality as part of normal ageing of haematopoietic stem cells. First findings of clonal hematopoiesis (CH) in healthy individuals were reported in X-chromosome inactivation studies of healthy women, in which clonality was age-associated and observed in over one third of women older than 60 years, later associated with somatic *TET2* mutations in a subset of cases (Busque et al., 1996, Busque, Patel et al., 2012, Champion et al., 1997). Further research of blood samples from apparently healthy or non-hematologic cancer patients has identified age-related clonal chromosomal aberrations (also known as clonal mosaicism) present in 2-3% of elderly individuals, many of which are associated with hematologic neoplasms, including chr9p UPD, chr8 trisomy, chr13q del and chr20q del. Some identified aberrations overlap with the chromosomal localization of cancer-related genes (ex. *TET2* on chromosome 4q, *DNTM3A* on chromosome 2p and *JAK2* on chromosome 9p). A positive correlation between the presence of clonal mosaicism and the development of hematological neoplasms over time has been observed, implicating that the detected clonal chromosomal anomaly might be an early event in the development of cancer (JacobsYeager et al., 2012, Laurie, Laurie et al., 2012). An interesting association in clonal mosaicism in healthy individuals has been

observed between the presence of inherited SNPs at the *FRA10B*, *MPL* and *TM2D3/TARSL2* loci (on chromosomes 10q, 1p and 15q, respectively) and subsequent acquisition of deletions, somatic mutations and copy neutral loss of heterozygosity (Loh, Genovese et al., 2018). In recent years, the rapid expansion of next-generation sequencing in research and clinical settings has revealed hematologic malignancy-associated somatic clonal mutations in blood cells of individuals with no diagnostic indications of a hematologic malignancy, as well as in apparently healthy individuals of advanced age. This condition was named Clonal Hematopoiesis of Indeterminate Potential (CHIP). With a lack of criteria necessary for the diagnosis of another hematologic or non-hematologic disease and in the presence of a normal blood count with no persistent cytopenia (≥ 4 months) CHIP can be diagnosed in an individual if the mutation allele frequency is ≥ 2% (Steensma, Bejar et al., 2015, Valent et al., 2017). The frequency of detected clonal mutations increases with age, with an occurrence of around 1% in individuals younger than 50 years, increasing to around 10% in those older than 70 years (Genovese et al., 2014, Jaiswal et al., 2014, Xie et al., 2014). In addition, the average number of somatic mutations is higher in older than in younger individuals with clonal haematopoiesis (Genovese et al., 2014). Clinically, an increase in red-cell distribution width is the single identifiable feature related to CHIP, detectable in a peripheral blood cell examination, but not present in all cases (Jaiswal et al., 2014). Clonal hematopoiesis is associated with an increased risk of subsequent diagnosis of hematologic malignancies and decreased survival, a close to doubled risk of coronary heart disease, as well as an increased risk of ischemic stroke, when compared to the age-matched population (Genovese et al., 2014, Jaiswal et al., 2014, Jaiswal, Natarajan et al., 2017, Xie et al., 2014). Higher variant allele frequencies (≥10%) correlate with higher risks of hematologic cancer and coronary heart disease, mediated by coronary-artery calcification and atherosclerosis (Jaiswal et al., 2014, Jaiswal et al., 2017). The association of clonal hematopoiesis and increased risk of atherosclerotic cardiovascular disease was confirmed in a genetic mouse model study where clonal expansion of *TET2* mutant cells led to NLRP3 inflammasome activation and increased IL-1 secretion and resulted in enlarged atherosclerotic plaques, a hallmark of atherosclerosis (Fuster, MacLauchlan et al., 2017). In humans, *TET2* mutations in blood cells resulted in elevated IL-8 levels in the plasma, linking the functional effect of these mutations to increased inflammatory responses and consequentially higher coronary heart disease risks (Jaiswal et al., 2017). The clonal evolution of clonal hematopoiesis was studied in single cases in which CHIP was identified prior to myeloid neoplasm diagnosis. Sequential DNA sequencing and variant allele frequency comparison demonstrated that the pre-malignant clone (carrying the mutations identified in CHIP) acquired additional mutations, resulting in

the subclone responsible for the clonal expansion and disease onset (Genovese et al., 2014). Despite CHIP being an obvious pre-malignant state in some cases, in others it remains benign and never progresses. The overall risk for evolution to hematologic cancer is low (0.5 - 1% per year), therefore no special therapy is recommended at the moment for the management of CHIP. The most frequently mutated genes in CHIP are epigenetic modifiers *DNMT3A*, *TET2* and *ASXL1* (Figure 4), with the majority of CHIP-carriers having a single somatic driver mutation (Genovese et al., 2014, Jaiswal et al., 2014, Xie et al., 2014). They tend to be early events initiating clonal expansion, consistent with previous findings of early occurring *DNMT3A* and *TET2* mutations in AML evolution (Jan, Snyder et al., 2012, Shlush, Zandi et al., 2014). Other recurrently mutated CHIP-associated genes include *TP53*, *PPM1D*, *SF3B1* and *JAK2*. Mutations of *ASXL1*, *DNMT3A*, *TET2* and *JAK2* are identified as independent risk factors for coronary heart disease (Jaiswal et al., 2017). The stability of clonal hematopoiesis and its low rate of transformation indicate that additional cooperating mutations or other events are necessary for the progression to hematologic malignancies. A sub-classification of clonal hematopoiesis is made in order to differentiate mutations which significantly increase risks for the development of hematologic malignancies and those which may remain stable in healthy individuals, with clonal hematopoiesis of indeterminate potential (CHIP) describing a more benign condition compared to clonal hematopoiesis of oncogenic potential (CHOP) with higher risks of developing a hematologic malignancy, in which mutations induce differentiation and/or proliferation of affected cells. Typical CHOP mutations are those in *JAK2, CALR, MPL, KIT, FLT3, NPM1, KRAS, NRAS* and *TP53* genes as well as fusion genes *BCR-ABL, FIP1L1-PDGFRA, RUNX1-RUNX1T1* and *CBFβ-MYH11* (Valent, Kern et al., 2019). Mutations associated with clonal hematopoiesis most likely occur early in the hematopoietic hierarchy, either in hematopoietic stem cells or in early progenitors, as they can further lead to the development of either myeloid or lymphoid neoplasms. Clonal haematopoiesis can also emerge as a result of neutral drift in the stem cell pool, when some cells die over time and others, carrying random mutations in non-driver genes, expand without proliferative advantage (Klein & Simons, 2011). More recently, targeted error-corrected sequencing enabled the detection of mutations at very low allele frequencies (median variant allele frequency of 0.2%) in healthy women between the age of 50 and 65, registering clonality in the haematopoietic system in 95% of the participants. More than half of the identified mutations were in *DNMT3A* and *TET2*, present in both myeloid and lymphoid haematological lineages, consistent with the previous hypothesis that mutations inducing clonal hematopoiesis occur in the haematopoietic stem cell pool (Young, Challen et al., 2016). The presence of *DNMT3A* and *TET2* mutations as part of clonal hematopoiesis was also observed in the blood

of non-hematologic cancer patients, as well as *TP53* and *PPM1D* mutations which were strongly associated with prior radiation or chemotherapy (Coombs, Zehir et al., 2017). The development of myeloid malignancies from a preexisting clonal hematopoiesis stage, despite the HSC origin of the initiating mutations, might be explained by myeloid skewing, which is a property of ageing hematopoietic stem cells, and is often linked with clonal dominance (Park & Bejar, 2018).

In summary, hematopoietic clonality is not necessarily linked to pathologies and can be a property of normal ageing, occurring in long-lived haematopoietic stem cells and early progenitor cells, at much higher frequencies in the population than estimated with the initial discovery of CHIP. Depending on the type and number of mutations, it carries low or higher risks of transformation to a myeloid neoplasm. Ageing of HSCs is not only driven by intrinsic factors (DNA damage, telomere attrition and epigenetic changes) but is a result of its combination with extrinsic factors (cytotoxic stress, autoimmunity, immunosenescence), which together contribute to the clonal selection (Park & Bejar, 2018). Further research is needed to better understand clonal hematopoiesis in ageing and its role in the development of hematologic cancers.

2. AIM

The aim of this thesis was to contribute to a better understanding of the genetics of leukemic transformation of MPN and MDS and to identify novel tumor suppressors involved in disease progression.

Specific aims:

- To investigate the profile of chromosomal aberrations in a cohort of 77 post-MPN AML and post-MDS AML patients, using genome-wide human high resolution SNP arrays.

- To compare the frequencies of detected chromosomal aberrations in sAML to those in chronic phase MPN and MDS.

- To apply deletion mapping for identifying of novel putative tumor suppressors involved in leukemic transformation.

- To assess the deletion profile and compare frequencies of deletions of novel tumor suppressor genes in other myeloid neoplasms, by studying a pan-myeloid cohort of 905 samples.

- To examine the frequency of aberrations of chromosome 6p in different disease entities among the pan-myeloid cohort of patient samples.

- To explore the mutational profile of genes located on chromosome 6p, in search for a common target of chromosome 6p aberrations, applying exome sequencing to four selected patients with chromosome 6p aberrations.

3. RESULTS

3.1. Manuscript: Frequent deletions of *JARID2* in leukemic transformation of chronic myeloid malignancies

Puda et al.
Published in the American Journal of Hematology, March 2012; 87(3): 245-250
(Puda, Milosevic et al., 2012)

In order to deepen our understanding of leukemic transformation and identify tumor suppressors involved in the process we applied deletion mapping in a cohort of 77 post-MPN and post-MDS AML patients, analyzed for chromosomal aberrations with the use of high-resolution SNP arrays, and identified recurrent aberrations of the short arm of chromosome 6 where the commonly deleted region mapped to a single gene – *JARID2*. A deletion followed by a uniparental disomy (UPD) led to a homozygous deletion of this gene in a PV patient in the accelerated phase of the disease. Other detected deletions covering this gene were hemizygous. We compared the frequency of deletions in the cohort of leukemic phase patients with the frequency of deletions detected in a cohort of 440 chronic phase MPN and MDS patients, and found a significant enrichment of chromosome 6p deletions in post-MPN and post-MDS AML patients (P=0.0033). The whole coding region of *JARID2* was sequenced in patients with deletions of chromosome 6p and no mutations were detected. Similarly, we detected a homozygous deletion of the *AEBP2* gene on chromosome 12, as a result of two independent deletion events. Both Jarid2 and Aebp2 are cofactors of the PRC2 complex, which represses gene transcription by trimethylating lysine 27 of histone 3, through the activity of its core members – Suz12, Ezh2 and Eed. No focal deletions of other PRC2 complex members were detected in the studied cohort. However, we found that larger deletions covering the regions of *SUZ12* and *EZH2* were more frequently deleted in the leukemic compared to the chronic disease phase (P=0.0011 for both regions). Furthermore, we found *AEBP2* often to be co-deleted with *ETV6*, and *SUZ12* with *NF1*. Five variants in PRC2 members (*SUZ12*, *EED*, *EZH2*, *EZH1* and *PHF19*) were identified in exome sequencing data from 40 patients, all in patients in the leukemic disease phase. The T598P mutation in *SUZ12* was amplified to homozygosity by a UPD and was confirmed to be of somatic origin, whereas control tissue DNA was not available for the remaining four patients.

Together with Jelena Milosevic Feenstra and Tiina Berg I performed the processing of microarrays in this study and analyzed the data together with Jelena

Milosevic Feenstra. I performed sequencing of single genes, and validations of variants from exome sequencing data, except for the *SUZ12* mutation which was identified and validated by Ashot S. Harutyunyan. The microarrays of chronic phase samples were processed and analyzed by Thorsten Klampfl, Ashot S. Harutyunyan and Tiina Berg. I performed the statistical analysis for the manuscript and made all the figures and tables. I wrote the manuscript together with Jelena Milosevic Feenstra and my supervisor Robert Kralovics.

Frequent deletions of *JARID2* in leukemic transformation of chronic myeloid malignancies

Ana Puda,[1] Jelena D. Milosevic,[1] Tiina Berg,[1] Thorsten Klampfl,[1] Ashot S. Harutyunyan,[1] Bettina Gisslinger,[2] Elisa Rumi,[3] Daniela Pietra,[3] Luca Malcovati,[3] Chiara Elena,[3] Michael Doubek,[4] Michael Steurer,[5] Natasa Tosic,[6] Sonja Pavlovic,[6] Paola Guglielmelli,[7] Lisa Pieri,[7] Alessandro M. Vannucchi,[7] Heinz Gisslinger,[2] Mario Cazzola,[3] and Robert Kralovics[1,2]

Chronic myeloproliferative neoplasms (MPN) and myelodysplastic syndromes (MDS) have an inherent tendency to progress to acute myeloid leukemia (AML). Using high-resolution SNP microarrays, we studied a total of 517 MPN and MDS patients in different disease stages, including 77 AML cases with previous history of MPN (*N* = 46) or MDS (*N* = 31). Frequent chromosomal deletions of variable sizes were detected, allowing the mapping of putative tumor suppressor genes involved in the leukemic transformation process. We detected frequent deletions on the short arm of chromosome 6 (del6p). The common deleted region on 6p mapped to a 1.1-Mb region and contained only the *JARID2* gene—member of the polycomb repressive complex 2 (PRC2). When we compared the frequency of del6p between chronic and leukemic phase, we observed a strong association of del6p with leukemic transformation (*P* = 0.0033). Subsequently, analysis of deletion profiles of other PRC2 members revealed frequent losses of genes such as *EZH2*, *AEBP2*, and *SUZ12*; however, the deletions targeting these genes were large. We also identified two patients with homozygous losses of *JARID2* and *AEBP2*. We observed frequent codeletion of *AEBP2* and *ETV6*, and similarly, *SUZ12* and *NF1*. Using next generation exome sequencing of 40 patients, we identified only one somatic mutation in the PRC2 complex member *SUZ12*. As the frequency of point mutations in PRC2 members was found to be low, deletions were the main type of lesions targeting PRC2 complex members. Our study suggests an essential role of the PRC2 complex in the leukemic transformation of chronic myeloid disorders. Am. J. Hematol. 87:245–250, 2012. © 2011 Wiley Periodicals, Inc.

Introduction

The hematopoietic stem cells maintain sufficient blood cell production that involves self-renewal and tight regulation of proliferation and differentiation. During the lifetime of an individual somatic mutations arise in hematopoietic stem cells that may cause dominance of one or more stem cell clones. Once a clone has been established, it undergoes further mutagenesis and the resulting impact on proliferation capacity and differentiation dynamics may give rise to hematological phenotypes with excessive or deficient production of terminally differentiated blood cells. These conditions in the myeloid compartment fall into two large phenotypic categories referred to as chronic myeloproliferative neoplasms (MPN) and myelodysplastic syndromes (MDS). MPN is characterized by excessive production of myeloid cells, whereas MDS often displays dysplasia and deficient myeloid cell production with or without the presence of blasts.

A proportion of BCR-ABL negative MPN cases, also named classical MPN, develop acute myeloid leukemia (AML; 5–7%). The average time from MPN diagnosis to transformation is ~6 years, with the transformation occurring faster in myelofibrosis then in polycythemia vera (PV) and essential thrombocythemia (ET) patients. Patients who develop AML have a poor prognosis with an average survival time after transformation less than 5 months [1]. Some patients experience disease progression characterized by either secondary myelofibrosis (post-PV or post-ET) or a so-called "accelerated phase" defined by pancytopenia and increased number of blasts in the bone marrow, but below 20% that is necessary to establish AML diagnosis. MDS patients are also prone to develop AML as a complication of the disease. Approximately 30% of MDS patients transform to AML within a few months to a few years [2]. Post-MPN and post-MDS AML share common phenotypic and molecular features, and have bad prognosis resulting in rapid progression of the terminal stage of the disease and death within only a few months after transformation. To date, no efficient therapy exists and the mechanism of transformation remains unclear.

During the clonal evolution of MPN and MDS, the acquisition of lesions that either abrogate differentiation or increase genome instability can transform patients from chronic phase of the disease to AML. In MPN, transformation to AML is often preceded by an accelerated phase characterized by variable degree of cytopenia and elevated

[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]Department of Hematology, University of Pavia, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy; [4]Department of Internal Medicine Hematology and Oncology, University Hospital Brno, Masaryk University, Brno, Czech Republic; [5]Division of Hematology and Oncology, Innsbruck University Hospital, Innsbruck, Austria; [6]Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia; [7]Section of Hematology, University of Florence, Florence, Italy

Additional Supporting Information may be found in the online version of this article.

Conflict of interest: Nothing to report.

*Correspondence to: Robert Kralovics, Center for Molecular Medicine of the Austrian Academy of Sciences, Lazarettgasse 14, BT25.3, A-1090 Vienna, Austria. E-mail: robert.kralovics@cemm.oeaw.ac.at

Contract grant sponsor: Austrian Science Fund; Contract grant number: P23257-B12; Contract grant sponsors: MPN Research Foundation, Fondazione Cariplo, Regione Lombardia; Contract grant sponsor: MPO; Contract grant number: FR-TI2/254; Contract grant sponsor: Associazione Italiana per la Ricerca sul Cancro (AIRC, Milano) "Special Program Molecular Clinical Oncology 5x1000" to AGIMM (AIRC-Gruppo Italiano Malattie Mieloproliferative - http://www-progettoagimm.it; studies in Pavia and Florence); Contract grant numbers: #1005, IG 9034;

Received for publication 15 November 2011; Accepted 15 November 2011

Am. J. Hematol. 87:245–250, 2012.

Published online 18 November 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/ajh.22257

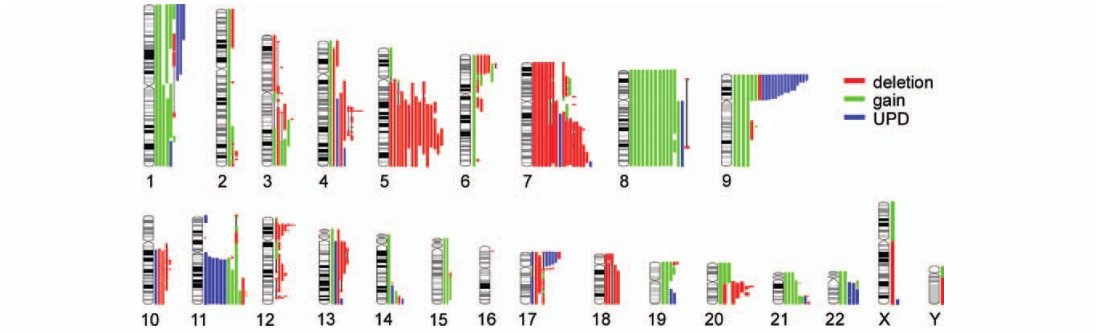


Figure 1. Karyoview of all chromosomal aberrations. Lesions were detected by Affymetrix SNP 6.0 arrays in 77 post-MPN (n = 46) and post-MDS (n = 31) AML patients. Physical positions and sizes of chromosomal aberrations are represented with green, red, and blue bars, indicating gains, deletions, and UPDs, respectively. The aberrations present in the same patient are depicted with thin black lines connecting the bars.

blasts in bone marrow (<20%) and in peripheral blood. As somatic deletions occur frequently in MPN and MDS, deletion mapping offers a powerful tool to identify tumor suppressor genes involved in the pathogenesis of these disorders. Recently, this approach allowed the identification of a number of novel myeloid tumor suppressors such as *IKZF1*, *CUX1*, and *FOXP1* [3–6]. In this study we applied deletion mapping in patients with post-MPN and post-MDS AML and identified frequent lesions of *JARID2*, a member of the polycomb repressive complex 2 (PRC2). Our study suggests that *JARID2*, and other PRC2 members, represent important tumor suppressors playing a crucial role in the leukemic transformation of chronic myeloid malignancies.

Patients and Methods

Patient samples. Peripheral blood samples from individuals diagnosed with MPN and MDS in different disease stages were obtained from several institutions in Italy, Austria, Serbia, and the Czech Republic. Written informed consent was obtained from all subjects in compliance with local ethics regulations. Genomic DNA was isolated according to standard procedures from granulocytes, mononuclear cell fractions, or the whole blood.

Microarray analysis. Microarray data were generated on a total of 517 samples. Of these samples, 77 were either post-MPN or post-MDS AML, 379 were chronic or accelerated phase MPN, and 61 chronic phase MDS. Genomic DNA from all patients was processed and hybridized to Affymetrix Genome-Wide Human SNP 6.0 arrays according to the manufacturer's instructions. Genotyping Console Version 3.0.2 software (Affymetrix) was used for the analysis of the raw data quality as well as identification of copy number alterations and losses of heterozygosity. The criteria used for definition of acquired (somatic) uniparental disomy (UPD) was telomeric position and the size of >1 Mb. In patients carrying numerous interstitial runs of homozygosity (>10 Mb) telomeric UPDs were excluded. Aberrations mapped to known copy number variation loci reported in the Database of Genomic Variants (DGV version 5, human reference genome assembly hg18) were not annotated.

Exome sequencing. DNA samples were processed and enriched for exons using Agilent SureSelect Human All Exon Kit (Agilent, Santa Clara, CA) and TruSeq DNA Sample Preparation and TruSeq Exome Enrichment Kit (Illumina, San Diego, CA) according to manufacturers' protocol. DNA libraries were sequenced using 51-bp paired end sequencing on HiSeq 2000 system (Illumina). Image analysis and base calling was performed using Real Time Analysis 1.12 software (Illumina). Resulting BCL files were converted to FASTQ format with the CASAVA 1.7 software (Illumina). Alignment of the reads and subsequent variant calling were performed in CLC Genomic Workbench 4.7 software (CLC bio, Aarhus, Denmark). For alignments, maximum of two mismatches or 2-bp insertions/deletions were allowed. Variants were called at coverage >10 and allelic frequency of a minimum of 15%. The variant lists were converted to MAQ format and GATK bed format and uploaded to SeattleSeq Annotation Server web tool (http://gvs.gs.washington.edu/ SeattleSeqAnnotation/). The annotated variants were filtered for those listed in dbSNP129 or 1,000 Genomes Project databases. Noncoding and synonymous variants were also removed. Finally, we applied Sanger sequencing for validating the variants in the genes encoding members of the PRC2 complex (*JARID2*, *AEBP2*, *EZH1*, *EZH2*, *SUZ12*, *PHF1*, *PHF19*, *EED*, *MTF2*, *RBBP4*, and *RBBP7*).

Single-gene mutational analysis and validation of exome sequencing. Exon sequencing was performed using 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). Primers were specifically designed for amplifying the regions of the variants.

All primer sequences and PCR conditions are available upon request.

Statistical analysis. Statistical significances of the distributions of frequencies of individual chromosomal aberrations between chronic phase and AML phase were determined using Fisher's exact test with the Bonfferoni correction for multiple testing.

Results

Identification of tumor suppressor genes by deletion mapping

To identify tumor suppressor genes that drive leukemic transformation in MPN and MDS, we aimed to map gene losses in transformed patients using high-resolution SNP array analysis (1.8 million probes per genome). We analyzed 77 AML cases with a previous history of either MPN (N = 46) or MDS (N = 31). In 16.9% of the patients, no chromosomal aberrations could be detected. We identified a number of recurrent deletions, gains, and acquired uniparental disomies (UPD) (Fig. 1). Deletions represented 59% of all cytogenetic lesions (total of 665 events). Because of the high frequency of recurrent deletion events in some genomic regions, it was possible to narrow down the common deleted regions (CDRs) to single genes. Among these recurrently deleted regions we confirmed known tumor suppressors such as *TET2*, *ETV6*, *IKZF1*, and *CUX1* [3–5,7–11].

In all analyzed samples, the vast majority of the deletions were hemizygous. The short arm of chromosome 6 acquired frequent hemizygous as well as a homozygous deletion, identifying a 1.1 Mb CDR. The CDR contained only a single gene *JARID2* (Fig. 2A). In addition to *JARID2*, we identified another homozygous loss as a result of two independent deletion events on the short arm of chromosome 12, forming a CDR that included the *AEBP2* gene (Fig. 2B). In both cases, the homozygous deletion arose by a two-step mechanism. In the first case, a 1.1-Mb deletion targeting *JARID2* was amplified through UPD of 36.5 Mb of the chromosome 6p (Fig. 2C). In the second case, the homozygosity was a consequence of two subsequent deletion events targeting *AEBP2*, the first one harboring a 22.7-

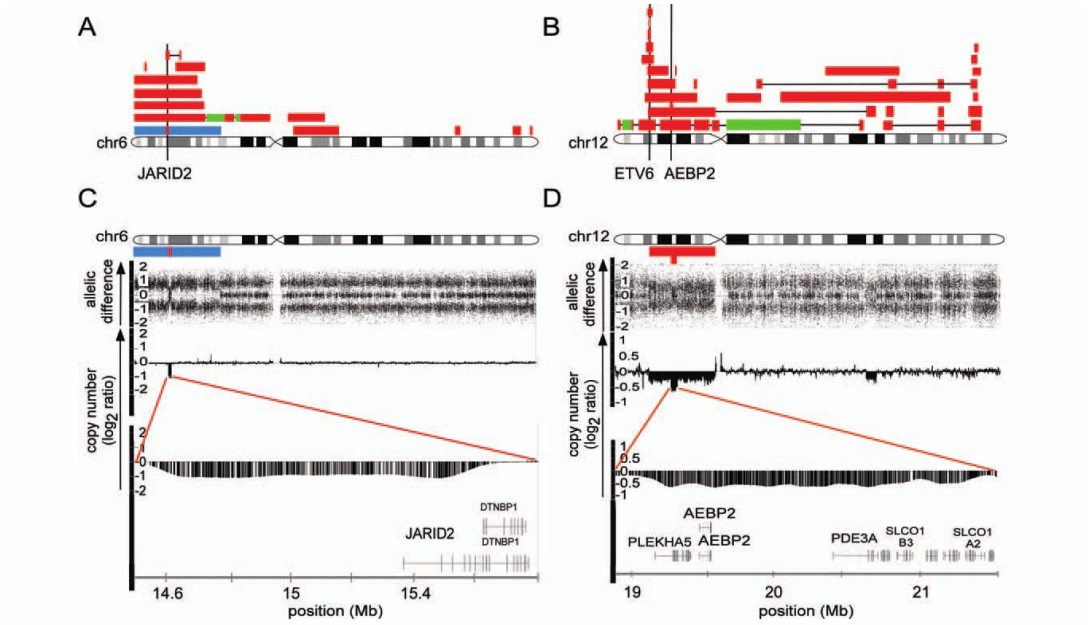


Figure 2. Deletions of *AEBP2* and *JARID2* genes. Positions and sizes of chromosomal aberrations are represented by horizontal bars; red indicating a deletion, green a gain, and blue an UPD. Bars connected by a black line indicate multiple aberrations present in the same patient. The output from the Genotyping Console Version 3.0.2. software represents the copy number data from Affymetrix SNP 6.0 arrays (log2 ratio to normal samples) as well as allelic difference. Chr, chromosome; Mb, megabase. (A) Chromosome 6 deletions detected in all analyzed patients. The commonly deleted region maps down to a single gene—*JARID2*. (B) Deletion profile of chromosome 12. The commonly deleted region harbors the *ETV6* gene; however, the homozygously deleted region in one patient identifies a secondary target of deletions—*AEBP2*, which is codeleted with *ETV6* in 40% of cases. (C) UPD of chromosome 6p with a homozygous deletion of ~1 Mb targeting a single gene *JARID2* in patient UPN_014 diagnosed with JAK2-V617F positive polycythemia vera in the accelerated phase. (D) Patient UPN_047 diagnosed with post-MDS AML carries a ~22.7-Mb deletion on chromosome 12p. Within this region lays a homozygous deletion ~1.8 Mb targeting *AEBP2*, suggesting a two-step deletion event.

Mb deletion, and the second one deleting 1.8 Mb of chromosome 12 (Fig. 2D). It is clear that, compared to single allele losses, deletion of both alleles could provide greater clonal advantage. Both loci also show frequent hemizygous deletion events in other patients in our cohort. In addition to the single case where *JARID2* was homozygously deleted in patient UPN_014, we found another five post-MPN AML cases where the deletion on chromosome 6p included *JARID2* (Fig. 2A). No other target gene on chromosome 6 has been reported in myeloid malignancies. Recurrent deletions are also present along the entire chromosome 12, mainly targeting *ETV6*, which has already been reported as a tumor suppressor frequently deleted in leukemia. We found that 40% of deletions targeting *ETV6* also codeleted *AEBP2*. Simultaneous loss of *ETV6* and *AEBP2* was present in a total of four patients, two diagnosed with post-MPN AML and two cases with post-MDS AML (Fig. 2B). We did not observe the deletion of both genes in any of the chronic phase samples. It is possible that the codeletion of *ETV6* and *AEBP2* provides stronger clonal advantage than the single gene deletions, accounting for the disease progression seen in patients.

Clinical phenotype associated with homozygous deletions of *AEBP2* and *JARID2*

The clinical data of patients UPN_047 and UPN_014, with homozygous losses of *AEBP2* and *JARID2*, respectively, are summarized in Table I. Patient UPN_047 with the homozygous *AEBP2* deletion had low levels of differentiated blood cells at diagnosis, accompanied with 6% blasts in peripheral blood and over 20% blasts in bone marrow. As the examination of blood and bone marrow indicated the existence of a previously unnoticed myelodysplastic phase, the patient was diagnosed as post-MDS AML. The patient had no history of prior cytotoxic therapy. Death occurred a few months after diagnosis.

TABLE I. Clinical Phenotype of Patients with Homozygous *JARID2* and *AEBP2* Deletions

	UPN_014	UPN_047
Diagnosis	PV	Post-MDS AML
Sex	M	F
Age at diagnosis (years)	68	70
Diagnosis at sample	Post-PV MDS (RAEB I)	Post-MDS AML
Age at sample (years)	75	70
Blood count at sample		
Leukocytes (10^9/l)	14.69	3.8
Hemoglobin (g/dl)	9.0	7.7
Hematocrit (%)	28.4	22.4
Platelets (10^9/l)	329	39
% blast in PB at diagnosis (%)	2	6
% blast in PB at sample (%)	5	6
Transfusion dependency	No	Yes
SPL at diagnosis	No	NA
SPL at sample	No	No
History of thrombosis	No	No
Secondary fibrosis	No	No
Therapy	Hydroxyurea Phlebotomy Anagrelide	Azacytidine
JAK2-V617F	Positive	NA
MPL-W515L	Negative	NA

UPN, unique patient number; PV, polycythemia vera; M, male; F, female; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; PB, peripheral blood; SPL, splenomegaly; NA, not applicable; RAEB I, refractory anemia with excess blasts.

TABLE II. Association of Chromosomal Deletions in PRC2 Loci, with Progression of Chronic Phase MPN/MDS to Acute Myeloid Leukemia

Gene name	Chromosome	Size of CDR	Number of genes in CDR	Chronic phase (MPN/MDS) (n = 440)	AML phase (n = 77)	P	P^*
JARID2	6p	1.1 Mb	1	1 (0.2%)	5 (6.5%)	0.0003	0.0033
AEBP2	12p	1.8 Mb	5	0	4 (5.2%)	0.0005	0.0055
SUZ12	17q	1.6 Mb	15	0	5 (6.5%)	0.0001	0.0011
EZH2	7q	15 Mb	>15	2 (0.5%)	17 (22%)	0.0001	0.0011

MPN, myeloproliferative neoplasm; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; CDR, commonly deleted region; Mb, megabase; *P*, *P*-value; *P**, *P*-value corrected for multiple testing using Bonferroni correction.

Patient UPN_014 was first diagnosed with JAK2-V617F positive PV and was treated with hydroxyurea. After 5 years of therapy, hematocrit levels were normalized and the patient continued on anagrelide for control of platelet count. Platelet count increased over time with a simultaneous decrease of hemoglobin levels. The patient was sampled at the "accelerated phase" of the disease characterized by anemia and 9% myeloblasts in the bone marrow.

Deletions of PRC2 loci in leukemic transformation

As *AEBP2* and *JARID2* encode proteins that are members of the PRC2 complex, we closely examined the aberration profile of the other PRC2 members (*EZH1/2*, *EED*, *SUZ12*, *RBBP4*, *RBBP7*, *MTF2*, *PHF1*, *PHF19*) in all 77 leukemic phase cases. Members of the complex are scattered across the genome and no focal deletions of individual members other than *JARID2* and *AEBP2* were found. Instead, the genes were often a part of larger deletions. Deletions covering the region of at least one member of the PRC2 complex were present in 35.6% of postchronic phase AML patients. Among the most frequently deleted were chromosomal regions containing *EZH2*, *JARID2*, *SUZ12*, and *AEBP2* (in 22, 6.5, 6.5, and 5.2% of patients, respectively; Table II). To investigate the presence of such deletions in the chronic phase, we included 440 chronic phase patient samples in this study. The results obtained for 61 MDS chronic phase patient samples were combined with previously reported MPN chronic phase cytogenetic aberration profiles for 379 patients (321 chronic phase MPN and 58 accelerated phase MPN or secondary myelofibrosis) [4]. Regions of *EZH2*, *JARID2*, and *SUZ12* that were deleted in the leukemic phase showed statistically significant differences compared to the frequencies found in chronic phase (Table II). Loss of the region including *EZH2* was detected in 0.5% of chronic phase samples (P = 0.0011), *JARID2* in only 0.2% (P = 0.0033) and loss of the chromosomal region covering *SUZ12* (P = 0.0011) was not found in any chronic phase case. Overall, deletions covering regions of PRC2 members were present in only 1.4% of all chronic phase cases and in 33.7% of the post-MPN and post-MDS AML cases (P = 0.0001). We did not observe mutual exclusivity of deletions of different PRC2 members. When we closely examined the *SUZ12* locus, a number of deletions were found in the region that targeted both *NF1* and *SUZ12* (Fig. 3A). Interestingly, in one patient with post-PV AML with a monosomy of chromosome 17, a homozygous deletion targeting only *NF1* was detected (Fig. 3B).

Point mutation frequency in PRC2 complex members

Next generation exome sequencing was used for sequencing 40 patient samples (31 chronic phase MPN, 6 post-MPN AML, and 3 post-MDS AML). We identified five variants in the PRC2 members *SUZ12*, *EED*, *EZH1*, *EZH2*, and *PHF19*. Variants were present in three MPN patients who had secondary myelofibrosis and one patient with post-MPN AML. The control tissue (T-cells) was available only from the patient with the *SUZ12* mutation in which we managed to show that the mutation is of somatic origin (Fig. 3C). This patient carried a somatic A to C substitution at the first position of codon ACA and the mutation was amplified to homozygosity by an acquired UPD of the long arm of chromosome 17 (Fig. 3C,D). The T598P mutation found in this patient is localized in the VEFS domain of Suz12 and is predicted to be possibly damaging with a high score (0.797) according to the PolyPhen-2 prediction tool. Unfortunately, patients with *EED*, *EZH1*, *EZH2*, and *PHF19* single nucleotide variants had no control tissue available; therefore, their somatic or germline origin could not be determined. The variant found in *EZH2* was predicted to be damaging based on the PolyPhen2 score and targets an amino acid residue that lies within the domain II of the protein, which is frequently targeted by somatic mutations in myeloid disorders [12]. We also performed sequencing of the entire coding region of PRC2 complex members affected by UPDs (Supporting Information Table I), as well as *JARID2* in all patients with a deletion of chromosome 6p, but could not identify any mutations. The overall frequency of mutations in PRC2 members was found to be low, suggesting that deletions are the main type of defects in *JARID2* and *AEBP2* in myeloid malignancies, and the same seems to be also true for other members of the PRC2 complex.

Discussion

PRC2 is a multimeric protein complex found in mammals that negatively regulates gene expression by trimethylating lysine 27 of histone H3 through the enzymatic activity of Ezh2 [13–15]. The core of the complex is formed by Ezh2, Suz12, and Eed, which are essential for its integrity and enzymatic function [16–18]. Other proteins identified to be part of the PRC2 complex are Aebp2, Jarid2, Ezh1, Rbbp4/7, Phf1, Mtf2, and Phf19 [19–27]. Both Jarid2 and Aebp2 contain DNA binding domains and have a role in the recruitment and binding of PRC2 to target genes. In addition, Aebp2 enhances the enzymatic activity of PRC2 by interacting with some of its components. Recent findings implicate that the PRC2 complex plays a role in hematopoiesis through epigenetic regulation of proliferative and self-renewal capacities of hematopoietic stem cells [28–30]. *EZH2* and *SUZ12* were found to be targeted by deletions or inactivating mutations in myeloid malignancies [31–33].

Our data show that *JARID2* and *AEBP2* are homozygously or hemizygously deleted in post-MPN and post-MDS progression stage patients, mainly AML. Patient UPN_014 is not a true chronic phase case, since the clinical data show clear progression of the disease with an increased number of blasts in the bone marrow. Other members of the PRC2 complex are also found affected by larger deletions and if shown to be targets of these deletions, could be novel tumor suppressors with a role in the disease progression from chronic phase to AML. PRC2 members could also be second targets of deletions, with an additive affect to the deletion of other tumor suppressors. Most of the patients acquire only hemizygous deletions covering the PRC2 complex members, suggesting that haploinsufficiency could already be sufficient to manifest a phenotypic effect. In the patient carrying the *JARID2*

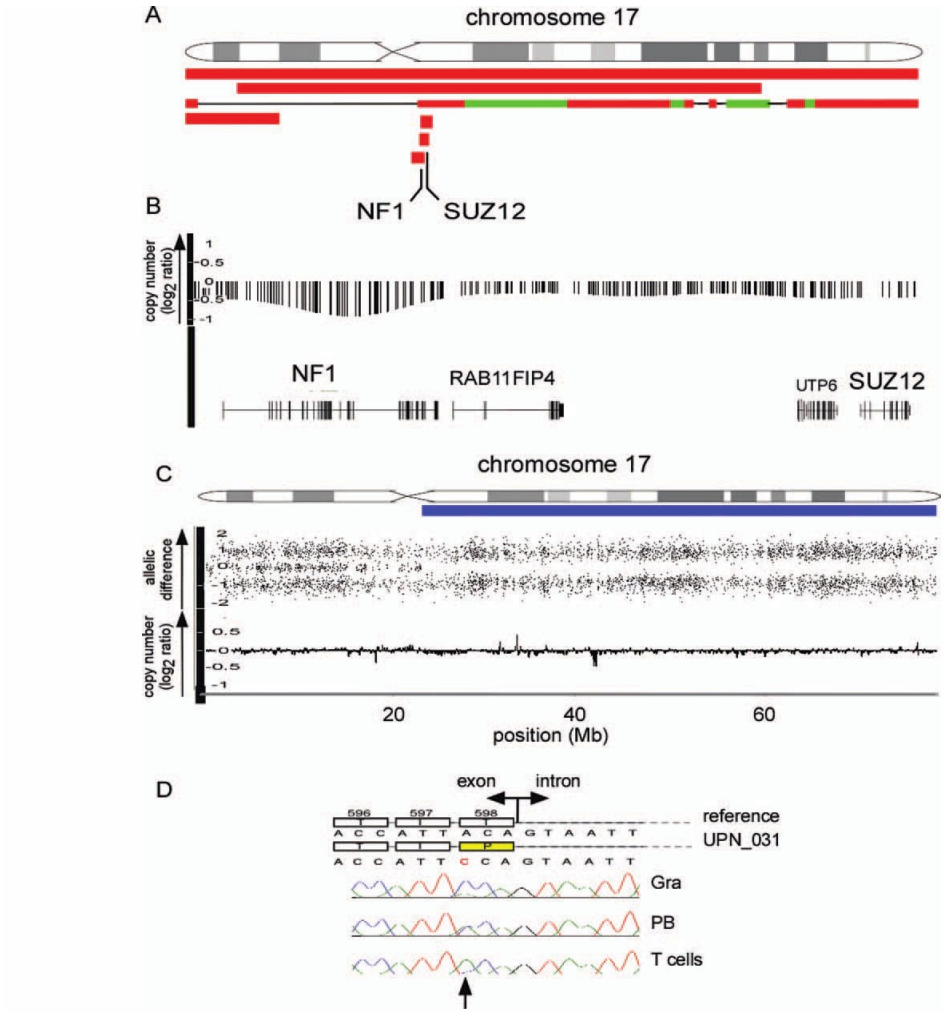


Figure 3. Summary of chromosome 17 defects. Chromosomal aberrations' sizes and positions are depicted as horizontal bars; green indicating gains, red deletions, and blue UPD. The outputs from the Genotyping Console Version 3.0.2. software represent the copy number data from Affymetrix SNP 6.0 arrays (log2 ratio to normal samples) as well as allelic difference. (A) Deletions of chromosome 17 in 77 analyzed post-MPN and post-MDS AMLs. Common deleted region maps to *NF1* locus. (B) Homozygous deletion targeting a single gene *NF1* on chromosome 17q in a post-MPN AML patient UPN_068. (C) UPD of chromosome 17q in patient UPN_031 diagnosed with secondary myelofibrosis. (D) Somatic mutation of SUZ12-T598P in patient UPN_031 carrying a UPD of chromosome 17q. Both mutated and wt alleles are present in the peripheral blood DNA sample. However, only the granulocytes carry the mutated allele, which is absent from the T-cells used as the control.

homozygous deletion, the *JAK2* mutation causing MPN accounted for the PV diagnosis, while the two subsequent aberrations resulting in the homozygous deletion of *JARID2* could have led to the progression of the disease, causing a phenotype switch from MPN to RAEB (refractory anemia with excess blasts), with a likeliness to further develop AML. Interestingly, the Jumonji mutant mice embryos (analog of human *JARID2*) die as a result of anemia due to the reduction of common myeloid and erythroid progenitor levels [34].

The results of this study show that *SUZ12* is targeted by both deletions and mutations. It has previously been shown that the loss of Suz12, the core component of PRC2 complex, enhances hematopoietic stem cell activity. The *SUZ12* locus is in a close proximity of the *NF1* tumor suppressor gene reported to be mutated in MPN [35]. Larger deletions may target both *SUZ12* and *NF1* simultaneously, likely resulting in a stronger effect on clonal progression or leukemic transformation. Same may be true for *EZH2* and *CUX1*, and *AEBP2* and *ETV6* codeletions, indicating that these PRC2 complex members could be secondary targets of deletions rather than drivers of the pathogenesis.

Our hypothesis is that disruption of these genes inactivates the function of PRC2 resulting in de-repression of its target genes, which leads to decreased differentiation potential of the myeloid progenitors that eventually can lead to leukemic transformation. This hypothesis corresponds to our finding that deletions of regions harboring the PRC2 complex members are present in one-third of post-MPN or post-MDS AML patients that we analyzed, and that their presence is significantly associated with leukemic transformation.

The leukemic transformation of chronic myeloid neoplasms is poorly understood. Recent studies implicated transcription factors (*ASLX1*, *CUX1*, *IKZF1*, *RUNX1*, *ETV6*), epigenetic regulators (*TET2*, *DNMT3A*, *IDH1/2*), *CBL*, and the p53 pathway in the leukemic transformation of chronic phase MPN and MDS [4,36–45]. In this study, we identified yet other tumor suppressors affecting transcriptional regulation. The diversity of lesions associated with the leukemogenesis in MPN and MDS suggests that many pathways are involved in this process. Identifying the relevant gene targets of the leukemia-associated transcription factors and early detection of these lesions are the prerequisites for novel therapeutic strategies capable of tackling the aggressive nature of leukemia arising from MPN and MDS.

Acknowledgments

The authors thank Martin Schalling for technical assistance.

References

1. 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;82:106–111.
2. Flach J, Dicker F, Schnittger S, et al. An accumulation of cytogenetic and molecular genetic events characterizes the progression from MDS to secondary AML: An analysis of 38 paired samples analyzed by cytogenetics, molecular mutation analysis and SNP microarray profiling. Leukemia 2011;25:713–718.
3. Jager R, Gisslinger H, Passamonti F, et al. Deletions of the transcription factor Ikaros in myeloproliferative neoplasms. Leukemia 2010;24:1290–1298.
4. Klampfl T, Harutyunyan A, Berg T, et al. Genome integrity of myeloproliferative neoplasms in chronic phase and during disease progression. Blood 2011;118:167–176.
5. Thoennissen NH, Lasho T, Thoennissen GB, et al. Novel CUX1 missense mutation in association with 7q- at leukemic transformation of MPN. Am J Hematol 2011;86:703–705.
6. 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;61:8820–8829.
7. Delhommeau F, Dupont S, Della Valle V, et al. Mutation in TET2 in myeloid cancers. N Engl J Med 2009;360:2289–2301.
8. Tefferi A, Pardanani A, Lim KH, et al. TET2 mutations and their clinical correlates in polycythemia vera, essential thrombocythemia and myelofibrosis. Leukemia 2009;23:905–911.
9. Bohlander SK. ETV6: A versatile player in leukemogenesis. Semin Cancer Biol 2005;15:162–174.
10. Cave 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;11:1459–1464.
11. 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;115:2882–2890.
12. Boyer LA, Plath K, Zeitlinger J, et al. Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature 2006;441:349–353.
13. Sewalt RG, van der Vlag J, Gunster MJ, et al. Characterization of interactions between the mammalian polycomb-group proteins Enx1/EZH2 and EED suggests the existence of different mammalian polycomb-group protein complexes. Mol Cell Biol 1998;18:3586–3595.
14. Kuzmichev A, Nishioka K, Erdjument-Bromage H, et al. Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev 2002;16:2893–2905.
15. Cao R, Wang L, Wang H, et al. Role of histone H3 lysine 27 methylation in polycomb-group silencing. Science 2002;298:1039–1043.
16. Cao R, Zhang Y. SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. Mol Cell 2004;15:57–67.
17. Pasini D, Bracken AP, Jensen MR, et al. Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. EMBO J 2004;23:4061–4071.
18. Montgomery ND, Yee D, Chen A, et al. The murine polycomb group protein Eed is required for global histone H3 lysine-27 methylation. Curr Biol 2005;15:942–947.
19. Margueron R, Reinberg D. The polycomb complex PRC2 and its mark in life. Nature 2011;469:343–349.
20. Shen X, Liu Y, Hsu YJ, et al. EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. Mol Cell 2008;32:491–502.
21. Peng JC, Valouev A, Swigut T, et al. Jarid2/Jumonji coordinates control of PRC2 enzymatic activity and target gene occupancy in pluripotent cells. Cell 2009;139:1290–1302.
22. Li G, Margueron R, Ku M, et al. Jarid2 and PRC2, partners in regulating gene expression. Genes Dev 2010;24:368–380.
23. Pasini D, Cloos PA, Walfridsson J, et al. JARID2 regulates binding of the polycomb repressive complex 2 to target genes in ES cells. Nature 2010;464:306–310.
24. Kim H, Kang K, Kim J. AEBP2 as a potential targeting protein for polycomb repression complex PRC2. Nucleic Acids Res 2009;37:2940–2950.
25. Sarma K, Margueron R, Ivanov A, et al. Ezh2 requires PHF1 to efficiently catalyze H3 lysine 27 trimethylation in vivo. Mol Cell Biol 2008;28:2718–2731.
26. Walker E, Chang WY, Hunkapiller J, et al. Polycomb-like 2 associates with PRC2 and regulates transcriptional networks during mouse embryonic stem cell self-renewal and differentiation. Cell Stem Cell 2010;6:153–166.
27. Boulay G, Rosnoblet C, Guerardel C, et al. Functional characterization of human polycomb-like 3 isoforms identifies them as components of distinct EZH2 protein complexes. Biochem J 2011;434:333–342.
28. O'Carroll D, Erhardt S, Pagani M, et al. The polycomb-group gene Ezh2 is required for early mouse development. Mol Cell Biol 2001;21:4330–4336.
29. Lessard J, Schumacher A, Thorsteinsdottir U, et al. Functional antagonism of the polycomb-group genes eed and Bmi1 in hemopoietic cell proliferation. Genes Dev 1999;13:2691–2703.
30. Kamminga LM, Bystrykh LV, de Boer A, et al. The polycomb group gene Ezh2 prevents hematopoietic stem cell exhaustion. Blood 2006;107: 2170–2179.
31. Ernst T, Chase AJ, Score J, et al. Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. Nat Genet 2010;42:722–726.
32. Nikoloski G, Langemeijer SM, Kuiper RP, et al. Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. Nat Genet 2010;42:665–667.
33. Brecqueville M, Cervera N, Adelaide J, et al. Mutations and deletions of the SUZ12 polycomb gene in myeloproliferative neoplasms. Blood Cancer J 2011;1,e33; doi:10.1038/bcj.2011.31.
34. Kitajima K, Kojima M, Nakajima K, et al. Definitive but not primitive hematopoiesis is impaired in jumonji mutant mice. Blood 1999;93:87–95.
35. Stegelmann F, Bullinger L, Griesshammer M, et al. High-resolution single-nucleotide polymorphism array-profiling in myeloproliferative neoplasms identifies novel genomic aberrations. Haematologica 2010;95:666–669.
36. Abdel-Wahab O, Pardanani A, Rampal R, et al. DNMT3A mutational analysis in primary myelofibrosis, chronic myelomonocytic leukemia and advanced phases of myeloproliferative neoplasms. Leukemia 2011;25:1219–1220.
37. Stegelmann F, Bullinger L, Schlenk RF, et al. DNMT3A mutations in myeloproliferative neoplasms. Leukemia 2011;25:1217–1219.
38. Lin P, Jones D, Medeiros LJ, et al. Activating FLT3 mutations are detectable in chronic and blast phase of chronic myeloproliferative disorders other than chronic myeloid leukemia. Am J Clin Pathol 2006;126:530–533.
39. Ding Y, Harada Y, Imagawa J, et al. AML1/RUNX1 point mutation possibly promotes leukemic transformation in myeloproliferative neoplasms. Blood 2009;114:5201–5205.
40. Taketani T, Taki T, Takita J, et al. Mutation of the AML1/RUNX1 gene in a transient myeloproliferative disorder patient with Down syndrome. Leukemia 2002;16:1866–1867.
41. Harutyunyan A, Klampfl T, Cazzola M, et al. p53 lesions in leukemic transformation. N Engl J Med 2011;364:488–490.
42. Green A, Beer P. Somatic mutations of IDH1 and IDH2 in the leukemic transformation of myeloproliferative neoplasms. N Engl J Med 2010;362:369–370.
43. Pardanani A, Lasho TL, Finke CM, et al. IDH1 and IDH2 mutation analysis in chronic- and blast-phase myeloproliferative neoplasms. Leukemia 2010;24:1146–1151.
44. Beer PA, Delhommeau F, LeCouedic JP, et al. Two routes to leukemic transformation after a JAK2 mutation-positive myeloproliferative neoplasm. Blood 2009;115:2891–2900.
45. Abdel-Wahab O, Manshouri T, Patel J, et al. Genetic analysis of transforming events that convert chronic myeloproliferative neoplasms to leukemias. Cancer Res 2010;70:447–452.

Supplemetary Table I. Association of uniparental disomies covering PRC2 loci, with progression of chronic phase MPN/MDS to acute myeloid leukemia.

gene name	chromosome	chronic phase (MPN/MDS) (n=440)	AML phase (n=77)	P	P*
EZH2	7q	4 (0.9%)	1 (1.3%)	0.5550	1
SUZ12	17q	2 (0.5%)	0	0.2759	1
JARID2	6p	1 (0.2%)	0	0.1489	1
AEBP2	12p	0	0	1	1
EZH1	17q	4 (0.9%)	1 (1.3%)	0.5550	1
MTF2	1p	3 (0.7%)	2 (2.6%)	0.1621	1
PHF19	9q	0	1 (1.3%)	0.1489	1
EED	11q	3 (0.7%)	6 (7.8%)	0.0005	0.0055
RBBP4	1p	5 (1.1%)	3 (3.9%)	0.1019	1
RBBP7	Xp	0	0	1	1
PHF1	6p	1 (0.2%)	0	0.1489	1
	Total:	23 (5.2%)	14 (18.2%)	0.0003	

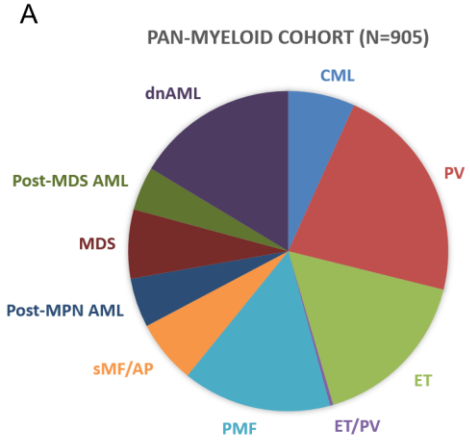
MPN, myeloproliferative neoplasm; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; P, P-value; P*, P-value corrected for multiple testing using Bonferroni correction

3.2. Deletions of genes encoding PRC2 members in a pan-myeloid cohort of patients

Following our findings of a commonly deleted region mapping to the *JARID2* gene and that chromosomal aberrations of the locus occur with higher frequencies in leukemic than in chronic stages of myeloid malignancies, we investigated the deletion profile of other important members of the PRC2 complex in a larger pan-myeloid cohort, hypothesizing that haploinsufficiency of PRC2 members is more frequent than mutations, and might result in dysregulation of gene repression of target genes.

Figure 9. Structure of the analyzed pan-myeloid cohort

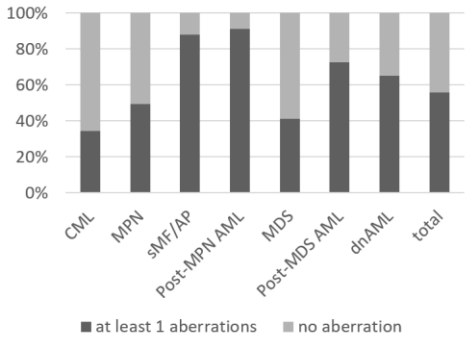
A



B

Diagnosis	Total number of patients	Patients with at least 1 detected aberration	Patients with no detected aberrations
CML	61	21	40
PV	200	142	58
ET	150	41	109
ET/PV	3	0	3
PMF	136	58	78
sMF/AP	58	51	7
Post-MPN AML	45	41	4
MDS	63	26	37
Post-MDS AML	40	29	11
dnAML	148	96	52
total	905	506	399

C



A. The representation of different disease entities in the pan-myeloid cohort used for the study. B. The table lists the number of patients in each disease sub-group with and without detected chromosomal aberration, as investigated by Genome-Wide Human SNP Arrays 6.0. C. The presence of chromosomal aberrations represented by column chart, for each of the disease sub-types.

Furthermore we were interested in seeing whether deletions of PRC2 members are specific to certain disease entities or occur across different myeloid neoplasms.

The analyzed cohort consisted of a total of 905 patients, including the cohort used for Manuscript 1 (Section 3.1). In addition to MPN, MDS and post-MPN and post-MDS AML samples, samples from CML, as well as from dnAML patients were included. The structure of the analyzed cohort by disease entity is described in Figure 9A and 9B. All data from the analyzed SNP arrays was combined and the copy number and loss of heterozygosity profile of the entire cohort was investigated. From the total number of analyzed samples 44% (N=399) had no detectable chromosomal aberration. The remaining 56% (N=506) of the patients carried at least 1 chromosomal aberration (Figure 9B and 9C).

Using the data from the performed SNP Arrays, we examined the frequency of deletions covering PRC2 member encoding genes and their distribution between different disease entities. We explored the deletion profile covering the core members important for the enzymatic function of the complex and the cofactors - *EZH2, SUZ12, EED, EZH1, JARID2* and *AEBP2* genes, on chromosomes 7q, 17q, 17q, 11q, 6p and 12p, respectively. The number of deletions in each locus was counted and is represented in Table 6. A total of 85 deletions in 60 patients were identified in our cohort, with 1 to 4 PRC2 member genes deleted in individual patients. In 36 patients a single PRC2 member was deleted, while deletions of multiple PRC2 components occurred in 24 patients. The presence of PRC2 member deletions and their co-occurrence in patients are graphically displayed in Figure 10D. A list of all deletions covering the investigated genes, with their exact positions, is provided in Table 21.

The frequency of patients harboring deletions of at least 1 analyzed PRC2 member was much higher in post-MPN AML (40%), post-MDS AML (22.5%) and dnAML (16.2%) than in chronic phase MPN and MDS (Figure 10A). Overall, 31.8% of sAML patients from our cohort had at least one PRC2 member deleted, compared to only 0.9% of chronic phase patients (MPN and MDS combined), with no such deletions detected in chronic phase MDS. Multiple PRC2 deletions were most frequent in dnAML, followed by post-MPN AML and post-MDS AML (Figure 10B). The enrichment of multiple deletions seen in dnAML cannot be explained by cytogenetic complexity alone as 64% of dnAML in our cohort harbored at least 1 cytogenetic aberration, compared to 91% and 71% of post-MPN and post-MDS AML, respectively.

Table 6. Deletions of genes encoding PRC2 components in the pan-myeloid patient cohort

		Deletions								
Gene	Chr	Total	MPN (n=489)	MDS (n=63)	AP/sMF (n=58)	Post-MPN AML (n=45)	Post-MDS AML (n=40)	Total sAML (n=85)	dnAML (n=148)	CML (n=61)
AEBP2	12p	13	0	0	0	2	4	6	7	0
JARID2	6p	12	2	0	1	6	0	6	3	0
EZH2	7q	36	1	0	1	10	8	18	14	2
SUZ12	17q	21	1	0	0	5	2	7	13	0
EED	11q	3	2	0	0	0	0	0	1	0
EZH1	17q	8	0	0	0	2	0	2	6	0
Total deletions		85	6	0	2	25	14	39	44	2
Total patients (n) (%)		60 (6.6%)	5 (1%)	0 (0%)	2 (3.4%)	18 (40%)	9 (22.5%)	27 (31.8%)	24 (16.2%)	2 (3.3%)

The deletions covering single genes were counted from data acquired by analyzing 905 patients with the use of Affymetrix Genome-wide Human SNP Arrays 6.0. In some patients deletions of chromosome 17q covered both *SUZ12* and *EZH1*. Chr, chromosome; MPN, myeloproliferative neoplasm; MDS, myelodysplastic syndrome; AP, accelerated phase MPN; sMF, secondary myelofibrosis; AML, acute myeloid leukemia; sAML, secondary acute myeloid leukemia; dnAML, *de novo* acute myeloid leukemia; CML, chronic myeloid leukemia; n, number

Table 7. Deletions of genes encoding PRC2 subunits in MPN and post-MPN AML

Gene	MPN (n=489)	post-MPN AML (n=45)	*P*	*P**
JARID2	2	6	0,0001	0,0006
EZH2	1	10	0,0001	0,0006
SUZ12	1	5	0,0001	0,0006
AEBP2	0	2	0,007	0,042
EZH1	0	2	0,007	0,042
EED	2	0	1	6
≥1 PRC2 del	**5**	**18**	**0,001**	**0,006**

Deletions covering specific genes, detected by Genome-Wide Human SNP Arrays 6.0, are counted in 60 MDS and 40 post-MDS AML samples. P-values are calculated using the Fisher's exact test. Bonferonni correction for multiple testing was introduced, resulting in a corrected P*-value. The last row presents the number of patients in each disease subgroup harboring a deletion covering at least 1 PRC2 gene.

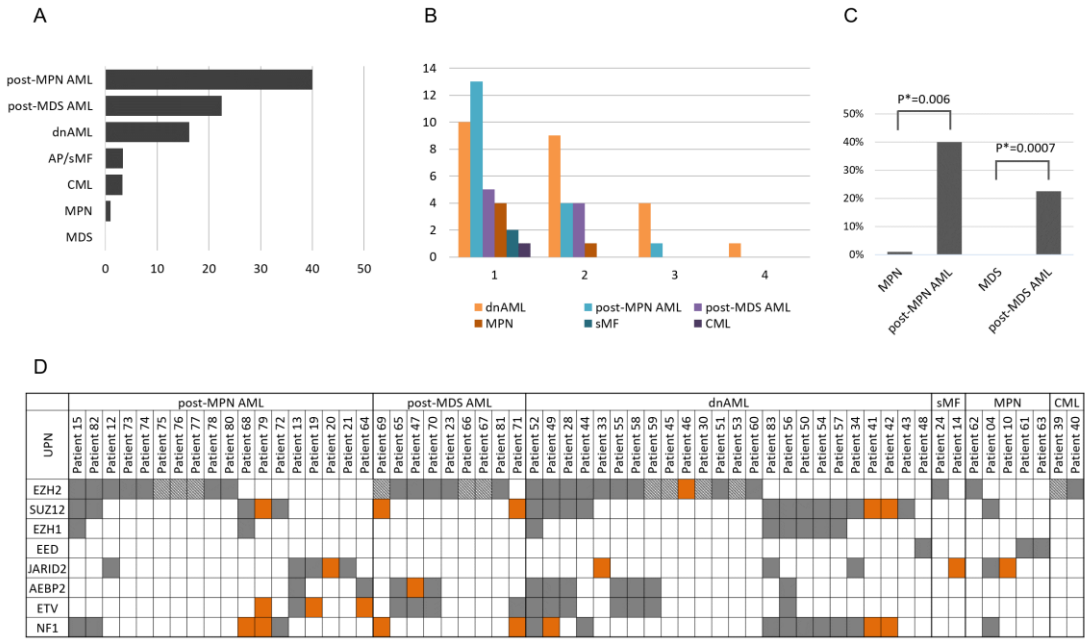
Table 8. Deletions of genes encoding PRC2 subunits in MDS and post-MDS AML

Gene	MDS (n=63)	Post-MDS AML (n=40)	*P*	*P**
EZH2	0	8	0,0003	0,0021
SUZ12	0	2	0,1485	1,0395
AEBP2	0	4	0,207	1,449
JARID2	0	0	1	7
EED	0	0	1	7
EZH1	0	0	1	7
≥1 PRC2 del	**0**	**9**	**0,0001**	**0,0007**

Deletions covering specific genes, detected by Genome-Wide Human SNP Arrays 6.0, are counted in 489 MPN and 45 post-MPN AML samples. P-values are calculated using the Fisher's exact test. Bonferonni correction for multiple testing was introduced, resulting in a corrected P*-value. The last row presents the number of patients in each disease subgroup harboring a deletion covering at least 1 PRC2 gene.

When we compared the occurrence of the deletions of interest in chronic and leukemic phase we observed an association of deletion covering *EZH2*, *JARID2* and *SUZ12* with leukemic transformation of MPN (*P**=0.006 for all three genes) (Table 7), whereas deletions covering *EZH2* are enriched with statistical significance in post-MDS AML compared to MDS (*P**=0.0021) (Table 8). The enrichment of the fraction of patients harboring at least 1 PRC2 deletion in leukemic compared to chronic disease stage is observed in both MPN and MDS, with statistical significance supported by the calculated (and corrected) *P* values of *P**=0.006 for MPN and *P**=0.0007 for MDS (Table 7, Table 8, Figure 10C). This data supports our hypothesis of the role of PRC2 haploinsufficiency in the progression of both MPN to post-MPN AML and MDS to post-MDS AML.

Figure 10. Frequencies and co-occurrence of deletions of PRC2-encoding genes detected in a pan-myeloid patient cohort



A. Occurrence of PRC2 deletions in different disease entities, represented as the % of patients carrying at least one deletion covering a PRC2 gene; X-axis: percentage. B. Number of simultaneous PRC2 genes deleted in a single individual, grouped by disease entity; X-axis number of deleted PRC2 members in single patients, Y-axis number of patients; C. Association of PRC2 deletions (at least 1 gene deleted) with leukemic transformation in MPN and MDS. The P-values with the Bonferonni correction are displayed (P*); D. Deletions of selected genes in all patients with at least 1 PRC2 deletion. Grey, deletion of the gene present; orange, focal deletion; dashed, monosomy chr7

Among all the detected deletions seven chr7 monosomies were observed (4 in dnAML, 3 in post-MPN AML, 3 in post-MDS AML and 1 in CML), with other deletions ranging in size from 0.35 Mb (Megabase) to 99.8 Mb. Eleven focal deletions were detected, defined as those smaller than 2Mb, 5 targeting *SUZ12*, 4 *JARID2*, 1 *AEBP2* and 1 targeting EZH2 (Figure 10D). In Patient 42 (dnAML) the only detected aberration was the focal deletion of *SUZ12* (also covering *NF1*). In two patients, the focal deletions of *EZH2* (Patient 46, dnAML) and *SUZ12* (and *NF1*) (Patient 69, post-MDS AML) co-occurred with one additional chromosomal aberration – chr 11 trisomy and chr 7 monosomy, respectively. In the remaining patients with focal PRC2 deletions at least three additional chromosomal aberrations were detected.

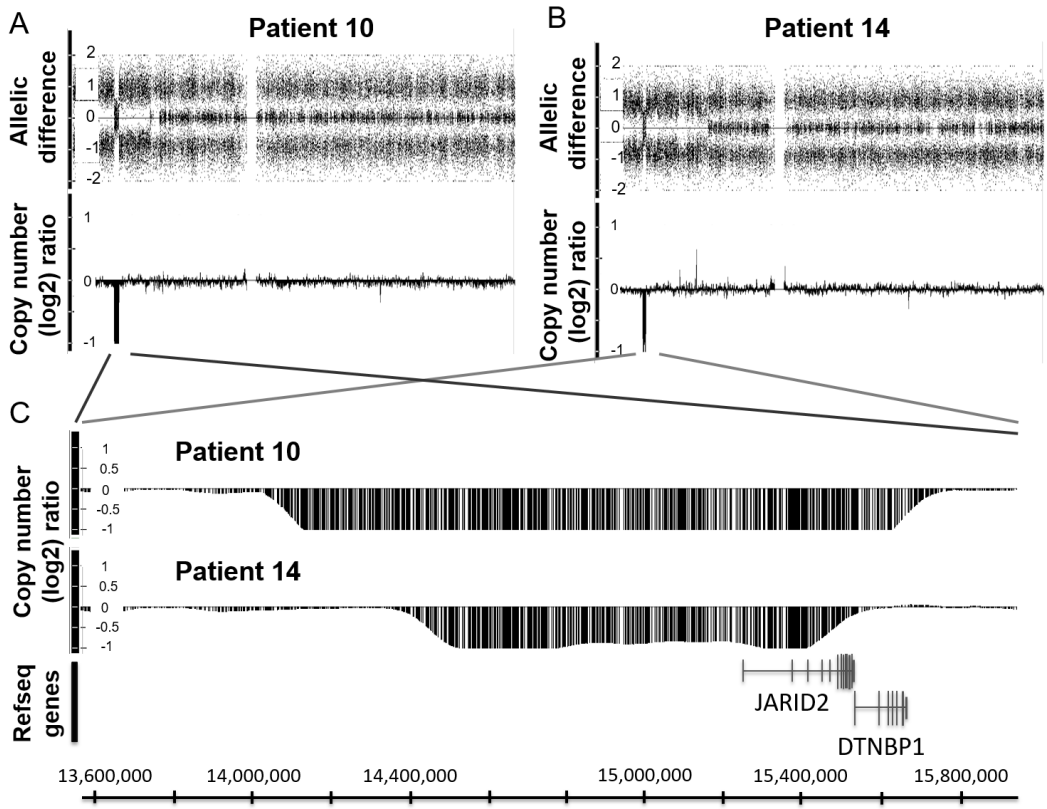
Both *SUZ12* and *EZH1* are located on chromosome 17q. Therefore, single larger deletions may result in the loss of both genes. An existence of 7 such deletions

was seen in our cohort, 5 in dnAML and 2 in post-MPN AML. All cases with *EZH1* deletions covered *SUZ12*, whereas 8/21 (38%) *SUZ12* deletions covered the *EZH1* locus. As reported in the study published in Manuscript 1. (Chapter 3.1.), we previously observed co-deletions of *SUZ12* and *NF1* which are in close proximity. Indeed, our previous findings were confirmed in the expanded cohort, with 19/21 (90%) *SUZ12* deletions covering *NF1*. Seven focal deletions of *NF1* are observed in our cohort, in sAML and dnAML. (Figure 10D), only two of which do not cover the *SUZ12* gene. The homozygous deletion of *NF1* in Patient 68, described in Chapter 3.1. is included. Of note, we have detected one more focal deletion of *NF1* in a post-MDS AML patient in our cohort but is not included in the presented results as no PRC2 deletions were present in the patient. We have already discovered a strong co-occurrence of *AEBP2* and *ETV6* deletions as a result of single deletion events on chromosome 12p (Chapter 3.1.). In the pan-myeloid cohort we confirm this co-occurrence with 12/21 (57%) *ETV6* deletions deleting *AEBP2*, as well as the association with leukemic progression as all deletions covering both *ETV6* and *AEBP2* were seen in leukemic samples (5 sAML, 7 dnAML). Of the remaining chromosome 12p deletions not covering *AEBP2* three *ETV6* deletions co-occurred with other PRC2 members – 2 with *SUZ12* and 1 with *JARID2* deletions. One post-MPN patient presented with loss of both *JARID2* and *AEBP2*. *EED* deletions were observed with lower frequencies than deletions of the other examined genes and showed mutual exclusivity with deletions of other investigated genes in our cohort.

3.3. Homozygous deletions of *JARID2* and *AEBP2* co-occur with other chromosomal aberrations in patients

In the analyzed cohort we have identified a second patient, Patient 10, with two aberrational events on chromosome 6 (a deletion and a UPD), resulting in a homozygous deletion covering the *JARID2* gene (Figure 11A and C), very similar to the case of Patient 14, described in Chapter 3.1 (Figure 11B and C). Clinical features of this *JAK2*V617F positive PV patient are summarized in Table 9. The detected loss of heterozygosity is 26.2 Mb long, starting from the p end of chromosome 6, whereas the deletion is 1.6 Mb long (chr6: 14100000-15700000; hg18 reference genome) and covers only two genes – *JARID2* and *DTNBP1*.

Figure 11. Chromosomal aberrations of chromosome 6 in Patients 10 and 14



Chromosomal aberrations are identified by analyzing patient samples with Affymetrix Genome-wide Human SNP Arrays 6.0. The output images were obtained by analyzing the data with the Genotyping Console 3.0.2. software. Allelic difference and copy number changes of chromosome 6 in Patients 10 and 14 are presented in panels A and B, respectively. C. Zoomed in copy number ratios of the homozygously deleted regions in Patients 10 and 14. The genes present in the locus and the genomic positions (hg18 reference genome) are shown on the bottom.

Table 9. Clinical properties of Patient 10

Diagnosis at sample	PV
Sex	M
Age at diagnosis of MPN (years)	35
Age at post chronic phase diagnosis (years)	45
Age at diagnosis of leukemia (years)	55
Blood count at sample	
Leukocytes (10^9/l)	7.04
Hemoglobin (g/dl)	8.8
Hematocrit (%)	28.2
Platelets (10^9/l)	275
Blasts in PB at sample (%)	16
Blasts in PB at progression phase (%)	30
Transfusion dependency	yes
SPL at diagnosis of MPN	yes
SPL at diagnosis of leukemia	splenectomy
History of thrombosis	yes
Secondary fibrosis	yes
Therapy	Phlebotomies, Interferon, Anagrelide, Azacytidine, Ruxolitinib
JAK2-V617F	Positive

PV, polycythemia vera; M, male; MPN, myeloproliferative neoplasm; PB, peripheral blood; SPL, splenomegaly.

In order to find out whether the deletions targeting *JARID2* and *AEBP2* occur as single events in the affected patients we looked into the aberrational profile of the patients and discovered that all three patients with homozygous deletions of the PRC2 cofactors harbor additional chromosomal lesions.

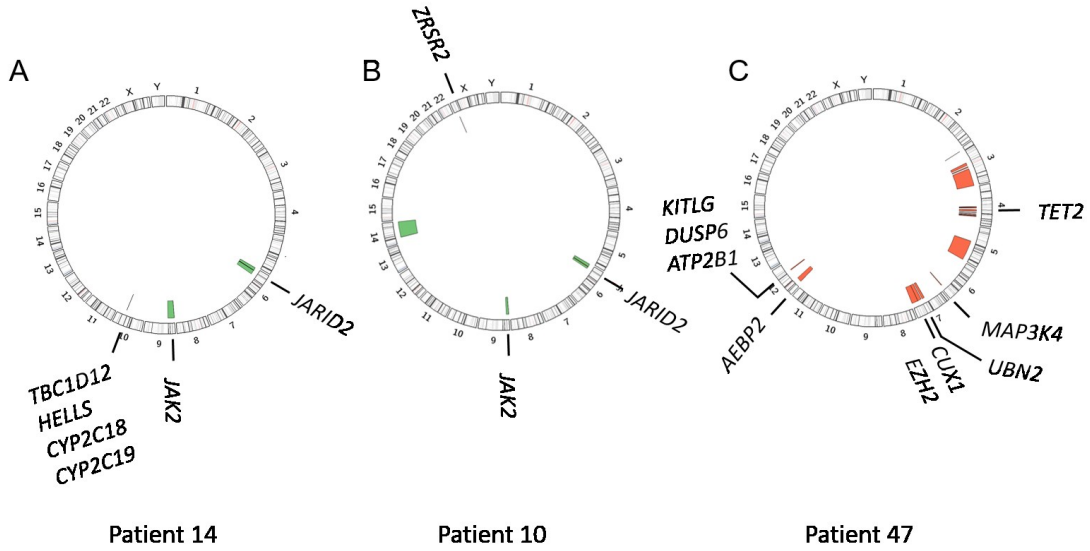
In the case of Patient 14, apart from the fully clonal chromosome 6 defects, we detected a UPD of chromosome 9p which amplified the *JAK2*V617F mutation, as well as a gain of the whole chromosome 9 which is not fully clonal, as detected by the SNP arrays. The patient also had a fully clonal focal deletion on chromosome 10q, covering the region of only 4 genes – *TBC1D12*, *HELLS*, *CYP2C18* and *CYP2C19* (Figure 12A). Based on the estimated fraction of cells with the chromosomal aberrations we can assume that chr6 and chr10 lesions, as well as the chr9p UPD happened first in the same background clone, and that the gain of chromosome 9 was an additional late event in the clonal evolution. In which order the first three aberrations were acquired is not possible to outline without having sequential DNA samples from the patient,

leaving it unclear to which stage of the disease onset or progression the chromosome 6 lesions contributed to in this patient.

In addition to the abnormal chromosome 6, Patient 10 carried a UPD of chromosome 9p amplifying the *JAK2* mutation, a UPD of chromosome 14q and a small deletion on the X chromosome covering the *ZRSR2* gene (Figure 12B). The 14qUPD is a recurrent aberration seen in MPN, targeting an imprinted locus, and has also been associated with clonal hematopoiesis in ageing individuals (Chase, Leung et al., 2015, Jacobs et al., 2012, Laurie et al., 2012). As previously described in Chapter 1.2.2 the spliceosome gene *ZRSR2* is recurrently mutated in MPN. In the case of Patient 10 the single copy of the gene on chromosome X is deleted in the male patient. All of the aberrations were fully clonal, as detected by SNP arrays, implying that the aberrations were part of the same clone, carrying all of the described genetic defects. However, due to the lack of sequential DNA samples from the patient the order in which the genetic changes were acquired cannot be delineated.

The homozygous deletion of *AEBP2* in patient 47 (described in Chapter 3.1) is also not a solitary chromosomal aberration detected in the patient. A complex pattern of deletions of chromosomes 3q, 4q, 5q and 7q were detected, together with small deletions of chromosomes 6q and 12q (Figure 12C). The large deletion of chromosome 5 associates with the chronic MDS stage which preceded the leukemic stage. The deletion of chromosome 4 includes the *TET2* gene, whereas the *EZH2* and *CUX1* genes are deleted as part of the chromosome 7 deletions. The focal deletion on chromosome 6q covers the *MAP3K4* gene, together with the *PARK2* and *PACRG* genes, and the *KITLG*, *DUSP6* and *ATP2B1* genes are deleted as part of the chromosome 12q deletion. Interestingly, in addition to the homozygous deletion on chromosome 12 the patient had another homozygously deleted region on chromosome 7 (chr7:138200000-138850000; hg18 reference genome). The region covers a small number of genes, one of which is *UBN2*, a member of the histone chaperone complex HIRA. In other patients this region is often co-deleted with *EZH2*, as part of long deletion which frequently occur in myeloid malignancies. All deletions in Patient 47, except for the focal deletions on chromosomes 7q and 12p are hemizygous and appear to be part of the same clone, based on clonal size estimations from the SNP array data.

Figure 12. Chromosomal aberrations in Patients 14, 10 and 47



The aberrational profile of patients was detected with the use of Affymetrix Genome-wide Human SNP Arrays 6.0. Circos plots are used for graphical presentations. Chromosomes are represented in a circular manner, with chromosomal aberrations of patients 14, 10 and 47 plotted in panels A, B and C, respectively. Single bars represent single chromosomal aberrations, with the position and length of the bar corresponding to the genomic position and length of the detected chromosomal aberrations. Deletions are represented in red, uniparental disomies in green. The positions of relevant single genes are marked with a line outside of the circle (Krzywinski, Schein et al., 2009).

The aberrational profiles of patients 14, 10 and 47 show that the *AEBP2* and *JARID2* homozygous deletions are not solitary genetic alterations in affected patients, providing insight into a putative collaborative action of PRC2 cofactor loss with other altered pathways in the disease evolution. *JARID2* homozygous deletions in both cases co-occurs with *JAK2*V617F mutations amplified by chr9p UPD. As the hemizygous deletions of the *JARID2* locus are enriched in the leukemic vs. chronic phase samples (as shown in Chapters 3.1 and 3.2) we speculate that the deletions occurred as late events in the disease evolution contributing to the progression rather than initiation of the disease.

3.4. Presence and frequency of chromosome 6 aberrations in a pan-myeloid cohort of patients

Deletions, gains and uniparental disomies of chromosome 6 are found in MPN, MDS and secondary AML (post-MPN or post-MDS AML) patients, and their presence is significantly enriched in the leukemic phase compared to the chronic phase, as described in Manuscript 1 (Chapter 3.1). In order to investigate whether chromosome

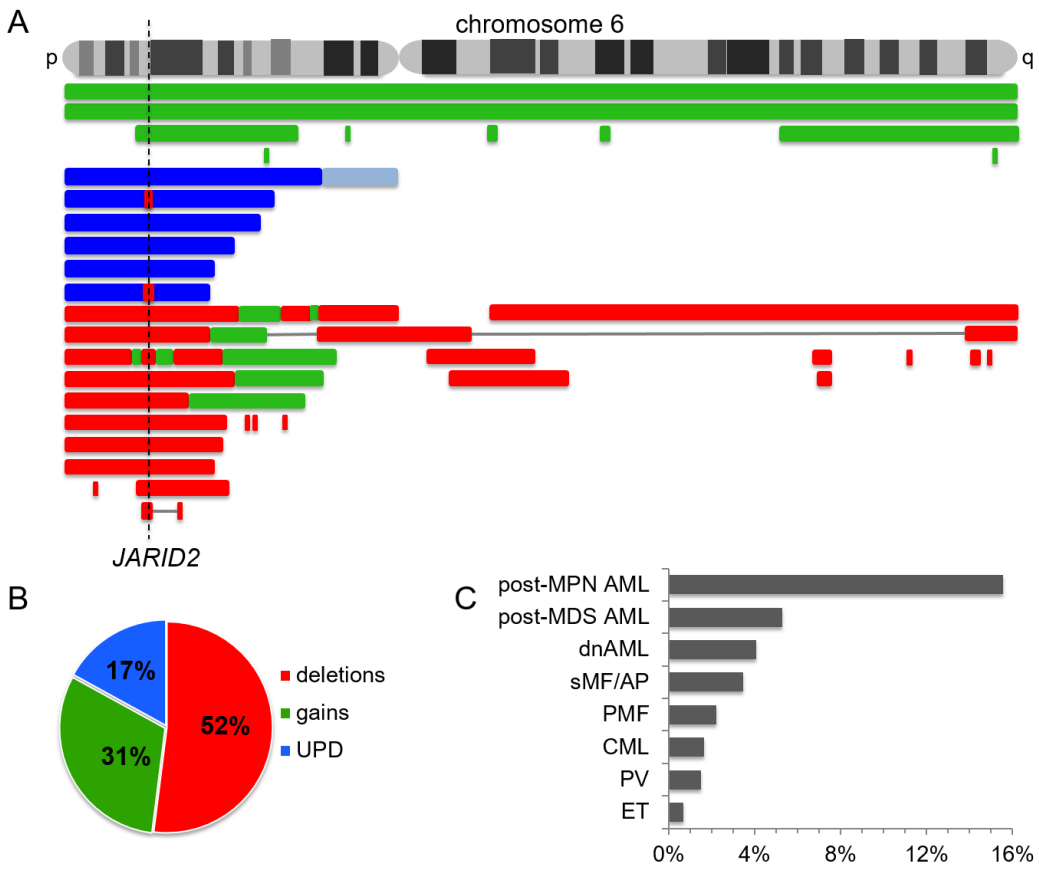
6p aberrations are restricted to these disease entities or also appear in patients with other types of myeloid malignancies we analyzed their presence in the pan-myeloid cohort of patients (described in Chapter 3.2). We detected 56 chromosome 6 aberrations in 37 patients, out of which 32 were deletions (57%), 17 gains (30%) and 7 UPDs (12.5%) (Table 10, Table 20). The majority of the lesions (N=42) mapped to the short arm of chromosome 6 (chr 6p). Seven UPDs (17% of all chr 6p events), 22 deletions (52%) and 13 gains (31%) were detected in 25 patients (Figure 13B), most of which covered the region of *JARID2* (Figure 13A). The aberrations were not restricted to MPN, MDS or leukemic phase patients, but were also detected in a number of dnAML patients, as well as in one CML patient (Table 10). However, when we analyzed the frequency of chromosome 6p aberrations in single disease entities we observed that the frequency of chr 6p aberrations is higher in AML and advanced stages of MPN than in chronic MPN (Figure 13C). 15.6% of post-MPN AML patients harbor chromosome 6p aberrations compared to 1.4% of MPN (*P*=0.0001) strongly suggesting that chromosomal aberrations of chr 6p might be associated with disease progression of MPN and severe phenotypes. Despite the lack of chromosome 6p aberrations in MDS in our cohort, we have not observed a significant association of their presence with evolution to post-MDS AML (*P*=0.148). These data implicate the role of lesions of the short arm of chromosome 6 in the pathogenesis and progression of MPN but not MDS.

Table 10. Detected chromosome 6 aberrations in the investigated pan-myeloid cohort

Diagnosis	Total number of patients	Patients with aberrations on chr 6	Patients with aberrations on chr 6p
CML	61	1	1
PV	200	5	3
ET	150	1	1
ET/PV	3	0	0
PMF	136	4	3
sMF/AP	58	2	2
Post-MPN AML	45	9	7
MDS	63	1	0
Post-MDS AML	40	3	2
dnAML	148	11	6
total	**905**	**37**	**25**

CML, chronic myeloid leukemia; PV, polycythemia vera; ET, essential thrombocythemia; PMF, primary myelofibrosis; sMF, secondary myelofibrosis; AP, accelerated disease phase; Post-MPN AML, post-myeloproliferative neoplasm acute myeloid leukemia; post-MDS AML, post-myelodysplastic syndrome acute myeloid leukemia; dnAML, *de novo* acute myeloid leukemia.

Figure 13. Chromosomal aberrations detected on chromosome 6



A. Graphical representation of detected chr 6 lesions. Blue bars represent uniparental disomies, red bars deletions and green bars gains of genomic regions. Bars connected with a grey line are detected in the same patient. The dashed vertical line depicts the position of *JARID2.* B. Distribution of chromosomal defects by type of aberration, presented as percentage from the total of 42 events detected on chr 6p. UPD, uniparental disomy. C. Frequency of chr 6p aberrations in different disease entities in the studied cohort of patient samples, presented as percentage of patients with chr 6p lesions from the total number of patients with the same diagnosis. Post-MPN AML, post-myeloproliferative neoplasm acute myeloid leukemia; post-MDS AML, post-myelodysplastic syndrome acute myeloid leukemia; dnAML, *de novo* acute myeloid leukemia; sMF, secondary myelofibrosis; AP, accelerated disease phase; PMF, primary myelofibrosis; CML, chronic myeloid leukemia; PV, polycythemia vera; ET, essential thrombocythemia.

3.5. Mutational profiling of *JARID2* in patients with chromosomal aberrations covering the genomic region of the gene

In order to investigate whether the aberrational profile of the *JARID2* locus on chromosome 6 is coupled with mutations of *JARID2*, and to supplement our previous findings where we showed that *JARID2* is not mutated in the 5 investigated patients with chr 6p deletions (Chapter 3.1), we expanded the sequencing of the whole coding

region of the gene to all patients with a deletion, UPD or gain of the *JARID2* genomic region. A total of 10 patients (including the 5 previously sequenced patients) were sequenced, 4 of which with a UPD, 4 with a deletion and 2 with a gain of the locus of interest (Table 11). Patients 10 and 14 were not included in the sequencing as the *JARID2* gene was completely deleted in both of the patients. In the 10 investigated patients we identified no mutations in the coding region of *JARID2*, showing that the deletions of the gene and its surrounding region are not coupled with mutations in the coding region of *JARID2* and that the target of 6p UPDs and gains are not mutations of *JARID2.*

Table 11. Mutational status of *JARID2* in patients with chromosome 6 aberrations, as detected by Sanger sequencing

UPN	Diagnosis	Type of aberration covering the *JARID2* locus	*JARID2* mutational status
Patient 01	ET	UPD	wt
Patient 04	PMF	Deletion	wt
Patient 05	PMF	UPD	wt
Patient 11	post-MPN AML	Gain	wt
Patient 12	post-MPN AML	Deletion	wt
Patient 13	post-MPN AML	Deletion	wt
Patient 19	post-ET AML	Deletion	wt
Patient 20	post-MPN AML	Deletion	wt
Patient 21	post-PV AML	Deletion	wt
Patient 23	post-MDS AML	UPD	wt
Patient 26	dnAML	UPD	wt
Patient 31	dnAML	Deletion	wt
Patient 33	dnAML	Deletion	wt
Patient 34	dnAML	Deletion	wt
Patient 38	post-MPN AML	Gain	wt

UPN, unique patient number; ET, essential thrombocythemia; PMF, primary myelofibrosis; AML, acute myeloid leukemia; post-MDS AML, post-myelodysplastic syndrome acute myeloid leukemia; dnAML, *de novo* acute myeloid leukemia; PV, polycythemia vera; UPD, uniparental disomy; wt, wild type.

3.6. Expression of *JARID2* in patients with chromosome 6 lesions

Furthermore we investigated the expression of *JARID2* in patients with chromosomal aberrations covering the genomic position of the gene. *JARID2* transcript levels were measured using a qPCR TaqMan MGB Gene Expression Assay, in 10 patients with chromosomal aberrations covering *JARID2*, where RNA or cDNA material was available - 4 patients with a UPD, 4 with a deletion and 2 with a gain of chr 6p. As controls we used samples from 11 patients with MPN diagnosis lacking a

chr6p aberration. Diagnosis, detected chromosome 6p aberrations and patient material used for the assay are shown in Table 12.

Table 12. Samples used for *JARID2* gene expression analysis

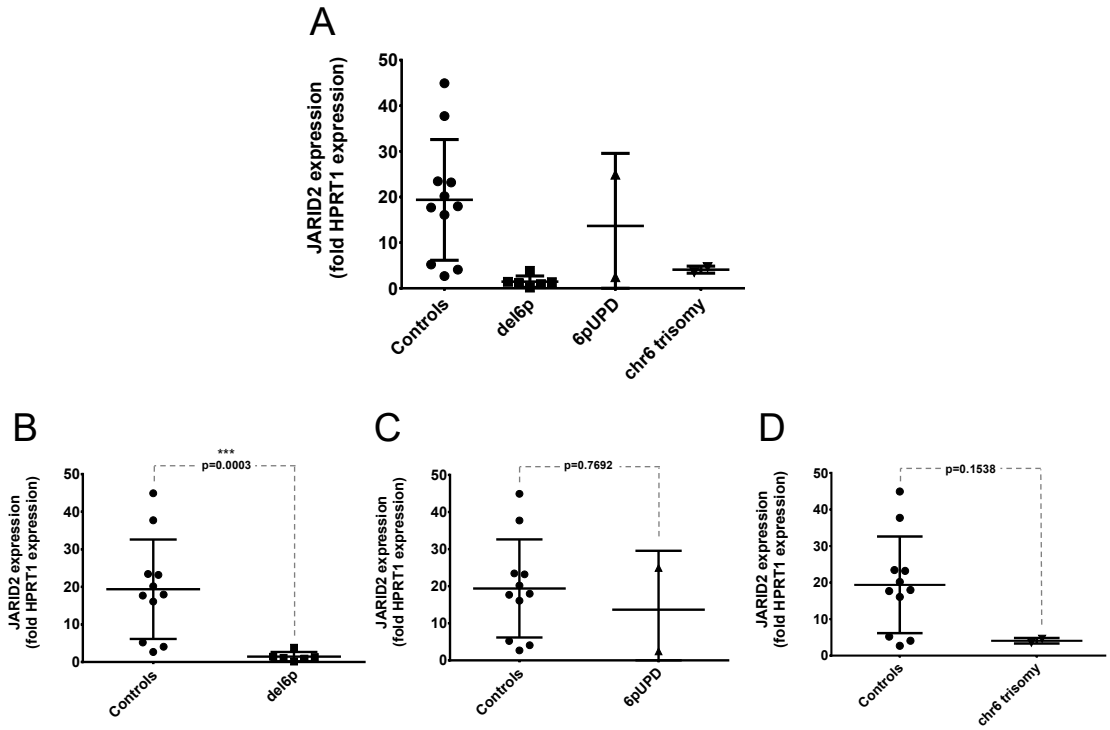
	Patient ID	Diagnosis	Aberration covering JARID2 locus	Material	Tissue
samples	Patient 01	post-MPN AML	trisomy	RNA	whole blood
	Patient 04	PMF	del	RNA	granulocytes
	Patient 05	PMF	UPD	RNA	granulocytes
	Patient 10	PV	UPD	RNA	granulocytes
	Patient 14	acc. phase PV	UPD	RNA	granulocytes
	Patient 17	post-MPN AML	trisomy	RNA	granulocytes
	Patient 19	post-MPN AML	del	RNA	granulocytes
	Patient 20	post-MPN AML	del	RNA	granulocytes
	Patient 21	post-PV AML	del	RNA	granulocytes
	Patient 26	dnAML	UPD	cDNA	mononuclear cells
controls	Patient 101	sMF	no	RNA	granulocytes
	Patient 102	ET	no	RNA	granulocytes
	Patient 103	ET	no	RNA	granulocytes
	Patient 104	ET	no	RNA	granulocytes
	Patient 105	sMF	no	RNA	granulocytes
	Patient 106	sMF	no	RNA	granulocytes
	Patient 107	PV	no	RNA	granulocytes
	Patient 108	PMF	no	RNA	granulocytes
	Patient 109	PV	no	RNA	granulocytes
	Patient 110	PMF	no	RNA	granulocytes
	Patient 111	PMF	no	RNA	granulocytes

UPN, unique patient number; post-MPN AML, post-myeloproliferative neoplasm acute myeloid leukemia; PMF, primary myelofibrosis; PV, polycythemia vera; ET, essential thrombocythemia; acc. Phase PV, accelerated phase polycythemia vera; dnAML, *de novo* acute myeloid leukemia; UPD, uniparental disomy; del, deletion.

Quantitative PCR was performed for all 21 samples in triplicates. Graphical representation of the results is shown in Figure 14, where the calculated delta-delta Cycle Threshold (ddCT) values are shown on the Y-axis, representing relative *JARID2* expression (fold HPRT1 expression). Patients with deletions of the *JARID2* locus show significantly reduced expression of *JARID2* (p=0.0003, Mann-Whitney U=1), with a median relative expression of 1.22 (n=6) compared to a median of 17.96 in the control samples (n=11) (Figure 14B). Expression of *JARID2* in patients with chromosome 6 gain shows a trend into the same direction (Figure 14D), however, the sample number (n=2) might be insufficient to detect formal statistical significance, and a *p*-value of 0.1538 is obtained (median 4.079, Mann-Whitney U=3). In the case of UPD patients a median of 13.67 compared to the control group results in a *p*-value of 0.7692 (Mann-

Whitney U=9), and no meaningful conclusion can be made based on the very big distribution of *JARID2* expression and the sample size of only 2 (Figure 14C).

Figure 14. Relative expression of *JARID2* in patients with chromosome 6 aberrations



A. expression of *JARID2* in patients with deletions, UPD and trisomy of chr6, as well as in control samples. B. expression of *JARID2* in patients with chromosome 6p deletion. C. expression of *JARID2* in patients with chromosome 6p UPD. D. expression of *JARID2* in patients with chromosome 6 trisomy. *P*-values are represented for single patient groups, compared to the control group. Del6p, chromosome 6p deletion; 6pUPD, chromosome 6p uniparental disomy; chr6, chromosome 6.

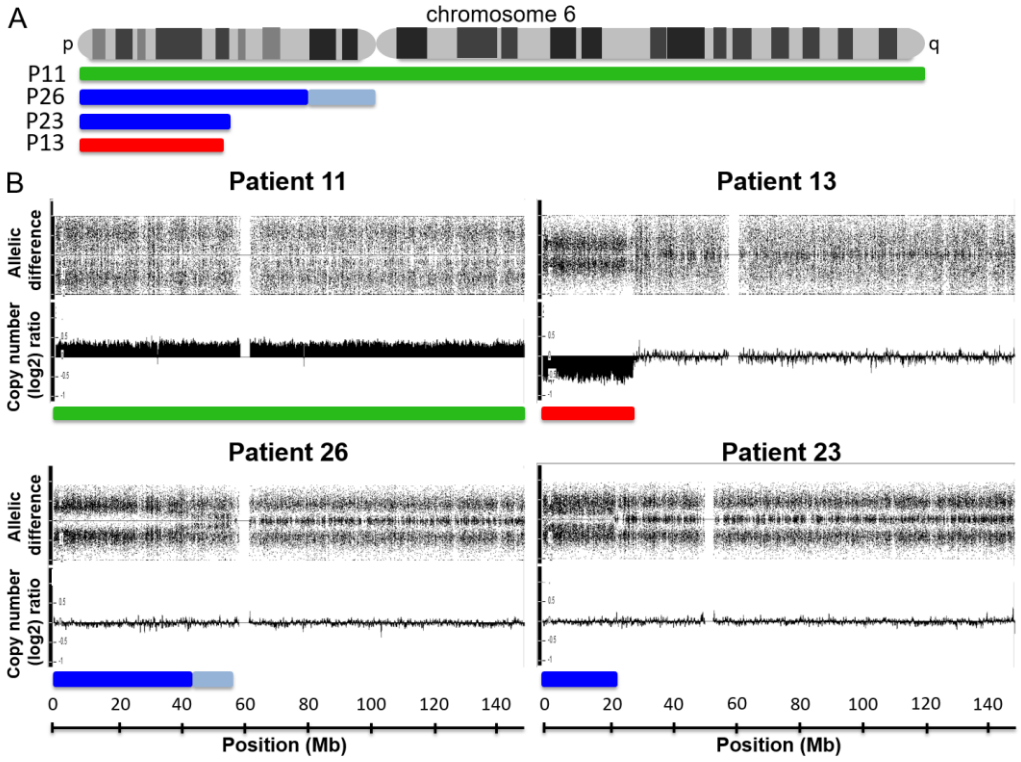
Despite the small sample size available and the big variance in the controls, this expression study reveals that *JARID2* expression is decreased in patients with chromosome 6p aberrations in our cohort, compared to those without such lesions. Therefore, haploinsufficiency instead of mutations of *JARID2* may be the mechanism of dysfunction, leading to decreased expression of the gene and disrupted functioning of the PRC2 complex.

3.7. Whole exome sequencing of patients with aberrations of chromosome 6

As demonstrated, chromosome 6 is affected by chromosomal aberrations in patients with different types of myeloid malignancies, with the frequencies being

highest in AML and advanced stage MPN patients. The deletion profile of patients points to *JARID2* as a target of the aberrations, however the gene is not mutated in patients with deletions, gains or UPDs covering the locus. In search for a potential second gene target of chr 6p aberrations we used whole exome sequencing (WES) of four selected patients. The criteria for patient selection were progression disease stage or AML diagnosis, fully or close to fully clonal chromosome 6 aberration (to allow easier filtering of variants) and availability of DNA. Based on the criteria a dnAML patient (Patient 26) and a post-MDS AML patient (Patient 23) with 6p UPD, as well as a post-MPN AML patient with a deletion (Patient 13) and a sMF patient with chromosome 6 trisomy (Patient 11) were selected for WES (Figure 15).

Figure 15. Aberrations of chromosome 6 in the four patients selected for WES.



A. Positions of the chromosomal lesions in the four patients. The green bar represents a gain, blue bars represent uniparental disomies, and the deletion is depicted by the red bar. B. Copy number and allelic difference ratios of the same four aberrations, presented as SNP 6.0 Array data analyzed using the Genotyping Console 3.0.1. software. P, Patient; Mb, mega base pair.

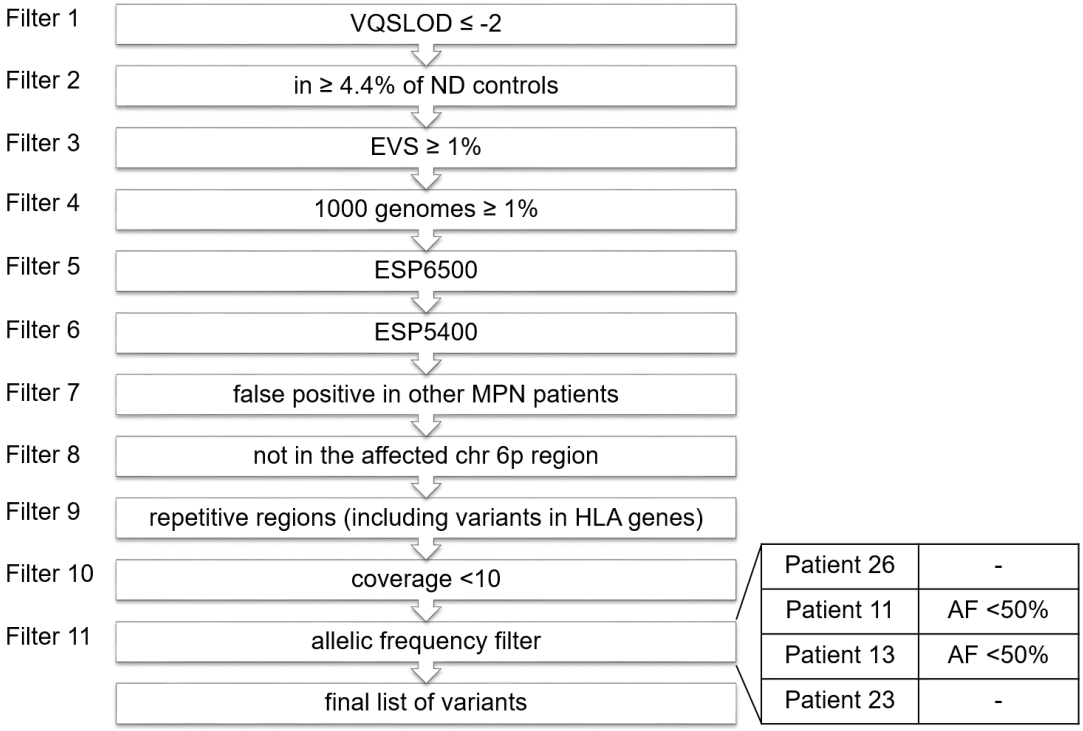
After applying quality filters to the exome sequencing data a median of 189862866 of total reads per sample was obtained, with a median of 164912225 unique reads per sample (86.5%) (Table 13). A median of 87.5% of the unique reads successfully aligned to the reference genome, and only less than 0.4% of the target in each patient was not covered by any read. The median coverage of the target was

124X, with less than 3% of the target being covered less than 2X. Alignment of the reads to the reference genome was followed by variant calling, and the resulting list of variants was filtered for only those positioned in exons and splice sites. The following filtering steps helped to reduce the number of variants for validation by Sanger sequencing (Figure 16, Table 14). As the aim of this study was to identify the targets of the detected chromosomal aberrations one of the filtering steps (Filter 8) aimed in filtering the variant list for only those positioned in the affected region of chromosome 6, defined individually for each of the four patients. Another patient-specific filtering step (Filter 11) based on the allelic frequency of the potential variant discarded all the variants which were located in the affected genomic region but were not targets of the detected aberration. In case of Patient 11, with a trisomy of chromosome 6, all variants with an allelic frequency lower than 50% were not considered. The same threshold was applied in the case of Patient 23, as the UPD in the patient was almost fully clonal and all variants with low allelic frequencies could not have happened before the UPD, and are therefore not potential targets of the aberration. This filter was not applied in the remaining two patients. In the case of Patient 13, the deletion coupled with a mutation which happened afterwards could potentially have meaningful consequences, irrespectively of the allelic frequency. Regarding Patient 26, who had two different UPD events detected, no filtering was applied because it is unknown whether the second UPD happened on the background clone carrying one 6p UPD already, or two different pools of cells exist in the patient, both with a different UPD on chromosome 6, but the overlap of the regions appears as a fully clonal UPD of the shorter affected region, as we detect by the used SNP Arrays. Including the above described filtering criteria, a number of additional filtering steps were performed, resulting in a final list of 24 potential variants in the four examined patients (range 1-13 per patient).

Table 13. Statistics of the whole exome sequencing data

Patient	GENOME SIZE	TOTAL READS	UNIQUE READS	UNIQUE READS (%)	ALIGNED READS	ALIGNED READS (%)	MEAN TARGET COVERAGE	ZERO TARGET COVERAGE (%)	TARGET COVERAGE (%)						
									min 2X	min 10X	min 20X	min 30X	min 40X	min 50X	min 100X
Patient 26	3137454505	229095240	195911805	86	171266023	87	150.6	0.11	97.47	95.31	93.36	91.33	89.06	86.46	67.23
Patient 11	3137454505	168400426	146426963	87	125895418	86	110	0.27	97.17	94.72	92.04	88.87	84.95	80.08	46.27
Patient 13	3137454505	143873606	124448254	86	109750419	88	94.5	0.33	96.93	94.27	91.24	87.38	82.38	76.13	36.99
Patient 23	3137454505	211325306	183397487	87	161681784	88	138.8	0.25	97.38	95.31	93.43	91.38	89.01	86.22	63.83
median	3137454505	189862866	164912225	86.5	143788601	87.5	124.4	0.26	97.27	95.01	92.70	90.10	86.98	83.15	55.05

Figure 16. Filtering steps used for analyzing the whole exome sequencing data



ND, non-disease; MPN, myeloproliferative neoplasm; AF, allelic frequency.

Table 14. Overview of the variant filtering steps applied on the exome sequencing data from the 4 analyzed patients

UPN	Exon/splicing variants	Median coverage	Variants after filtering											Validated variants	Somatic mutations	Germline mutations
			Filter 1	Filter 2	Filter 3	Filter 4	Filter 5	Filter 6	Filter 7	Filter 8	Filter 9	Filter 10	Filter 11			
Patient 26	1960	128	1364	1173	792	574	499	496	493	34	9	9	9	8	N/A	N/A
Patient 11	2014	93	1364	776	633	489	456	456	453	54	20	19	13	13	1	12
Patient 13	2037	83	1393	801	663	545	507	507	505	1	1	1	1	1	0	1
Patient 23	2050	116	1145	564	441	322	293	293	289	5	4	4	1	1	N/A	N/A

UPN, unique patient number; N/A, not available

Except for a 43 bp frameshift insertion in the *TRERF1* gene, at position 64, in Patient 26, all other variants were validated by Sanger sequencing. None of the identified genes were mutated in more than one patient (Table 15 and Figure 17). For two of the patients (Patient 11 and Patient 13) DNA isolated from T cells was available and used as control tissue DNA. The origin of all of the identified mutations in these two patients was investigated, and as a result only one mutation was found to be somatic. Other mutations were present in the control DNA as well, and were therefore described as germline. From Patient 23 and Patient 26 no control DNA was available and consequently the origin of the identified mutations remains unknown.

Table 15. Mutations validated by Sanger sequencing in the 4 analyzed patients

UPN	Gene	Mutation	Chr	Start position	End position	Reference base	Altered base	Origin of mutation	Allelic frequency	SIFT score	PolyPhen-2 score
Patient 26	OR2B3	F40L	6p	29054908	29054908	A	G	N/A	89.8%	1	0.015
	NCR3	R96Q	6p	31557660	31557660	C	T	N/A	87.6%	0.49	0.001
	UHRF1BP1	S506P	6p	34825190	34825190	T	C	N/A	81.5%	0.27	0.005
	DNAH8	I4044T	6p	38917229	38917229	T	C	N/A	29.9%	0	0.571
	APOBEC2	F52L	6p	41029089	41029089	T	C	N/A	78.0%	0	0.996
	C6orf132	L683F	6p	42073603	42073603	G	A	N/A	71.1%	0.19	0.364
	TJAP1	R359Q	6p	43473025	43473025	G	A	N/A	67.1%	0.07	0.602
	CYP39A1	G410R	6p	46554836	46554836	C	T	N/A	59.2%	0	1
Patient 11	RREB1	L1649P	6p	7249083	7249083	T	C	germline	54.9%	1	0
	FAM65B	**L521R**	**6p**	**24843298**	**24843298**	**A**	**C**	**somatic**	**66.2%**	**0**	**1**
	ZNF165	R69H	6p	28053464	28053464	G	A	germline	71.2%	0.05	0.44
	PSORS1C1	P38fs	6p	31106501	31106501	-	C	germline	67.6%		
	ZBTB9	T163A	6p	33423364	33423364	A	G	germline	65.9%	0.48	0.017
	RSPH9	A94V	6p	43618165	43618165	C	T	germline	69.0%	0.27	0
	MRPS18A	R173L	6p	43639572	43639572	C	A	germline	73.9%	0.11	0.011
	KLHL31	S117T	6p	53519722	53519722	A	T	germline	63.8%	0.2	0.371
	ZNF292	R2094Q	6q	87969628	87969628	G	A	germline	67.9%	0.66	0.042
	CLVS2	S178N	6q	123332273	123332273	G	A	germline	65.7%	0.28	0.006
	TMEM200A	R402W	6q	130762771	130762771	C	T	germline	65.5%	0.07	0.999
	STX11	E206K	6q	144508380	144508380	G	A	germline	68.2%	0.03	0.921
	THBS2	P816L	6q	169626366	169626366	G	A	germline	67.1%	0.01	1
Patient 13	C6orf195	M33V	6p	2623960	2623960	T	C	germline	94.3%	1	0
Patient 23	FAM217A	E240Q	6p	4069739	4069739	C	G	N/A	77.0%	0.02	0.971

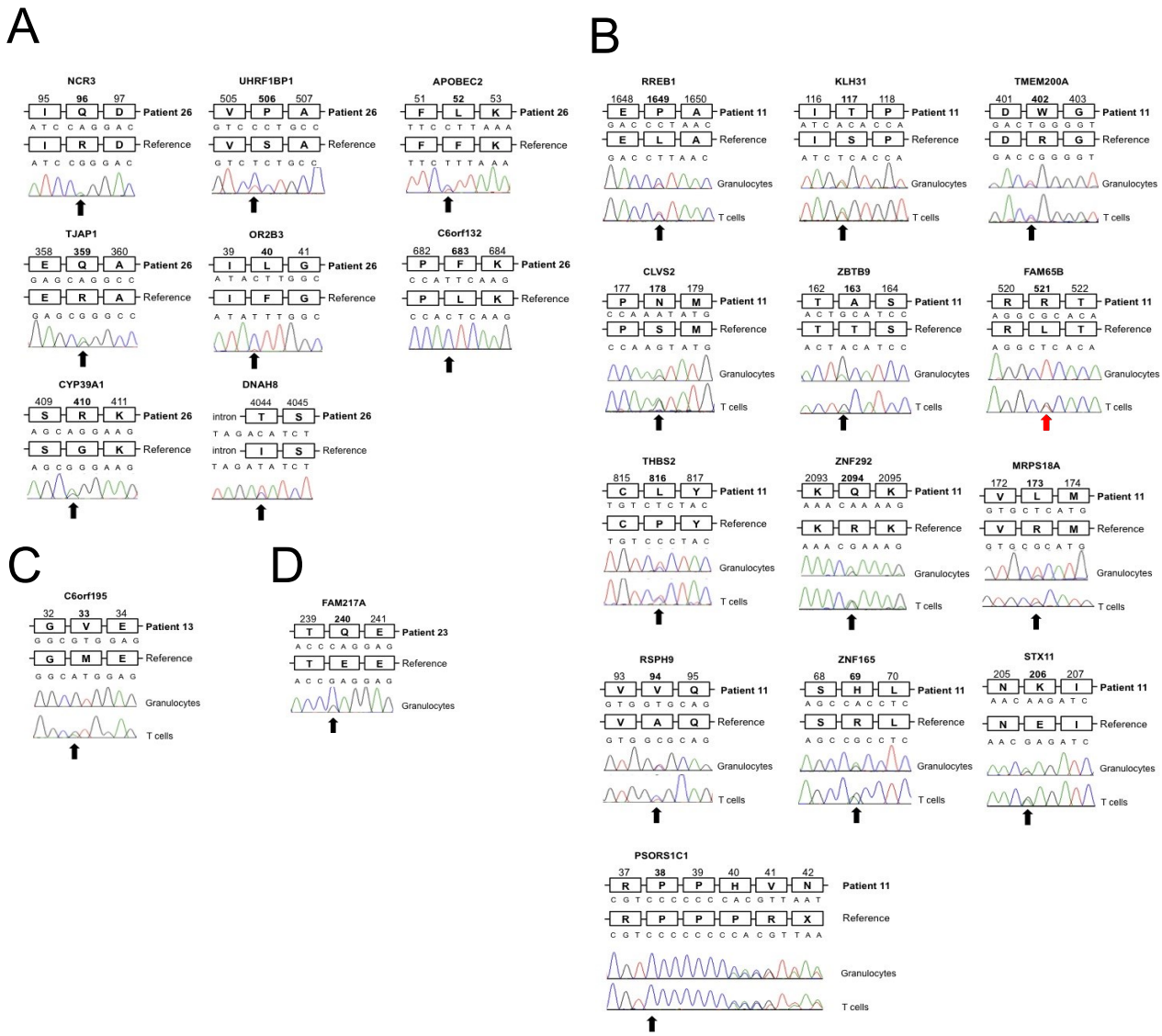
The identified somatic mutation is highlighted in red. The genomic positions are mapped to the hg19 reference genome. The allelic frequency shown in the table is taken from the whole exome sequencing results. UPN, unique patient number; Chr, chromosome; N/A, not available; SIFT, Sorting Intolerant from Tolerant; PolyPhen-2, Polymorphism Phenotyping v2; The SIFT and Polyphen-2 scores were calculated using the Ensembl Variant Effect Predict.

The identified somatic mutation in Patient 11 is a L521R change in the *FAM65B* gene (Family With Sequence Similarity 65, Member B), which was amplified by trisomy of chromosome 6. According to the SIFT and PolyPhen-2 scores as predictions of the functional effect of variants this Leucine to Arginine change is predicted to be probably damaging with the strongest possible score. Other mutations with predicted damaging effect, for which the origin of variant could not be established, are those in genes *DNAH8, APOBEC2, TJAP1, CYP39A1* and *FAM217A* (Table 15).

Taken together, whole exome sequencing of the four selected patients harboring chromosome 6 aberrations did not identify recurrent mutations in any single gene. Variants in multiple genes were identified in the patients, with unknown origin in two out of four patients, and a single somatic mutation in the *FAB65B* gene identified in a sMF patient. This data provides a list of candidate genes located on chromosome 6p with a role in the pathogenesis of myeloid neoplasms. However, mechanisms other than point mutations, such as epigenetic regulation of transcription, might be

responsible for gene disturbances and should be studied in patients with chromosomal lesions of chromosome 6p for a deeper understanding of the complexity of chromosome 6 genetics in hematological malignancies.

Figure 17. Validated mutations in the four analyzed patients



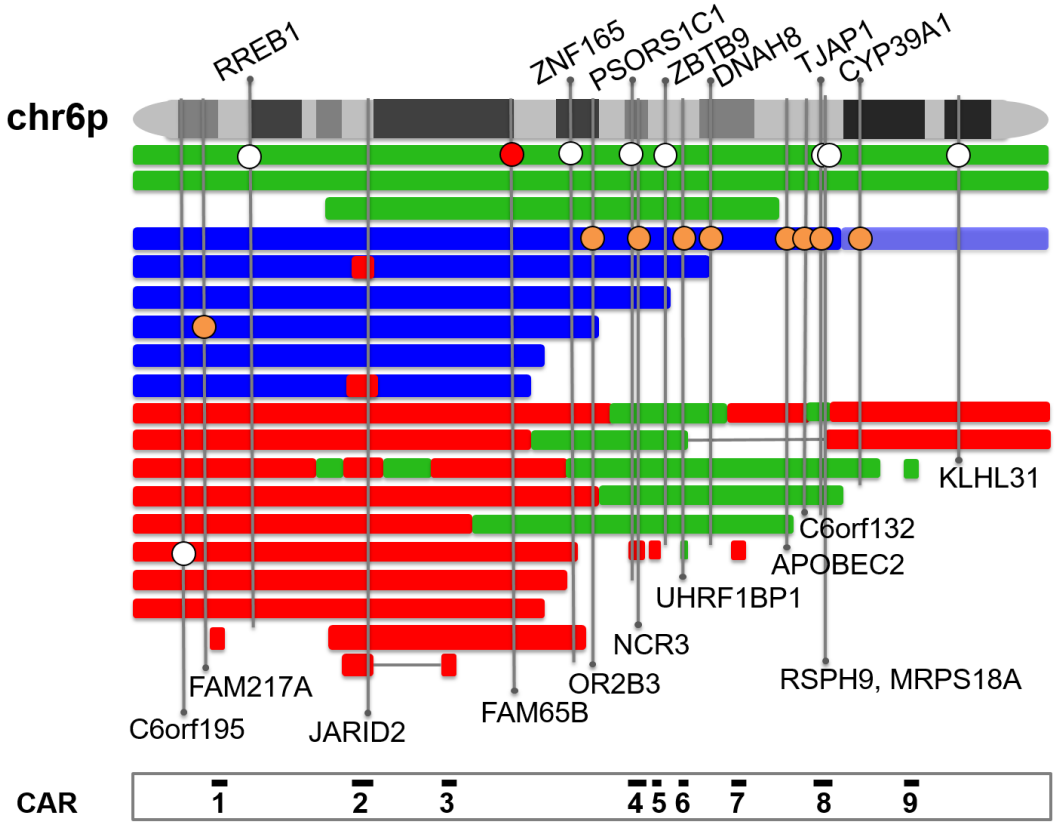
A. Mutations in Patients 26; B. Mutations in patient 11; C. Mutations in Patient 13; D. Mutations in Patient 23. Traces obtained from Sanger sequencing raw data (Sequencher software) are supplemented with DNA and amino acid sequence in the patient and the reference. For Patients 11 and 23 sequencing traces in both tumor tissue DNA (granulocytes) and control tissue DNA (T cells) is shown. The position of the identified somatic mutation is indicated by a red arrow, whereas germline mutations or mutations of unknown origin are depicted with a black arrow.

3.8. Intersection of exome sequencing data with the aberrational profile of chromosome 6p

Exome sequencing of the four patients gave more insight into the mutational profile of genes located in the aberration regions of chromosome 6 and identified several genes which were mutated in the analyzed patients. However, the data did not provide evidence that a single gene is targeted by the chromosomal lesions on chromosome 6 found in our cohort of patients. Therefore, we intersected the data obtained from SNP arrays with the results of the whole exome sequencing in order to map genomic regions or genes which are commonly affected in patients, either by a chromosomal aberration or mutation. The aberration map of chromosome 6p (Figure 13A) was supplemented with names of genes which were found to be mutated in the 4 analyzed patients (Figure 18). Some of the mutated genes in Patient 11 were located on the long arm of chromosome 6, as the patients had a chr 6 trisomy and was investigated for variants across the whole chromosome. These genes were not plotted on the resulting intersecting map as the aim was to identify commonly affected regions on the short chromosome 6 arm.

Focal chromosomal aberrations, which we defined as those smaller than 2Mb in length and found in genomic regions where at least 5 patients with aberrations were identified, were used to define commonly affected regions (CAR). Nine such regions were identified across chromosome 6p. The size of the lesion defining the CAR ranged from 0.31 to 1.87 Mb (mega base pairs) and they contained between 1 and 43 genes. The position of the CARs is graphically presented on the bottom of Figure 18, and the size, genomic position and list of containing genes is shown in Table 16.

Figure 18. Intersection of chromosomal aberrations and mutations detected on chromosome 6p



Green bars represent gains of genomic regions, blue bars uniparental disomies and red bars deletions, all detected in patients using the Affymetrix Genome-Wide Human SNP Array 6.0. Bars connected with a grey line are detected in the same patient. The position of genes is depicted with circles and connected to the gene names with vertical grey lines. Aberrations and mutations plotted on the same bar are found in the same patient. The somatic mutation is presented in red, germline mutations in white and mutations of unknown origin in orange. Commonly affected regions (CAR) are shown on the bottom. The black bar represents the genomic locus defined by the focal lesion. CARs are numbered from 1 to 9.

The intersection of the data identified a number of genes which were found mutated in single patients, but also in regions which are commonly affected by aberrations – *PSORS1C1, NCR3, UHRF1BP1, TJAP1, RSPH9 and MRPS18A*. The origin of the mutations identified in *NCR3, UHRF1B1* and *TJAP1* is unknown, whereas the other two were found to be germline mutations.

Table 16. Commonly affected regions on the short arm of chromosome 6

CAR	Size of CAR (Mb)	Type of aberration defining CAR	Genomic position start (chr6)	Genomic position end (chr6)	Number of genes	List of genes	Number of overlapping aberrations	UPD	del	gain
1	0.31	deletion	5150000	5460000	2	LYRM4, FARS2	18	7	9	2
2	1.06	deletion	14540000	15600000	1	JARID2	20	7	11	2
3	0.87	deletion	19955000	20820000	3	MBOAT1, E2F3, CDKAL1	19	7	10	2
4	0.7	deletion	31200000	31900000	43	PSORS1C1, PSORS1C2, CCHCR1, TCF19, POU5F1, HCG27, HLA-C, HLA-B, MICA, HCP5, MICB, MCCD1, DDX39B, ATP6VaG2, NFKBIL1, LTA, TNF, LTB, LST1, NCR3, AIF1, PRRC2A, BAG5, APOM, C6orf47, GPANK1, CSNK2B, LY6G5B, LY6G5C, ABHD16A, LY6G6E, LY6G6D, LY6G6C, C6orf25, DDAH2, CLIC1, MSH5, SAPCD1, VWA7, VARS, HSPA1L, LSM2, HSPA1A	12	4	1	7
5	1.1	deletion	32200000	33300000	36	FKBPL, PRRT1, PRT2, EGFL8,AGPAT1, RNF5, AGER, PBX2, GPSM3, NOTCH4, C6orf10, HCG23, BTNL2, HLA-DRB5, HLA-DRB6, HLA-DRB1, HLA-DQA1, HLA-DQB1, HLA-DQA2, HLA-DQB2, HLA-DOB, TAP2, PSMB8, TAP1, PSMB9, HLA-DMB, HLA-DMA, BRD2, HLA-DOA, HLA-DPB1, HLA-DPA1, COL11A2, RXRB, SLC39A7, HSD17B8, RING1	12	4	1	7
6	0.17	gain	34850000	35020000	3	UHRF1BP1, TAF11, ANKS1A	10	3	0	7
7	0.6	deletion	38200000	38800000	4	ZFAND3, BTBD9, GLO1, DNAH8	9	2	2	5
8	1.26	gain	42790000	44050000	33	TBCC, GLTSCR1L, PTCRA, CNPY3, GNMT, PEX6, PPP2R5D, KLHDC3, MEA1, CUL7, KLC4, MRPL2, PTK7, SRF, CUL9, DNPH1, TTBK1, SLC22A7, CRIP3, ZNF318, ABCC10,DLK2, TJAP1, LRRC73, YIPF3, POLR1C, XPO5, POLH, GTPBP2, RSPH9, MAD2L1BP, MRPS18A, VEGFA	6	1	0	5
9	0.9	gain	49200000	50100000	12	MUT, CENPQ, GLYATL3, C6orf141, RHAG, CRISP2, CRISP3, PGK2, CRISP1, DEFB133, DEFB114, DEFB110	6	1	2	3

The genomic positions defining the commonly affected regions are mapped to the hg18 reference genome. CAR, commonly affected region; Mb, megabase; UPD, uniparental disomy; del, deletion

On the other hand, CAR 1-3 are located in a region where most of the chromosomal aberrations occur, indicating that genes in these commonly affected regions might potentially be involved in myeloid malignancies. In addition to *FAM65B*, which is detected as a somatic mutation in one patient, and *JARID2* which is homozygously deleted in two patients, some of these genes might have a role in the pathophysiology of the disease but analyzing the frequency of mutations in larger cohorts and investigating the functional effect of the gene disruptions *in vitro* is necessary for elucidating their role and impact in hematological malignancies.

4. DISCUSSION

4.1. General discussion

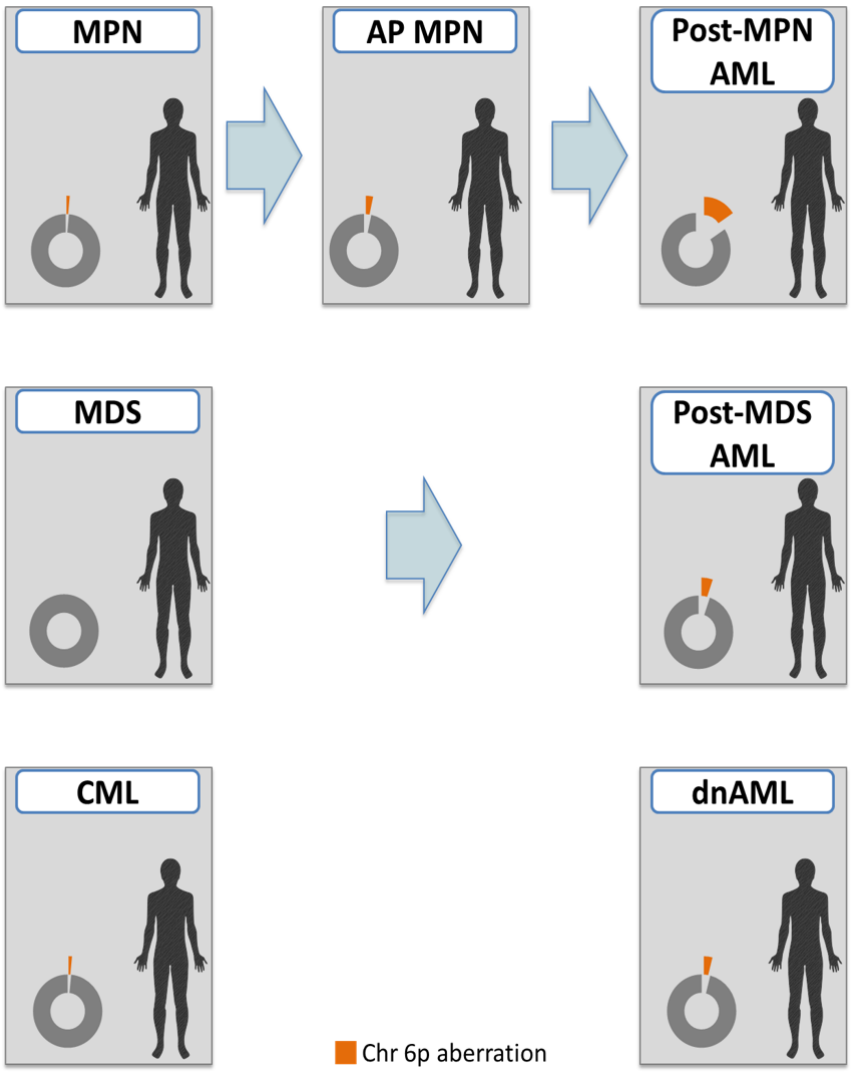
The results published in Manuscript 1 have already been discussed as part of the manuscript. Their interpretation will not be repeated but they will be put into a wider picture of understanding the genetics of myeloid malignancies, and will be discussed in the context of the subsequent results. In addition, a general discussion focusing on the contributions of the results of this thesis to the knowledge in this field, and putting the results in the context of novel finding is elaborated in this chapter.

Chromosome 6p aberrations are recurrent in myeloid malignancies, and associate with leukemic stages of MPN

With the use of high resolution genome wide SNP Arrays we have shown that chromosomal aberrations occur frequently in sAML, by identifying at least one chromosomal aberration in 83.1% of 77 sAML patients, the majority of the events being deletions. Recurrent chromosomal aberrations are detected in the studied cohort, among others large aberrations of chromosomes 1, 5q, 6, 7, 8, 9p, 11q, 17p and 21, as well as smaller aberrations mapping to single genes already associated with myeloid malignancies, including those on chromosomes 4q (*TET2*), 7p (*IKZF1*), 7q (*CUX1*) and 12p (*ETV6*) (Figure 1 from Manuscript in Chapter 3.1) (Delhommeau et al., 2009, Klampfl et al., 2011). In addition to the known tumor suppressors targeted by focal deletions, we report *JARID2* as a putative novel tumor suppressor, a gene positioned on the short arm of chromosome 6 at p23-p22.3. Assessing the frequencies of chromosome 6p abnormalities, including deletions, uniparental disomies and gains, in a large pan-myeloid cohort of 905 patients, we discovered low rates in chronic phase MPN (1.5%), CML (1.6%) and MDS (0%), increased rates in accelerated phase MPN (3.4%), dnAML (4%) and post-MDS AML (5.3%), with highest frequencies in post-MPN AML (15.6%). The combined frequency off chromosome 6p aberrations in sAML is 10.6%, however we have detected statistically significant enrichment of chr 6p aberrations in leukemic transformation of MPN but not MDS. This strong association implicates a role of genes located on chromosome 6p in the progression of MPN to post-MPN AML.

Following these findings this thesis has focused on two specific topics which will further be discussed – the deletion profile of *JARID2* and other PRC2 member encoding genes in relation to disease progression in MPN and MDS; and the mutational profile of genes located on chromosome 6 in patients with chromosomal aberrations of the locus, in a pan-myeloid cohort of patients, in search for other potential gene targets of the recurrent genetic lesions.

Figure 19. Presence of chromosome 6p aberrations during disease progression of MPN and MDS and in different myeloid neoplasms



Chromosome 6p aberrations were detected using Affymetrix Genome-Wide Human SNP Arrays 6.0 in our pan-myeloid cohort of 905 patients. Frequencies are calculated for each disease entity and represented as cut-outs from the circle, depicted in orange. MPN: 1.5%; AP MPN: 3.4%; post-MPN AML: 15.6%; MDS: 0%; post-MDS AML: 5.3%; dnAML: 4%; CML: 1.6%. MPN, myeloproliferative neoplasm; AP, accelerated phase; AML, acute myeloid leukemia; dnAML, *de novo* acute myeloid leukemia; CML, chronic myeloid leukemia

Deletions of genes encoding PRC2 members are frequent in sAML and dnAML

The Polycomb Repressive Complex 2 (PRC2) is one of the two polycomb repressive complexes in mammals, together with PRC1. Through regulation of chromatin modifications PRC2 represses target genes during different cellular processes, including differentation. The core of the complex consists of Ezh2, Suz12 and Eed, all of which are required for efficient PRC2 activity. Ezh2 is a methyltransferase responsible for thrimethylation of Lysine 27 of Histone H3 through its catalytic SET domain, leaving repressive H3K27me3 marks at promotor sites of target genes, which results in gene silencing. The activity of Ezh2 is partially compensated by Ezh1 upon Ezh2 loss (Aoyama, Oshima et al., 2018, Margueron & Reinberg, 2011). Both Ezh2 and Ezh1 contain a SRM domain (stimulatory responsive domain) which differ in one residue among each other, causing allosteric activation, with stronger activation seen in PRC2-Ezh2 compared to PRC2-Ezh1 (Lee, Yu et al., 2018a). Suz12 has a role in maintaining structural stability of the complex through binding to both Ezh2 and Eed, but also to Rbbp4, Jarid2 and Aebp2 (Kasinath et al., 2018). The complex consists of two lobes – the top catalytic lobe formed by Ezh2, Eed and the VEFS domain of Suz12, and the bottom lobe containing Jarid2, Aebp2, Rbbp4 and the remaining part of Suz12. A regulation loop between PRC2 and Jarid2 exists, with Jarid2 being a substrate of the PRC2 complex, which in turn, when methylated activates the complex to methylate H3K27 at other gene loci (Sanulli, Justin et al., 2015). The region of Jarid2 containing the K116 residue contributes to the formation of the active conformation of the complex, through binding to Eed and Ezh2 (Kasinath et al., 2018). Aebp2 binds to Rbbp4 and recruits PRC2 to DNA methylated sites and stimulates PRC2 binding to histones in both PRC2-Ezh2 and PRC2-Ezh1 complexes (Lee et al., 2018a, Sun, Li et al., 2018, Wang, Paucek et al., 2017). Jarid2 and Aebp2 in synergy regulate PRC2 activity, both inducing its enzymatic activity with their loss resulting in reduced stability of the complex (Kasinath et al., 2018, Lee et al., 2018a, Wang et al., 2017). In addition to its main role in regulating PRC2 activity, Jarid2 directly binds to H2AK119u1 histone marks, which are a result of PRC1 activity, interconnecting the functions of PRC1 and PRC2 complexes in the regulation of target gene expression (Cooper, Grijzenhout et al., 2016).

The research conducted in this thesis identifies *JARID2* as one of the possible tumor suppressors involved in MPN disease evolution. We found the gene homozygously deleted in two patients, both in progressive phases of MPN. In both cases a focal deletion of the *JARID2* locus was coupled with a uniparental disomy, resulting in a full loss of the *JARID2* gene. In another study a homozygous *JARID2*

deletion was identified in a sMF patient with mutations of *IDH2* and *SF3B1*, which later transformed to AML (Rampal et al., 2014). No association of chromosome 6p aberrations and *JARID2* mutations has been observed in our study, independent of disease stage and disease entity. Other studies screening for *JARID2* mutations in myeloid malignancies revealed no mutations or found mutations at very low frequencies (2%) in chronic phase MDS and MPN (Khan et al., 2013, Score, Hidalgo-Curtis et al., 2012). A recent study of MPN exploiting RNA sequencing as a tool for variant identification has discovered *JARID2* mutations in 3.8% of the 104 analyzed patients (Schischlik, Jager et al., 2019). A study of PMF with prior exposure to ionizing radiation (IR) identified 2 *JARID2* mutations in 13 analyzed 17 patients exposed to IR and 1 in unexposed PMF patients (Poluben et al., 2019). Overall, our results give evidence that mutations of *JARID2* occur unfrequently, whereas deletions of the JARID2 containing locus occur with higher frequencies and are enriched in the leukemic phase compared to chronic phase of myeloid malignancies (in 7% vs. 0.3%, respectively). The majority of detected deletions are hemizygous, therefore haploinsufficiency of the gene might be the mechanism of action involved in disease progression, supported by our findings that *JARID2* expression is lower in patients with deletions compared to controls. Recently, an extensive functional study of *JARID2* knockout in mouse models and patient derived xenografts showed that *JARID2* loss results in de-repression of self-renewal in multipotent myeloid progenitor cells, upregulating genes characteristic for HSC, such as *RUNX1T1* and *MYCN*, as well as the *HOXA* genes. *JARID2*-KO mice develop a MPD/MPN-like phenotype with longer latency period in heterozygous *JARID2* mice (Celik, Koh et al., 2018).

PRC2 haploinsufficiency in disease progression

Deletions of genes encoding PRC2 complex members other than *JARID2* were also observed frequently in our study. With a crucial role of both core members and the co-factors of PRC2 for its enzymatic activity, we can hypothesize that haploinsuffuciency of any of the genes may contribute to reduced activity of the complex. Our data shows that deletions of PRC2 member encoding genes *EZH2, EED, SUZ12, EZH1, JARID2* and *AEBP2* occur across different myeloid malignancies. Uniparental disomies covering PRC2 member genes also occur in hematologic malignancies involving the myeloid lineage, as shown in Manuscript 1, but are not associated with disease progression, neither are they coupled with mutations of the containing genes encoding PRC2 members. Overall, 6.6% of patients from the analyzed pan-myeloid cohort had deletions of at least 1 PRC2 complex member detected. The frequencies differed greatly between different disease entities with an

overrepresentation in leukemic compared to chronic stages of myeloid neoplasm. In chronic phase MDS no deletions were detected affecting PRC2 members, with a strong enrichment in post-MDS AML (22.5%) (*P*=0.0007). The same trend was observed in MPN (1%) and post-MPN (40%) (*P*=0.006). Our data shows that deletions covering *EZH2* are significantly associated with leukemic transformation of both MDS (*P*=0.0021) and MPN (*P*=0.006), whereas *JARID2* and *SUZ12* containing deletions are specific for the leukemic transformation of MPN (P=0.006). Taken together, the results of this thesis demonstrate that deletions of genes encoding PRC2 subunits are frequently seen in secondary AML and implicate that PRC2 inactivation may have an important role in disease progression rather than in disease initiation. PRC2 deletion rates were also high in dnAML (16.2%) but much lower in sMF (3.4%) and CML (3.3%). However, the size of the deletions should be kept in mind when interpreting the results of our study as some of the deletions are long, affecting many genes, sometimes the whole chromosome, with only 11 focal deletions (<2Mb) targeting PRC2 members identified in our cohort. In addition to hemizygous deletions of *AEBP2*, which were detected exclusively in sAML in our cohort, we have identified a focal homozygous deletion in a post-MDS AML patient. Focal deletions of *AEBP2* were also reported in pediatric AML, as well as mutations of the gene in pediatric ALL and AML, indicating that loss of PRC2 co-factors may contribute to leukemic development not only in adult- but also in childhood leukemias (Huether, Dong et al., 2014, Olsson, Zettermark et al., 2016).

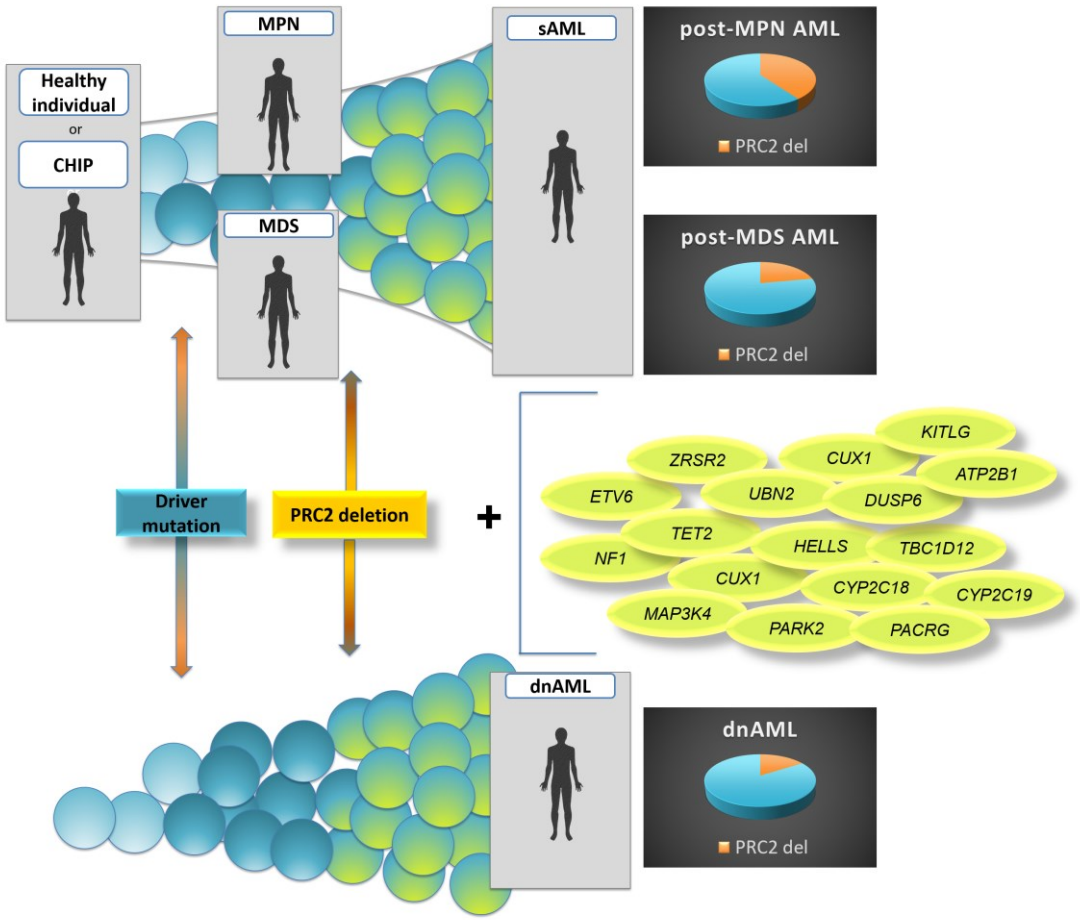
The loss of PRC2 subunits likely cooperate with each other or with other genes to promote disease evolution

Deletions of PRC2 member genes are most often larger deletion covering a various number of additional genes. Therefore, the possibility of synergy in disease progression cannot be excluded and should be further functionally investigated in order to better understand consequences of codeletions of epigenetic modifiers and other genes in the hematopoietic system. Here we report frequent codeletions of neighboring genes, *SUZ12* and *NF1* on chromosome 17q and *AEBP2* and *ETV6* on chromosome 12p. The *NF1* gene encodes a GTPase-activating protein which activates Ras and its downstream signaling pathway (Cichowski & Jacks, 2001). The loss of *NF1* in mice induces MPN and *NF1* loss cooperates with the inactivation of other genes, such as *ICSBP* and *KRAS*, in the progression of MPN and the development of AML (Cutts, Sjogren et al., 2009, Koenigsmann, Rudolph et al., 2009, Largaespada, Brannan et al., 1996, Le, Kong et al., 2004, Sachs, Been et al., 2016). Mutations of *NF1* are frequently found in neurofibromatosis type 1, a benign disease which in some cases

progresses to malignant peripheral nerve sheath tumor (MPNST), but also predisposes to the development of different tumor types including juvenile myelomonocytic leukemia (JMML), a MPN/MDS overlap disease (Cichowski & Jacks, 2001, Loh, 2011). Recent studies of neurofibromatosis type1 have associated PRC2 inactivation on a *NF1*-mutant background with disease progression to MPNST or the development of JMML (Caye, Strullu et al., 2015, De Raedt, Beert et al., 2014, Lee, Teckie et al., 2014, Suresh, Kliot et al., 2016, Zhang, Wang et al., 2014). In one study more than 60% of *NF1*-mutated JMML harbor at least one inactivated PRC2 member, often resulting from a deletion secondary to the *NF1* mutation, leading to haploinsufficiency of the affected gene (Caye et al., 2015). In line with the results of this thesis, showing frequent co-deletions of *NF1* and other PRC2 members, particularly its neighboring gene *SUZ12*, a similar synergy of *NF1* and PRC2 loss may lead to disease progression of myeloid neoplasms. In addition, we have detected *ETV6* deletions co-occuring with deletions of various PRC2 members. The Etv6 protein belongs to the ETS family of transcription factors, binds to DNA and acts as a transcriptional repressor, with a role in regulating HSC survival and megakaryocytic differentiation (Hock, Meade et al., 2004, Hollenhorst, McIntosh et al., 2011). Chromosomal translocations resulting in fusion genes involving the *ETV6* gene often occur in hematological malignancies, including MPN, MDS, AML and ALL, with a variety of different fusion partners of *ETV6* identified, most of which fall into functional groups of receptor tyrosine kinases, non-receptor tyrosine kinases, transcription factors or homeobox proteins (De Braekeleer, Douet-Guilbert et al., 2012). Focal deletions of chr12p covering the *ETV6* gene have previously been identified in hematological malignancies but the rate of co-deletion with *AEBP2* and other PRC2 genes has first been investigated in our study (Baens, Wlodarska et al., 1999, Feurstein, Rucker et al., 2014, Sato, Suto et al., 1995). In our cohort we identified 21 deletions covering the locus of the *ETV6* gene. More than 70% of *ETV6* deleted patients had a PRC2 member gene co-deleted, the majority of which presented with *AEBP2* loss, but *SUZ12* or *JARID2* loss was also simultaneously with *ETV6* loss identified in our cohort. *ETV6* mutations also occur in a variety of hematological malignancies, at low frequencies, and have been associated with shorter overall survival in MDS (Barjesteh van Waalwijk van Doorn-Khosrovani, Spensberger et al., 2005, Bejar et al., 2011, Wang, Dong et al., 2014). In MDS *EZH2* and *ETV6* mutations were identified in the same patient, providing more evidence of the impairment of both PRC2 and Etv6 functions in patients (Bejar et al., 2011). To which extent and in which way the simultaneous loss affects disease initiation and evolution is unknown. In our study all double deletions of *ETV6* and PRC2 genes were present in AML (both sAML

and dnAML), suggesting a synergetic role of double haploinsufficiency preferably in leukemic transformation than in disease initiation.

Figure 20. Clonal evolution and PRC2 deletions in the progression of chronic myeloid neoplasms



Chronic myeloid malignancies (MPN and MDS) arise upon a mutation in one of the known driver genes, in some cases subsequent to a previous CHIP phase. Inactivation of the PRC2 complex through deletions of the encoding genes might have a role in leukemic transformation as deletions occur in 40% and 22.5% of post-MPN AML and post-MDS AML, respectively, as presented by pie charts. PRC2 deletions may cooperate with deletions of other genes in the disease progression. The genes detected codeleted with PRC2 subunits in our cohort are represented in the figure.

We were able to demonstrate that deletions of PRC2 members are not mutually exclusive, with co-deletions of PRC2 members being significantly enriched in leukemic compared to chronic phase myeloid malignancies, providing insight into a collaborative

effect of multiple deletions in disease progression. Single cases from other studies also describe a complex pattern of acquisition of PRC2 mutations and deletions in MPN disease progression, as described in a post-ET MF patient with a complex karyotype, including deletions of *TET2*, *EZH2*, *AEBP2*, *NF1* and *SUZ12*, in addition to a *SUZ12* mutation and a *TET2* mutation (Brecqueville, Cervera et al., 2011). The only PRC2 complex member showing mutual exclusivity of deletions with other complex members in our study was *EED*. This might be random due to a low frequency and low number of observed *EED* deletions, but a synthetic lethality of *EED* with other related genes may also be the explanation of this observation with further studies needed to understand this relation in more detail.

In addition to multiple PRC2 deletions, the loss of PRC2 members co-occurs with deletions or mutations of other genes. In the three patients from our cohort with homozygous deletions of PRC2 cofactors *JARID2* or *AEBP2* deletion profiling detected simultaneous deletions of genes recurrently seen mutated in myeloid neoplasms, such as *ZRSR2, CUX1* and *TET2* (Delhommeau et al., 2009, Lindsley et al., 2015, McNerney et al., 2013, Ogawa, 2019, Thol et al., 2012), as well as deletions of other genes with potential roles in disease progression such as *UBN2, HELLS* and *TBC1D12*. The disruption of the spliceosome and epigenetic machineries, as presented here, co-occur in patients. Expression of *EZH2*, the catalytic unit of the PRC2 complex, is shown to be reduced in patients with spliceosome mutations (*U2AF1* and *SRSF2*), with an effect on H3K27me3 levels as in cases with *EZH2* mutations or haploinsufficiency (Khan et al., 2013). Mutations of both EZH2 and spliceosome genes were also found in MDS/MPN. *ZRSR2* and *SRSF2* mutations were detected in 20% and 15% of *EZH2*-mutant MDS/MPN, respectively, as well as *U2AF1* in 2% of *EZH2*-mutant MDS/MPN (Rinke, Muller et al., 2017). As can be the case in Patient 10 harboring both *JARID2* and *ZRSR2* deletions, loss of both a PRC2 member and a spliceosome component might have synergetic mechanistic functions in leukemic transformation, where single lesions may not be enough for disease progression, with a milder effect in the regulation of proliferation and differentiation. *TBC1D12* is a binding partner of Rab11, which is a Ca^{2+}-dependent GTPase involved in formation and secretion of exosomes from the plasma membrane during erythroid maturation (Fader, Savina et al., 2005, Oguchi, Noguchi et al., 2017, Savina, Furlan et al., 2003). However, up to date there is no association of this gene with myeloid malignancies. Recurrent mutations in the promoter of *TBC1D12* have been reported in breast cancer but not in other cancers, including MDS and AML (Rheinbay, Parasuraman et al., 2017, Son, Kim et al., 2019). *HELLS* is a epigenetic regulator expressed in hematopoietic progenitor cells, involved in transcription regulation of a large number of target genes, including *MLL1* and *KRAS* (Myant & Stancheva, 2008,

Prasad, Ronnerblad et al., 2014, von Eyss, Maaskola et al., 2012). Hells interacts with the transcription factor E2f3, as well as with the DNA methyltransferase Dnmt3b, which in synergy with Dnmt3a regulates hematopoietic stem cell self-renewal and differentiation and when overexpressed is associated with poor prognosis in AML (Challen, Sun et al., 2014, Hayette, Thomas et al., 2012, Schulze, Rohde et al., 2016). Ubn2 is part of the HIRA histone chaperone complex and has a role in epigenetic regulation of gene transcription by actively taking part in nucleosome assembly (Xiong, Wen et al., 2018). Recently, it has been shown that Ubn2 regulates the transcription of *KRAS*, activating the downstream Ras/MAPK signaling pathway (Zhao, Zhong et al., 2019). Giving strong evidence that these genes are deleted in single patients from our cohort, and hypothesizing that homozygous loss of PRC2 cofactors in combination with the loss of other epigenetic regulators or other genes may have collaborative effects in disease pathogenesis, further investigation in cell lines would be required for elucidating the effect of multiple gene inactivation.

PRC2 loss also cooperates with the MPN driver mutation *JAK2*V617F in the pathogenesis of MPN. Both cases of *JARID2* homozygous deletions in patients in our study co-occurred with *JAK2*V617F mutations, amplified by 9pUPD. Mice with co-occurring *JAK2V617* mutation and Jarid2 deletion developed a MPN phenotype with reduced median survival compared to *JAK2*V617F-only mutant mice, with a dose-dependent effect of *JARID2* loss. The reduction of median survival is milder in mice with heterozygous than in those homozygous *JARID2* loss. *JARID2* loss on a *JAK2*V617F background causes stronger JAK-STAT activation and stimulates faster disease development (Celik et al., 2018). The cooperation of *JAK2*V617F is seen with *EZH2* as well. These 2 genes cooperate in accelerating the development of a MPN-like phenotype, resulting in shortened survival (Sashida et al., 2016, Shimizu et al., 2016, Yang et al., 2016). A skewage of hematopoietic differentiation to megakaryopoiesis occurs, as well as an increase of the HSC and progenitor cell pool. The reduction of H3K27me3 marks at promotor regions of target genes results in overexpression of several genes, including Hmag2, S100a8, S100a9 and Ifi27l2a (Sashida et al., 2016, Shimizu et al., 2016, Yang et al., 2016). *EZH2* loss also cooperates with the loss of *TET2* or *RUNX1* mutations to induce a MDS or MDS/MPN phenotype in mice, with more severe clinical features and reduced overall survival compared to mice with retained *EZH2* (Muto, Sashida et al., 2013, Sashida et al., 2016). The order of mutation acquisition seems to also play a role in PRC2-dependant leukemogenesis, as demonstrated in a study in which *JARID2* knockout mice on a *IDH2*-mutant background developed leukocytosis, splenomegaly and an AML phenotype, but only when *IDH2* mutations occurred prior to *JARID2* loss (Celik et al., 2018).

Point mutations of PRC2 members

Somatic mutations of genes encoding the PRC2 subunits, although rare, occur in myeloid malignancies. The most frequently mutated of them is *EZH2* with mutations occurring in 3% of MPN, more frequently in PMF than in PV and ET (Lundberg et al., 2014). The reported frequencies of *EZH2* mutations, which most often affect the catalytic SET domain, are 10%-13% in PMF, 5% in sMF, 2.5%-6% in MDS, 8%-12% in MDS/MPN and 2% in AML (Ernst, Chase et al., 2010, Haferlach et al., 2014, Khan et al., 2013). In our research group, in a cohort of 40 patients for which exome sequencing was performed for other purposes (data not shown, see Manuscript 1, Chapter 3.1), we detected a *EZH2* R249X nonsense mutation in a PMF patient, which results in a truncated protein lacking the SET domain. In addition to this *EZH2* mutation we detected another 5 mutations of PRC2 subunits - two *EZH1* mutations (*EZH1* P78T in ET and *EZH1* C696F in PMF), a *SUZ12* T598P mutation in sMF, *EED* R302S in sMF and a *PHF19* T225P mutation in sMF. The *EZH1* C696F mutation affects the catalytic SET domain of Ezh1, the *SUZ12* T598P is located in the VEFS domain of Suz12, while the PHD domain of PHF19 is affected by the *PHF19* T225P mutation. The mutations we detected were found in higher numbers of sMF than chronic phase patients, but with the numbers being too small we cannot draw any conclusions on their frequencies. Except for the *SUZ12* somatic mutation we were not able to determine the origin of other mutations as control DNA from patients was not available. *EZH2* mutations most often co-occur with *TET2, ASXL1, RUNX1 DNMT3A, JAK2, NRAS, KRAS* and *IDH1/2* mutations (Haferlach et al., 2014, Khan et al., 2013, Rinke et al., 2017). Reduced *EZH2* expression, followed by a reduction of H3K27me3 repressive marks can be a result of mutations, haploinsufficiency (del 7q) or spliceosome mutations (Khan et al., 2013). *SUZ12* mutations are not as frequent and occur with or without an additional deletion of the genetic locus (Brecqueville et al., 2011). The hotspot of *SUZ12* mutations targets the VEFS-box domain of the protein which binds to both Ezh2 and Eed and stabilizes the active conformation of PRC2 (Jiao & Liu, 2015). The somatic mutation identified in our study affected the VEFS domain and was coupled with a chromosome 17q UPD in a sMF patient (Manuscript 1, Chapter 3.1) *SUZ12* and *NF1* are reported co-mutated in a MDS/MPN patient, implicating that despite the higher frequency of deletions as a mechanisms of *SUZ12* and *NF1* inactivation, double mutations also occur at very low frequencies (Score et al., 2012). *NF1* mutations, uncoupled with 17q del or SUZ12 mutations, are also found in MPN (Brecqueville et al., 2011). Nonsense and missense *EED* mutations are rare events,

identified in 1-3% of MDS and MDS/MPN (Score et al., 2012, Ueda, Sanada et al., 2012). Both *SUZ12* and *EED* mutations result in a decrease of H3K27me3 marks, and may co-occur in patients (Khan et al., 2013, Score et al., 2012). Mice with a heterozygous loss of *EED* develop a MPN phenotype (Lessard, Schumacher et al., 1999). As shown in our study the presence of chromosome 11q UPDs is significantly enriched in leukemic vs. chronic phase MPN, but not associated with EED mutations. A study conducted by our group showed that chromosome 11q UPD is a recurrent aberrations in myeloid malignancies and often, but not exclusively, associates with CBL mutations (Klampfl et al., 2013b). We have also demonstrated that other UPDs detected in our cohort, other than the chromosome 17q mutation previously described, were not coupled with mutations of PRC2 members (Manuscript 1, Chapter 3.1).

Overall, genes encoding polycomb repsessive complex 2 subunits are infrequently mutated in myeloid malignancies, with the exception of *EZH2*, and are targeted by deletions to a much higher extent, with an association of deletions with advanced and leukemic stages of myeloid neoplasms.

The complexity of chromosome 6 genetics in myeloid malignancies

Exome sequencing of selected patients performed within the scope of this thesis has confirmed our previous findings that point mutations of *JARID2* are not present in the investigated patients with UPD, gain or deletion of chromosome 6. Even though *JARID2* seems to be a target of a number of the aberrations, other genes are potentially affected in other patients. Our results provide a list of genes in which variants were detected, coupled with a chromosomal aberration. In addition, a list of commonly affected chr 6p regions was assembled using an integrative approach of aberration mapping combined with variant mapping. *FAM65B* is the single gene found somatically mutated in our study, identified in a sMF patient with trisomy of chromosome 6. The majority of chromosomal aberrations detected on chromosome 6p covered the region of *FAM65B*. The identified mutation caused a leucine to arginine amino acid change at position 521, and was amplified by chromosomal gain. The structural and functional domains of the protein are not well characterized and limited amount of research indicates some of its functions. Fam65b acts as a RhoA (Ras homologue family member A) inhibitor, with a known role in neutrophil polarization and in proliferation and migration of T-lymphocytes (Froehlich, Versapuech et al., 2016, Gao, Tang et al., 2015, Megrelis, El Ghoul et al., 2018, Rougerie, Largeteau et al., 2013). Fam65b directly binds to RhoA through its RHOA-binding motif (Gao et al., 2015). RhoA is a Rho GTPase, an activator of ROCK (Rho-associated coiled coil-containing protein kinases), which leads to downstream phosphorylation and activation

of its various targets, with Jak2 being one of them (Haga & Ridley, 2016, Huang, Kong et al., 2012). This might link the function of Fam65b to the JAK/STAT signaling pathway, responsible for the pathogenesis of MPN in the majority of patients. Oncogenic *RHOA* mutations also frequently occur in T-cell lymphomas (Palomero, Couronne et al., 2014, Sakata-Yanagimoto, Enami et al., 2014, Vallois, Dobay et al., 2016, Yoo, Sung et al., 2014). Its overexpression is observed in breast cancer, correlating with the malignancy grade and proliferation state of the cancer, while inactivation of *RHOA* has been described in colorectal cancer (Chan, Lee et al., 2010, Dopeso, Rodrigues et al., 2018, Fritz, Brachetti et al., 2002). Constitutive activation of the RhoA/ROCK signaling cascade regulates Stat5-mediated transcription, and is involved in MPN development in vivo in cooperation with oncogenic mutations in KIT, FLT3 and BCR-ABL (Ling & Lobie, 2004, Mali, Ramdas et al., 2011). RhoA and ROCK have an important role in megakaryocytic development and platelet production, through regulation of endomitosis (Avanzi, Goldberg et al., 2014, Suzuki, Shin et al., 2013). This knowledge of the function of Fam65b and its interacting partners and our findings of a somatic mutation in a sMF patient as a novel gene potentially involved in MPN disease evolution suggest that prospective studies of the mutational rate of this gene in a cohort of MPN, MDS and AML patients might lead to significant discoveries relevant for understanding the underlying mechanisms of disease evolution. Other variants with predicted damaging effects were found in *DNAH8, APOBEC2, TJAP1, CYP39A1* and *FAM217A*. However, due to lack of control DNA the origin of mutations could not be delineated. Follow up studies could reveal more about the frequency of mutations in larger cohorts. Of note, as part of clonal hematopoiesis prior to any malignancy recent studies discovered somatic mutations in multipotent HSC which may give rise to both myeloid and lymphoid cells. Therefore, the use of T-cell DNA as control tissue might be misleading in cases where unrecognized clonal hematopoiesis preceded the myeloid disease. With this kept in mind the origin of variants detected in exome sequencing studies might be reconsidered and should potentially be confirmed with the use of a different tissue as control, when possible. Therefore, in a wider picture all genes found mutated in our study, somatic and germline, should be kept in sight as potential targets of chromosome 6 mutations.

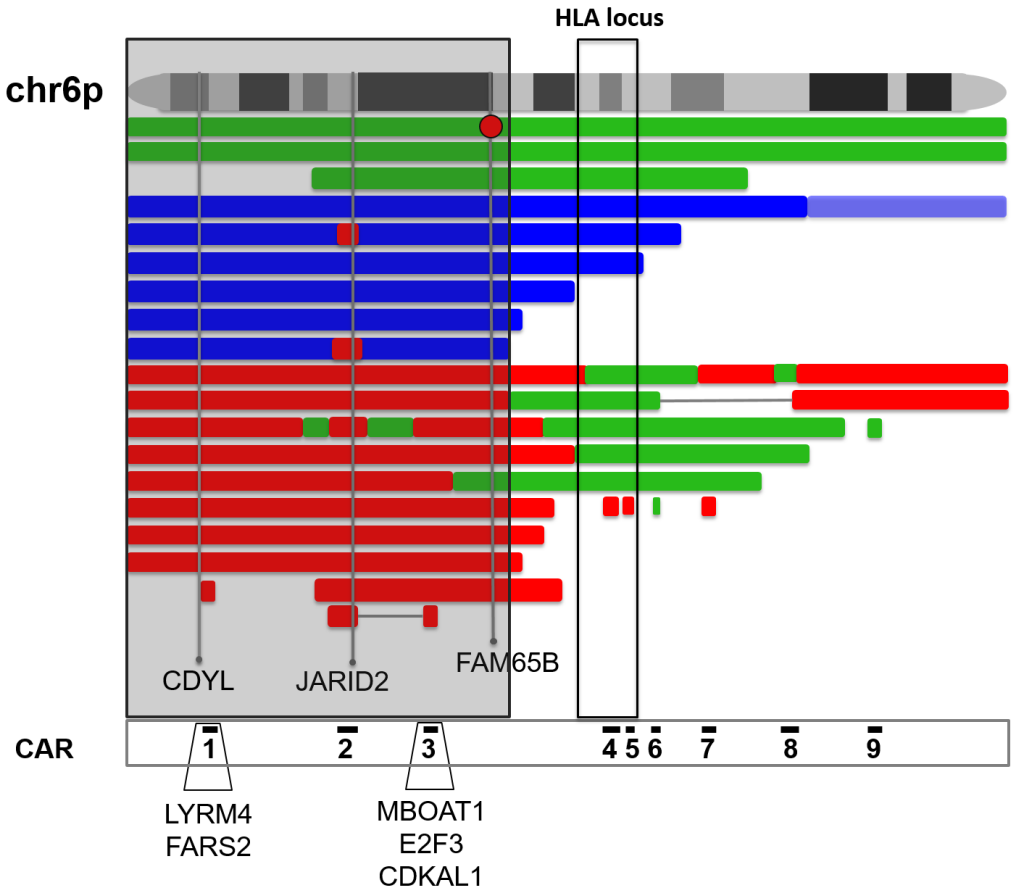
In addition to *JARID2* and *FAM65B*, the intersection of exome sequencing data with the aberrational profile of chromosome 6p allowed the identification of several commonly affected regions (CAR), with a list of other putative tumor suppressor genes. The region affected in the majority of patients is the one of highest interest, covering the genomic position chr6: 1-36450000 (hg18), highlighted in Figure 21. This includes genes *LYRM4*, *FARS2*, *MBOAT1*, *E2F3* and *CDKAL1* as parts of CAR1 and CAR3, in addition to *JARID2* and *FAM65B*. The E2f3 transcription factor regulates the cell cycle,

fine tuning transcription dependent of the differentiation context of the cell, and regulate cell survival in a p53-dependant manner (Chen, Pacal et al., 2009, Chong, Wenzel et al., 2009). E2f3 has a significant role in the development of myeloid hematopoietic cells, as well as in cancer (Gao, Li et al., 2017, Pulikkan, Dengler et al., 2010, Trikha, Sharma et al., 2011, Trikha, Sharma et al., 2016). *LYRM4*, *FARS2*, *MBOAT1* and *CDKAL1* have so far not been associated with hematological malignancies. Interestingly, the *CDYL* gene, encoding a PRC2-interacting protein is also located on chromosome 6p (chr6 p25.1), very close to the CAR1, and is found deleted in our cohort in 1 MF, 3 dnAML and 4 sAML patients, with all of these deletions covering the locus of the JARID2 gene as well. *CDYL* is involved in epigenetic regulation through complexes with REST and G9a, HDAC1 and C-terminal binding protein (CtBP) (Caron, Pivot-Pajot et al., 2003, Mulligan, Westbrook et al., 2008, Shi, Sawada et al., 2003). *CDYL* regulates PRC2 activity by interacting with both Ezh2 and repressive epigenetic histone marks (H3K27me2 and H3K27me3), and recruits Ezh2 to the DNA replication fork by interacting with the replication machinery and chromatin assembly factors (Liu, Liu et al., 2017, Zhang, Yang et al., 2011). Whole exome sequencing results from our study have not identified mutations of this genes, but simultaneous haploinsufficiency of *CDYL* and *JARID2* may have functional effects on AML development and should further be investigated.

The *HLA* (human leukocyte antigen) locus is also located on chromosome 6p and should not be overseen when investigating the genetics of chromosome 6. In 11-13% of aplastic anemia cases chromosome 6pUPD occurs, in which HLA (human leukocyte antigen) genes are identified as targets of these chromosomal aberrations (Babushok, Perdigones et al., 2015, Betensky, Babushok et al., 2016, Katagiri, Sato-Otsubo et al., 2011, Zaimoku, Takamatsu et al., 2017). Aplastic anemia is a rare nonmalignant bone marrow failure disorder, characterized by bone marrow hypocellularity, caused by eradication of hematopoietic stem cells mediated by immune cells, and is accompanied with pancytopenia in the peripheral blood. Complications of aplastic anemia include progression to MDS or AML (Shallis, Ahmad et al., 2018). In addition, clonal hematopoiesis occurs in almost half of aplastic anemia patients, with frequent mutations in a variety of MDS- and AML-specific genes (Yoshizato, Dumitriu et al., 2015). Escape from activated cytotoxic T cells (CTL) due to the loss of heterozygosity of the *HLA* locus on chromosome 6 and uniparental expression of *HLA* genes leads to aplastic anemia in cases with chr 6p UPD (Katagiri et al., 2011). Clonal hematopoiesis is sustained in aplastic anemia in patients with chr 6p LOH, without additional mutational events. However, if the secondary somatic mutation occurs in a HSC with chr 6pUPD it provides proliferative advantage to the affected clone (Imi, Katagiri et al., 2018). The same mechanism of immune

surveillance escape has been observed in leukemic relapse after hematopoietic stem cell transplantation (Vago, Perna et al., 2009, Villalobos, Takahashi et al., 2010). Somatic mutations of *HLA* genes are also described in aplastic anemia, both coupled and not coupled with a chr6p UPD (Zaimoku et al., 2017). *HLA* genes located on the short arm of chromosome 6 are clustered in a 3.6Mb MHC (major histocompatibility complex) region and are one of the most polymorphic genes of the human genome (MungallPalmer et al., 2003). In the exome sequencing performed in this thesis variants in the *HLA* genes were filtered out during data analysis due to the highly repetitive nature of the genomic region. However, the aberration profiling does not point to *HLA* genes as main targets, as the majority of the detected aberrations do not cover the HLA cluster located at chr6 p21-p22. Out of 42 chromosomal aberrations on chromosome 6p we detected in the large studied cohort 16 were covering this locus - 4 UPDs in 3 patients, 8 gains, 2 focal deletions (in a dnAML and CML patient), and 2 deletions with breakpoints in the region. It is unlikely that the *HLA* locus is targeted by aberrations of chromosome 6p in our cohort but without further studies no conclusions can be reached about their role in the disease development.

Figure 21. Chromosome 6p: putative target genes of chromosomal aberrations in myeloid malignancies



Chromosomal aberrations on chromosome 6p, detected in the studied panmyeloid cohort, are represented with bars. Blue bars, red bars and green bars represent uniparental disomies, gains and deletions, respectively. Aberrations connected with a grey line are detected in the same patient. The position of three single genes are depicted with vertical grey lines. Commonly affected regions identified in this study are represented with black bars in the bottom. The region affected in the majority of patients, as well as the position of the HLA locus are highlighted. Names of genes CAR1 and CAR3 cover are displayed.

4.2. Conclusion and future prospects

The work performed in the scope of this PhD project and presented in this thesis contributes to the knowledge and understandings of the genetics of MPN and MDS, with a focus on genetic factors contributing to disease progression and evolution to secondary AML, investigating the presence of detected aberrations in dnAML as well. We have shown that the presence of chromosomal aberrations is associated with leukemic transformation of MPN and MDS and for the first time identified *JARID2* as a putative tumor suppressor in MPN development, providing evidence that the gene is preferentially targeted by deletions and not mutations, and that the presence of these deletions is associated with disease progression. In addition, by means of deletion mapping, we have demonstrated that deletions of PRC2 subunits are strongly enriched in sAML, and are present in dnAML as well, but rarely occur in chronic phases of MPN and MDS. The detection of frequent codeletions of single PRC2 subunits, as well as their codeletion with other genes (*NF1*, *ETV6*, spliceosome factors, epigenetic regulators) in our cohort implies a possible cooperating role in disease evolution. We have shown that chromosome 6p is affected across a broad range of myeloid malignancies and that lesions of the chromosomal arm associate with MPN evolution to sAML. Furthermore, we found no association with *JARID2* mutations, and our study did not reveal any other single gene recurrently targeted by mutations in patients with aberrant chr6p.

As a follow up of our study a number of experiments could fulfill our findings of the effect of PRC2 loss and chromosome 6 aberrations in patients with myeloid malignancies and their effect in disease evolution. In order to investigate the expression of *JARID2* upon chr6p deletion RNA sequencing in a larger number of patients could be performed, with an additional objective of investigating the expression profile of PRC2 target genes upon *JARID2* loss. Despite the low number of investigated samples, our data shows a trend of reduced *JARID2* expression in patients with *JARID2* loss. In addition, due to the lack of a common target in chromosome 6p aberrations based on DNA sequencing performed as part of this thesis, but also in a separate study performed by our research group where exome sequencing of DNA from a PMF patient with chromosome 6p UPD revealed no somatic mutations (Milosevic Feenstra et al., 2016), DNA methylation profiling of patients with chromosome 6p aberrations can be a powerful tool for identifying epigenetic changes leading to inactivation of genes, and might complement the findings of exome sequencing and facilitate the detection of a common target gene on chromosome 6p. Perturbations of PRC2 genes by the means of CRISPR/Cas9 technology (Ran, Hsu et al., 2013) in cell lines may provide more information about the effect of gene loss on

proliferation and cell survival, whereas knockouts of multiple PRC2 members can add additional layers of knowledge to the understanding of the effect of simultaneous haploinsuficiency observed in patients. Functional studies of homozygous knockouts or PRC2 cofactors *JARID2* and *AEBP2* would be of particular interest, contributing to the understanding of our findings that these two genes are recurrently found homozygously deleted in patients. These functional studies should also address the effect of simultaneous loss of a PRC2 gene and other genes detected in patients from our cohort. Of great importance is the sensitivity to and effect of drugs in cells lacking one or more PRC2 subunits, therefore a drug screen on knockout cells is recommended in follow up studies with the aim of gaining more knowledge and opening up possibilities of applying precision medicine in the management of patients with PRC2 haploinsuficiency.

In pediatric AML an association between PRC2 inactivation due to mutations or deletions has been made with poor clinical outcome (Bond, Labis et al., 2018). Investigating the clinical impact of PRC2 haploinsufficiency in non-pediatric myeloid cancers might show a similar correlation and identify a high-risk subcategory of patients with specific treatment requirements. Therefore, the clinical impact of PRC2 deletions should be assessed in follow up studies, comparing the overall survival and outcome in patients with and without PRC2 haploinsufficiency, delineating the prognostic impact of PRC2 haploinsufficiency in MPN, MDS and AML. Intensive and extensive investigation of the genetic landscape of myeloid malignancies in the last years has significantly broadened the knowledge in this field and recognizes the need for incorporating genetics in the classification and prognostification of patients, with the aim of personalized tailoring of therapeutic decisions (Dohner, Estey et al., 2017, Grinfeld, Nangalia et al., 2018, Lindsley et al., 2015, Papaemmanuil et al., 2016, Schischlik et al., 2019, Yoshizato et al., 2017). On the other hand, understanding the mechanism behind leukemic transformation of chronic myeloid malignancies is of great importance as the prognosis and outcome of patients in the leukemic phase are very poor (Abdulkarim, Girodon et al., 2009, Hulegardh et al., 2015). PRC2 defects are potential candidates to be incorporated as biomarkers in the new classification of myeloid malignancies, if an association with clinical properties and outcome is found. We strongly believe that such an association exists as the enrichment of PRC2 defects in leukemic stages is clearly observed in the research of this thesis. A whole new era of genomics, transcriptomics, high-throughput screens and cancer immunology will allow precision diagnostics, patient management and monitoring upon therapy introduction, and therefore, our contribution to the knowledge in this field is of major importance for bridging the gaps in knowledge and improving the quality and personalization of treatment each patient in the future receives.

5. MATERIAL AND METHODS

5.1. Patient samples

Upon diagnosis of MPN, MDS, AML or CML in institutions in Austria, the Czech Republic, Italy or Serbia, peripheral blood was obtained from a total of 905 patients included in the study, accompanied by written informed consent, following regulations of the local ethics committees. Following standard operating procedures genomic DNA was extracted from whole blood samples, or from granulocytic or T-cell fractions from patient blood samples.

5.2. Microarray analysis

For the purpose of identifying copy number alterations (deletions and gains) and loss of heterozygosity Genome-Wide Human SNP Arrays 6.0 (Affymetrix) were used. Processing and hybridizing of genomic DNA from all 905 patients was done according to the manufacturer's protocol. Analysis of raw data was performed using Genotyping Console Version 3.0.2 software (Affymetrix). Copy number alterations and loss of heterozygosity were annotated as previously described. (Puda, AJH, 2012)

5.3. Whole exome sequencing

DNA extracted from whole blood (Patient 26) or granulocytes (Patients 11, 13 and 23) was used for preparing DNA libraries applying the TruSeq DNA Sample Preparation Kit (Illumina). For whole exome enrichment the TruSeq Exome Enrichment Kit was used (Illumina). Prior to sequencing using 51bp paired end sequencing on the HiSeq 2000 platform (Illumina) samples were hybridized to the Illumina flowcell V2 Primary data analysis providing base calls was performed by the Realtime Analysis software (Illumina). DNA sequence alignment to the human genome reference b37 was processed with the BWA MEM algorithm. (McKenna, 2010; Li, 2013) Mark duplicates was used for removing PCR duplicates (Picard tools 1.118) Variant calling was performed using the HaplotypeCaller tool (GATK). The resulting unfiltered SNP and indel calls were annotated using the snpEff tool and the GATK Variant Annotator for selecting only the most severe consequence prediction. Functional annotation of genetic variants was performed using the Annovar tool version 2012May25 (Wang, Li et al., 2010) and custom scripts. SNPs annotated in the dbSNP129 database were filtered out, as well as synonymous polymorphisms and non-coding RNA, leaving exonic and/or splicing mutations. Further filtering steps included discarding variants with VQSLOD score ≤2 and those present in at least 3/67 non-disease control samples. For the purpose of this project only variants in the affected region of chromosome 6 were left for further analysis. The affected regions are patient specific,

derived from the Genome-Wide Human SNP Arrays 6.0 (Affymetrix). Furthermore, all variants present in at least 1% in the EVS (Exome Variant Server) and 1000 Genomes databases were filtered out, as well as those present in the ESP6500 and ESP5400 databases. Variants in repetitive regions and those with coverage <10 were filtered out. A filtering step including the allelic frequency was included, based on the expected frequency from the estimated clone size harboring the chromosomal aberration on chromosome 6, as estimated from the analysis of data obtained from the Genome-Wide Human SNP 6.0 Arrays. The filtering steps were described and outlined in Results section 3.7 (Figure 16).

Exome sequencing of 40 samples, from which only variants in PRC2 encoding genes were validated for the purposes of this thesis, was performed as previously reported (Puda et al., 2012).

5.4. Sequencing for mutational analysis of single genes and validation of variants from exome sequencing

Sanger sequencing was used for the validation of all variants from whole exome sequencing, as well as for sequencing the whole coding region of genes encoding PRC2 subunits (JARID2, AEBP2, EZH2, SUZ12, EZH1, EED, RBBP4/7, PHF1, PHF19 and MTF2). Primers were designed using the Primer3 tool (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The sequences of all used primers are listed in Tables 17-19. Genomic DNA was used for PCR when available, whereas whole genome amplification using the REPLI-g Mini Kit (Qiagen) was performed on DNA samples with limited quantity. For PCR reactions the AmpliTaq Gold 360 Mastermix (Applied Biosystems) and a touchdown program [95°C, 5min; 10x (94°C, 30sec; 67°C, 30sec (-1°C/cycle); 72°C, 30sec); 30x (94°C, 30sec; 57°C, 30sec; 72°C, 30sec); 72°C, 10min] were used. The BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems) was used for sequencing on the 3130xl Genetic Analyzer (Applied Biosystems). The Sequencher Version 4.9 software (Gene Codes) was used for sequencing analysis.

5.5. Gene expression assay

For assessing the level of *JARID2* expression qPCR was performed on an ABI Prism 7900HT using TaqMan Gene Expression Assays (Applied Biosystems) Hs99999909_m1 (HPRT1) and Hs01004460_m1 (JARID2). Patient material used for the assay was RNA or cDNA, as described in Results section 3.6 (Table 12). Where RNA was available it was transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions.

Delta-delta CT (ddCT) was calculated from means of triplicate measurements of *JARID2* and *HPRT1* (housekeeping gene used as the internal control).

5.6. Statistical analysis

Calculations of statistical significance were performed using the Fisher's exact test, in combination with the Bonferroni correction for multiple testing, in cases where multiple tests were performed.

Table 17. Primers used for Sanger sequencing of *JARID2*

		Forward primer sequence	Reverse primer sequence	Product size
JARID2	Exon1	caagatcaaccaccaccaac	AGAAACAGCCTTATCGGACG	285
	Exon2	GGTCGTGGTCACACAGTACG	AAGCACAAGCTGCCAGAATAC	344
	Exon3	TTGCTTTTCTCCACCCATTC	AAAGCAGGGCATTTGATACG	458
	Exon4	CCTTAATTAGGAAGAGTTTGCTTG	TGAACATGGCAGAGAGTAAAGC	399
	Exon5	TTTTCTCCTCGGTTTTGTTTC	GAACGAGCTTTGCCTTCATC	374
	Exon6	GTTCCCTCGCTAACACATGG	TCACCTCCTGCTTCTTGAGC	475
	Exon7_1	CAGGGTCCTTTCTCACGGG	TCCTCTTGGAGTTCCGCAG	510
	Exon7_2	GCAAACAGGTGCTATCCCTC	GGATCGTGGAACTCCTTGG	550
	Exon7_3	AGACAAGCACATGGCAAGG	CAAGGGAAGAGAACGTGAAG	507
	Exon8_1	AGCCTGTCAGAGTCCTGGC	GGAACTTGTGGTGGTCGTTC	416
	Exon8_2	TCTCCTACGACTCCCTGTCC	TCAGAGAACACACCGTCCAG	431
	Exon9	GAATACAAGGTGGCCCACAG	CTGAGTCCTTTCCGTTCACC	331
	Exon10-11	TCTGTTCTGAGGGGTGTGTG	GCAGTGCTTAGAAGGGATTTC	490
	Exon12	TTCCTTCTTGTTTTAGCGGAAG	GGAACCACCTGGACAAGAAG	313
	Exon13	GAGCAGAGCCTCTCGTGTG	CTGTGTCCTCATTGCCTTTG	320
	Exon14	TTGAGAGCAGGCCTCTTAGG	ATAGACACGCACACGCAGG	378
	Exon15	TTTCTCACAGGGAGGCAAAG	CTGCAGACACACCCTCCTG	392
	Exon16	CTGACAGGAGGGTGTGTCTG	CTCCAGAGATGGGAGCACAG	420
	Exon17	AGTCCTCAAAGCCCTTGGC	GCCTAAGAACAGGGCACCTC	408
	Exon18	CTCTGCCCTAAACTTGCCC	tgcaaagacagcttgaatcc	370

Table 18. Primers used for Sanger sequencing of PRC2 genes

		Forward primer sequence	Reverse primer sequence	Product size
AEBP2	exon 1F	AGGCAGAGACGATGTCGGAG	ttccgagttgagagtctggg	578
	exon 2F	tggagcagaatgtcaagtgg	aaactaaaatccagcacataccc	510
	exon 3F	ttttgattggcttgtacattcag	caagatggcaccattgcac	360
	exon 4F	cccttatgcttcctttctgg	cccttatgcttcctttctgg	450
	exon 5F	cccacattcactcatttgttg	ggtgtgtcatcccattattcc	574
	exon 6F	ctgaccttatgatccacccg	tctttaatcctgacaacctcaaaac	357
	exon 7F	tcaaagtagcagaaaatgccag	tgcttggtattaaaattcagcac	318
	exon 8F	tgatcatcacattgcctgtatc	aaggggttgactctaatgtgc	237
	exon 9F	tgtcttgacagtgtttattgatttg	AAGGGAGCCACCAGACATAC	267
EED	exon 1F	AAGGGAAGGAGCCAGGAAG	ccacatcaaaatccccgtag	524
	exon 2F	tcttaggtcagtcagcatcttcc	tttacaacttgacagagtatcctgag	346
	exon 3F	cttgtgatttcagcctttgg	accagcttcacaaaatgcac	426
	exon 4F	gctcacaggaggtattttaaggc	gggtgatagtgaagaaatcgg	334
	exon 5F	ttttagacctcaaatcaccattg	cgtctcaaaagactcatccaaag	440
	exon 6F	tttcacctcaagtttgttggg	caagcatgtggactctcttctc	353
	exon 7F	ggctttactgtgcataacttacatc	cctgacaggctacatgaaacac	310
	exon 8F	tggcctatttagatgaaaccc	ttctaaactcattgttggggc	378
	exon 9F	tggttggttatgtaggaacacag	ataaagtgctccctgccaag	352
	exon 10F	gagctgtgaattccaaaacaatag	aacatcagggtagatttgtaaagg	371
	exon 11F	aagagcacagaggctggaac	aaaatgacttaaggatggcactg	302
	exon 12F	tgcttttcgtatgacttggaac	TCTAAATGCTCTACGTGCCC	331
EZH1	exon 1F	tctgttgaactcacacatttgg	gagaatggcgtgaacctgc	460
	exon 2F	gggtgacaacagagcaagac	gtgtgagccaccataccttg	339
	exon 3F	catgagccactatgcccag	ctagctgtgttaattactggacaatc	327
	exon 4F	caagcaacaaacaaagctcag	ggggcagaagataaaagcaag	313
	exon 5F	atatctgggcctctggagtg	aacaagaagcaccaacagcc	382
	exon 6F	aatcaaaatgctatgacaaaagg	tgcccttaccataaacaaagc	303
	exon 7F	ctcccttggcagaccgtag	cagaagccaggaactctatacg	369
	exon 8F	actgaattttgtgcaccctg	gctccttaagagcaaaagcc	353
	exon 9F	ttgtagacagatgttgtaaagatgaaa	gatagcaccactgcactcca	498
	exon 10F	gggaatattttcattcctaactgttc	ctccagacccagacacacag	423
	exon 11F	tttctcagaaccctcccaag	cggttgctaaagagggagg	286
	exon 12F	tccccagtcatcaaacacag	cacagggtcaacaagtgcag	247
	exon 13F	gttaggaccagcctcctctg	ggccataagcagtaaagggc	324
	exon 14F	tgccaaacatccttgaacac	cctcaaaagaaccagtggaaag	385
	exon 15F	agttgctggttctttccacc	gagattaattcaaagaagcctgg	296
	exon 16F	atgtgagataccacccagcc	aagatcgcaccattgcacc	281
	exon 17F	acactgggcttcaacattcc	ggggcctaaaatgataccag	353
	exon 18F	tttgattcatgttttcactggg	catctccattaccctccctg	289
	exon 19F	agtgggaggggaagtgaaac	GACACAGTGCAGGAGACTCG	246
EZH2	exon 1F	tgattgttagtttgctgcgg	ctaagcttccaagtattcactcatc	353
	exon 2F	ttttgtattatttgaatgtgggaaac	aagatggacaccctgaggtc	408
	exon 3F	aaatctggagaactgggtaaagac	gcccaggttcagtcccttatag	375
	exon 4F	aggctatgcctgttttgtcc	aaaagagaaagaagaaactaagccc	332
	exon 5F	ctgactggcattccacagac	aagtgtagtggctcatccgc	380
	exon 6F	catcaaaagtaacacatggaaacc	gcaatcctcaagcaacaaag	437
	exon 7F	ggaaagttgtatgaagtaacgtaaacc	ggtgcagacaacattaacgc	323
	exon 8F	gattatttgtgataaatggataatgtg	tctggtctttatactgaaactaaccaa	489
	exon 9F	gatgggttagtgttttgccg	tcattcaattccctcttggg	458
	exon 10F	atccccagcatctagcagtg	ttcccagtggaaataagattcc	374
	exon 11F	tcttggctttaacgcattcc	caaattggtttaacatacagaaggc	289
	exon 12F	aggccagctacactccacag	agggagtgctcccatgttc	333
	exon 13F	gagagtcagtgagatgcccag	tttgccccagctaaatcatc	371
	exon 14F	tgtgcccaattactgccttag	tttccaatcaaacccacagac	334
	exon 15F	gcgttttctccagaaggtcc	tctggtcacctcactgacctc	292
	exon 16-17F	gcaaaccctgaagaactgtaac	gacccatcaaaagaagcaaag	530
	exon 18F	atgaatgtgccgttatgcag	AAAACACTTTGCAGCTGGTG	442

		Forward primer sequence	Reverse primer sequence	Product size
SUZ12	exon 1F	CTCCCCTCTCTCCTCCTTCC	gacatcccctcaattcctcc	533
	exon 2-3F	aaatagtgcgtgatttccatagc	caattcaaccagtcactctctcac	481
	exon 4F	aatctggtttccatcttgcc	ttggaccaaactgacctctaag	297
	exon 5F	tgggcctgaataacagttgag	aaacatcagaaccaacatattttcc	318
	exon 6F	gctgtaagtttggaactgatttg	acacagatatgcgagttggc	422
	exon 7F	cccgtgtttcctatagctgtg	tttgctttaagactcagcacc	433
	exon 8F	agcatttgcaaagatgtggg	agtaagtttaactgagctatctatccg	525
	exon 9F	catcaggactggagtgtagctg	tgctctgactagatcatttacttgtg	416
	exon 10F	actttgtttgcctagccgtc	catctgaacccacaaggcag	535
	exon 11F	tcctgcgatcatcagttgag	agtgagccaagactgcacc	413
	exon 12F	cgcgtcactgggcatattta	gagttggttgaggattttgtga	589
	exon 13F	tgtttaccatgctggtgtgc	caaccccatagacacctaatactg	426
	exon 14F	tttataaaagggtgatgttgcag	aaacctgtacatttccggattc	443
	exon 15F	gaaatgttgccactttgctg	cattggaatagaataaaatttggg	350
	exon 16F	tcatcttttattttactggaattgga	AACATGGGGTTAGAGCTTTTCA	475
RBBP4	exon 1F	cctccatttcatggttaggc	ctccgggtgactgaagagc	344
	exon 2F	aaaacctggttgtattagtgactttg	ttccctcaaccttaaatattgtttc	356
	exon 3F	atgccagtttgttgctaagg	cacatatcaatacaaatcacaagatg	407
	exon 4F	ttatctatttgatcataacggttcc	GATGTCCCACAGGCAGATG	393
	exon 5F	GACCATgtgtgtatccttccc	TGGCTTGGTTTGGAAGTATTG	421
	exon 6F	TCTGTTTGGGTCAGTTGCTG	GATCCCACAAGGCAACAGTc	411
	exon 7F	ATTCTTGCCACAGGATCAGC	tagctgggactacgggcatc	449
	exon 8-9F	aataccttgacttcctcttccc	ATCTTGGCAGTATGACCACC	491
	exon 10F	GCCACCAGAGTTGTTGgtatg	aaaatgaacagagatggtagctg	321
	exon 11F	tttactctctggcttcctgg	TTGGGCTAATAGCATTATCGG	299
RBBP7	exon 1F	CCTCTTCCCTTCCCTCCTC	actgctcttccagaaggtgg	464
	exon 2F	tctccaccttctggaagagc	tcatcccacaaatcacgtaag	456
	exon 3F	ggagaagatacacagttgatgctaag	catgtgggtgggaagttctg	424
	exon 4F	ttctgccaggtcaaaatctg	acttgaggatggttatgggac	411
	exon 5F	cagatctcgttcgtgtgcag	ggggaatgttaccacaataatcag	359
	exon 6F	gggtgacagactgagacactg	tcagcttcccaaggtattgag	413
	exon 7-8F	tgaaggggagcacatagtgg	gcatctccatgtaacaccacg	563
	exon 9F	gtcggggacaagagagtgag	gaacaaaggctctttaggaagatg	373
	exon 10F	gttttaagattactttctgggctg	caataaccttcagggcctcc	290
	exon 11F	ccaccttctaaaaccagacagc	cggtttctgtaaatgtttggc	360
	exon 12F	ctggctagcctaccatttcc	AAAGAAGCCATAAGCCACCC	336
MTF2	exon 1F	tgttttaaactgctttgtttggag	TCTTCAAATATGATGAAGCAGCTC	526
	exon 2F	TGGCTTGTTTTATCTTGGCAC	ttatggtctataataacgactatgctg	392
	exon 3F	cgcatatgtgctgctcactg	aagaatgcattttagcattatttcc	331
	exon 4F	CAAGgtaaccattgatttcttttaatc	aattactgcaccaattcccg	523
	exon 5F	tttggaaaggaaatttctccag	gcaattcaccatcactcacac	347
	exon 6F	aattagaacagtattgtggattaggc	ttttccctctgagacaaccc	336
	exon 7F	ttggaaaagaacttccttcaatc	acttagtgaagatgacatcaggc	405
	exon 8F	gcaatcagcgaaactctgtc	acaggcaacaaacacacagg	591
	exon 9F	tttcctctgcatcacattgc	cctccaatccttgagaatcac	337
	exon 10F	tgtagtattgggtgggctttc	aaaatcagagtaactgccacttg	401
	exon 11-12F	gtgcacttgactaaacttgtgg	TGAATGTGCAGTTCCCTCAG	573
	exon 13F	TCCTACTTTGGATTTACCTTGTTC	ctgatagacacaacttacatcactttc	362
	exon 14_1F	tttattgtttgggtttggctc	TTCGCCACATGCTATTCTACC	414
	exon 14_2F	TTCCAAGAAGAGCACTCCAG	CATGTTAAGGCTAGTATCATCCCC	423

		Forward primer sequence	Reverse primer sequence	Product size
PHF1	exon1-2F	agagcaaaactccatctcagg	GAGGGCAGctgtgagagaac	562
	exon 3F	TCTATGGAAAGACATTAGCCCTG	gataaggctaagcagcctgg	346
	exon 4F	tcagggattaaatgagatggg	ctgcagagacaggacaggtg	305
	exon 5F	tccctgttacccttcctgtg	cctgagtgctaccctcatcc	346
	exon 6F	ggatgagggtagcactcagg	ttgggaagagactggagtgg	298
	exon7-8F	gttacctcacctgtttgccc	TGAAAGctgggagaaatgaag	541
	exon 9F	aaggggtagtgcagttttgg	acagtcttccaggtgggttg	274
	exon 10F	aacccacctggaagactgtg	CCCTGAAGGGAAGctgtaag	344
	exon 11F	ctcagggtgttagtcctggg	GATGGTGGAGACACTGAGGC	395
	exon 12F	CACTGCAGgtactggagcag	caagaagtgggtagccaagg	304
	exon 13F	gtattctggggaagggagtc	gagcacatgtgaatcagagaaac	291
	exon 14F	tcccctctatgtgctcaagg	CTCTCCAGGTACATCCCAGC	485
PHF19	exon1F	atggtggtttccttccttcc	gaattctccagcaatcccc	384
	exon2F	tgctttctggaacatacggag	ccaggagtaagagatggaagg	287
	exon 3F	ggcctagcagacattagaggc	aagagaaacaggaagccagc	306
	exon 4F	tcctaggacttccctcagcc	ggcgctatctgtctccaaag	333
	exon 5F	ttccttctgggatagagggg	gccctcaggtccaatcttc	610
	exon 6F	ctcttccctttctgccagtg	gagcctggcagtcaacctg	298
	exon7-8F	aaggtaggtggggaatgagg	agggatgcagcaactcaag	597
	exon 9F	aagcatcaggagatggaagg	cagagaggcagggagcatag	262
	exon 10F	gaggcagagaggcttgtgtc	ggtcctacagctgggactgg	360
	exon 11F	tctccccaactgctgtgac	gagagtcctctgggcctgg	295
	exon 12F	ggacttggaataaaggtgatgg	ttcagacagacctgcacagc	335
	exon 13F	tgaaggatgcatgttagggg	ccgccagagactgagacac	304
	exon 14F	cctggtactgaggggaggac	TCTGTCCTGCTTCCTTCTCC	540

Table 19. Primers used for validation of whole exome sequencing hits by Sanger sequencing

UPN	Gene	Mutation	Chr	Start position	End position	Forward primer sequence	Reverse primer sequence	Product size
	OR2B3	F40L	6	29054908	29054908	CTAGGGCCAGGAAGATGATG	GAAAATGAGAGCTCCCCAAA	366
	NCR3	R96Q	6	31557660	31557660	TCCCAGCATCTCACCTTTCT	CCTGAGATTCGTACCCTGGA	197
	UHRF1BP1	S506P	6	34825190	34825190	GAAATGGAGGGCTGCTCTAA	AAGGCTGACAAATGGGAAAA	150
	DNAH8	I4044T	6	38917229	38917229	TGACCCTCTCAGGTGATTCAG	CGGTTTTTGCCGTTACTTTC	360
Patient 26	APOBEC2	F52L	6	41029089	41029089	CCAAATTCTCCTCTCCTTCAGA	CGCATGCTCATCCTCTAGGT	404
	C6orf132	L683F	6	42073603	42073603	GGGAGGGAGTGGAAACTTCT	GGGGCTGATGATGACAAACT	225
	TJAP1	R359Q	6	43473025	43473025	TCTCCACCACACCCACTGTA	CTGACCAGCAGGTCCTCTTC	185
	CYP39A1	G410R	6	46554836	46554836	CCAAAGTTGTTCCCCTCTGA	GTTTTCTTCCCACAGGAACG	360
	TRERF1	G64fs	6	42237137	42237137	CTGGCCTGGGAGTAGGTGTA	TTCACAACCAAGGCAGACAG	404
	RREB1	L1649P	6	7249083	7249083	AAGGACAGCGACAAGGAAGA	TCTGCTGGCTTTTGTCTGTG	310
	KLHL31	S117T	6	53519722	53519722	TAAATTTCTGGGCTGCTGCT	TGGCCTTAGCTGCATTTCTT	194
	TMEM200A	R402W	6	130762771	130762771	GGATTCCCTTGTGGTTCCTT	GGCACAAGCAACCTATCCAT	226
	CLVS2	S178N	6	123332273	123332273	GGATATTTTGCGTGCCATCT	TCTCTTCCTTCAGCGTTTCTTC	282
	ZBTB9	T163A	6	33423364	33423364	GGATGCTCTTCCTGCTCATC	CCCTGGTCCTCATCATCATC	203
	FAM65B	L521R	6	24843298	24843298	AGAAAGGATCTGCAGCCTTC	AAGGAGGAAGACCCAGAGGA	430
Patient 11	THBS2	P816L	6	169626366	169626366	TCTGATGAGGACCTCCAACC	TGGCTGTAAGCAATTTCGTG	171
	PSORS1C1	P38fs	6	31106501	31106501	CCGTTCACTTGACCCACTTT	GCATCTGGCTCACCAGAAAT	177
	ZNF292	R2094Q	6	87969628	87969628	GAGCAGAGACCCAAAATACCC	GTAAAGGCAGCAAAGCATCC	262
	STX11	E206K	6	144508380	144508380	CCATGCACGACTACAACCAG	TTGAGCTCGATGACGTTCAG	333
	RSPH9	A94V	6	43618165	43618165	AAAGGTGGCCATTTCAACAG	AGCCCAGGCTCTCTTTTAGG	261
	ZNF165	R69H	6	28053464	28053464	TATCCATGGGCAGGACACTT	TAGATCTCCCATCCCCTGTG	361
	MRPS18A	R173L	6	43639572	43639572	TAGTCCCACTGTCCCCTGTC	CAGGCACCTCTTTGTTCTCC	250
Patient 13	C6orf195	M33V	6	2623960	2623960	GGCCTGGTCACTGTTGATCT	CCTGTGGTCCCTGATGATCT	225
Patient 23	FAM217A	E240Q	6	4069739	4069739	GCAGGGTCCACTGTCACTTT	TGGAAACAGCAGTGTGGAAG	283

6. REFERENCES

Abdel-Wahab O, Adli M, LaFave LM, Gao J, Hricik T, Shih AH, Pandey S, Patel JP, Chung YR, Koche R, Perna F, Zhao X, Taylor JE, Park CY, Carroll M, Melnick A, Nimer SD, Jaffe JD, Aifantis I, Bernstein BE et al. (2012) ASXL1 mutations promote myeloid transformation through loss of PRC2-mediated gene repression. *Cancer Cell* 22: 180-93

Abdulkarim K, Girodon F, Johansson P, Maynadie M, Kutti J, Carli PM, Bovet E, Andreasson B (2009) AML transformation in 56 patients with Ph- MPD in two well defined populations. *Eur J Haematol* 82: 106-11

Akada H, Yan D, Zou H, Fiering S, Hutchison RE, Mohi MG (2010) Conditional expression of heterozygous or homozygous Jak2V617F from its endogenous promoter induces a polycythemia vera-like disease. *Blood* 115: 3589-97

Albiero E, Madeo D, Ruggeri M, Bernardi M, Giorgetti A, Rodeghiero F (2008) Loss of the JAK2 intramolecular auto-inhibition mechanism is predicted by structural modelling of a novel exon 12 insertion mutation in a case of idiopathic erythrocytosis. *Br J Haematol* 142: 986-90

Anand S, Stedham F, Beer P, Gudgin E, Ortmann CA, Bench A, Erber W, Green AR, Huntly BJ (2011) Effects of the JAK2 mutation on the hematopoietic stem and progenitor compartment in human myeloproliferative neoplasms. *Blood* 118: 177-81

Anderson LA, Duncombe AS, Hughes M, Mills ME, Wilson JC, McMullin MF (2012) Environmental, lifestyle, and familial/ethnic factors associated with myeloproliferative neoplasms. *Am J Hematol* 87: 175-82

Antoniani C, Romano O, Miccio A (2017) Concise Review: Epigenetic Regulation of Hematopoiesis: Biological Insights and Therapeutic Applications. *Stem Cells Transl Med* 6: 2106-2114

Aoyama K, Oshima M, Koide S, Suzuki E, Mochizuki-Kashio M, Kato Y, Tara S, Shinoda D, Hiura N, Nakajima-Takagi Y, Sashida G, Iwama A (2018) Ezh1 Targets Bivalent Genes to Maintain Self-Renewing Stem Cells in Ezh2-Insufficient Myelodysplastic Syndrome. *iScience* 9: 161-174

Araki M, Yang Y, Imai M, Mizukami Y, Kihara Y, Sunami Y, Masubuchi N, Edahiro Y, Hironaka Y, Osaga S, Ohsaka A, Komatsu N (2019) Homomultimerization of mutant calreticulin is a prerequisite for MPL binding and activation. *Leukemia* 33: 122-131

Araki M, Yang Y, Masubuchi N, Hironaka Y, Takei H, Morishita S, Mizukami Y, Kan S, Shirane S, Edahiro Y, Sunami Y, Ohsaka A, Komatsu N (2016) Activation of the thrombopoietin receptor by mutant calreticulin in CALR-mutant myeloproliferative neoplasms. *Blood* 127: 1307-16

Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, Bloomfield CD, Cazzola M, Vardiman JW (2016) The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood* 127: 2391-405

Arnaudeau S, Frieden M, Nakamura K, Castelbou C, Michalak M, Demaurex N (2002) Calreticulin differentially modulates calcium uptake and release in the endoplasmic reticulum and mitochondria. *J Biol Chem* 277: 46696-705

Asada S, Goyama S, Inoue D, Shikata S, Takeda R, Fukushima T, Yonezawa T, Fujino T, Hayashi Y, Kawabata KC, Fukuyama T, Tanaka Y, Yokoyama A, Yamazaki S, Kozuka-Hata H, Oyama M, Kojima S, Kawazu M, Mano H, Kitamura T (2018) Mutant ASXL1 cooperates with BAP1 to promote myeloid leukaemogenesis. *Nat Commun* 9: 2733

Avanzi MP, Goldberg F, Davila J, Langhi D, Chiattone C, Mitchell WB (2014) Rho kinase inhibition drives megakaryocyte polyploidization and proplatelet formation through MYC and NFE2 downregulation. *Br J Haematol* 164: 867-76

Babushok DV, Perdigones N, Perin JC, Olson TS, Ye W, Roth JJ, Lind C, Cattier C, Li Y, Hartung H, Paessler ME, Frank DM, Xie HM, Cross S, Cockroft JD, Podsakoff GM, Monos D, Biegel JA, Mason PJ, Bessler M (2015) Emergence of clonal hematopoiesis in the majority of patients with acquired aplastic anemia. *Cancer Genet* 208: 115-28

Baens M, Wlodarska I, Corveleyn A, Hoornaert I, Hagemeijer A, Marynen P (1999) A physical, transcript, and deletion map of chromosome region 12p12.3 flanked by ETV6 and CDKN1B: hypermethylation of the LRP6 CpG island in two leukemia patients with hemizygous del(12p). *Genomics* 56: 40-50

Balasubramani A, Larjo A, Bassein JA, Chang X, Hastie RB, Togher SM, Lahdesmaki H, Rao A (2015) Cancer-associated ASXL1 mutations may act as gain-of-function mutations of the ASXL1-BAP1 complex. *Nat Commun* 6: 7307

Balligand T, Achouri Y, Pecquet C, Chachoua I, Nivarthi H, Marty C, Vainchenker W, Plo I, Kralovics R, Constantinescu SN (2016) Pathologic activation of thrombopoietin receptor and JAK2-STAT5 pathway by frameshift mutants of mouse calreticulin. *Leukemia* 30: 1775-8

Barak Y, Juven T, Haffner R, Oren M (1993) mdm2 expression is induced by wild type p53 activity. *EMBO J* 12: 461-8

Barbui T, Tefferi A, Vannucchi AM, Passamonti F, Silver RT, Hoffman R, Verstovsek S, Mesa R, Kiladjian JJ, Hehlmann R, Reiter A, Cervantes F, Harrison C, Mc Mullin MF, Hasselbalch HC, Koschmieder S, Marchetti M, Bacigalupo A, Finazzi G, Kroeger N et al. (2018a) Philadelphia chromosome-negative classical myeloproliferative neoplasms: revised management recommendations from European LeukemiaNet. *Leukemia* 32: 1057-1069

Barbui T, Thiele J, Gisslinger H, Finazzi G, Carobbio A, Rumi E, Luigia Randi M, Bertozzi I, Vannucchi AM, Pieri L, Carrai V, Gisslinger B, Mullauer L, Ruggeri M, Rambaldi A, Tefferi A (2014) Masked polycythemia vera (mPV): results of an international study. *Am J Hematol* 89: 52-4

Barbui T, Thiele J, Gisslinger H, Kvasnicka HM, Vannucchi AM, Guglielmelli P, Orazi A, Tefferi A (2018b) The 2016 WHO classification and diagnostic criteria for myeloproliferative neoplasms: document summary and in-depth discussion. *Blood Cancer J* 8: 15

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 et al. (2011) Survival and disease progression in essential thrombocythemia are significantly influenced by accurate morphologic diagnosis: an international study. *J Clin Oncol* 29: 3179-84

Barjesteh van Waalwijk van Doorn-Khosrovani S, Spensberger D, de Knegt Y, Tang M, Lowenberg B, Delwel R (2005) Somatic heterozygous mutations in ETV6 (TEL) and frequent absence of ETV6 protein in acute myeloid leukemia. *Oncogene* 24: 4129-37

Barraco D, Cerquozzi S, Hanson CA, Ketterling RP, Pardanani A, Gangat N, Tefferi A (2017) Prognostic impact of bone marrow fibrosis in polycythemia vera: validation of the IWG-MRT study and additional observations. *Blood Cancer J* 7: e538

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, Cancer Genome P (2005) Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet* 365: 1054-61

Bejar R, Lord A, Stevenson K, Bar-Natan M, Perez-Ladaga A, Zaneveld J, Wang H, Caughey B, Stojanov P, Getz G, Garcia-Manero G, Kantarjian H, Chen R, Stone RM, Neuberg D, Steensma DP, Ebert BL (2014) TET2 mutations predict response to hypomethylating agents in myelodysplastic syndrome patients. *Blood* 124: 2705-12

Bejar R, Papaemmanuil E, Haferlach T, Garcia-Manero G, Maciejewski JP, Sekeres MA, Walter MJ, Graubert TA, Cazzola M, Malcovati L, Ogawa S, Fenaux P, Hellstrom-Lindberg E, Kern W, Boultwood J, Pellagatti A, Bowen D, Tauro S, Groves MJ, Vyas P et al. (2015) Somatic Mutations in MDS Patients Are Associated with Clinical Features and Predict Prognosis Independent of the IPSS-R: Analysis of Combined Datasets from the International Working Group for Prognosis in MDS-Molecular Committee. *Blood* 126: 907-907

Bejar R, Stevenson K, Abdel-Wahab O, Galili N, Nilsson B, Garcia-Manero G, Kantarjian H, Raza A, Levine RL, Neuberg D, Ebert BL (2011) Clinical effect of point mutations in myelodysplastic syndromes. *N Engl J Med* 364: 2496-506

Bejar R, Stevenson KE, Caughey B, Lindsley RC, Mar BG, Stojanov P, Getz G, Steensma DP, Ritz J, Soiffer R, Antin JH, Alyea E, Armand P, Ho V, Koreth J, Neuberg D, Cutler CS, Ebert BL (2014) Somatic mutations predict poor outcome in patients with myelodysplastic syndrome after hematopoietic stem-cell transplantation. *J Clin Oncol* 32: 2691-8

Benton CB, Boddu PC, DiNardo CD, Bose P, Wang F, Assi R, Pemmaraju N, Kc D, Pierce S, Patel K, Konopleva M, Ravandi F, Garcia-Manero G, Kadia TM, Cortes J,

Kantarjian HM, Andreeff M, Verstovsek S (2019) Janus kinase 2 variants associated with the transformation of myeloproliferative neoplasms into acute myeloid leukemia. *Cancer* 125: 1855-1866

Betensky M, Babushok D, Roth JJ, Mason PJ, Biegel JA, Busse TM, Li Y, Lind C, Papazoglou A, Monos D, Podsakoff G, Bessler M, Olson TS (2016) Clonal evolution and clinical significance of copy number neutral loss of heterozygosity of chromosome arm 6p in acquired aplastic anemia. *Cancer Genet* 209: 1-10

Birnbaum RA, O'Marcaigh A, Wardak Z, Zhang YY, Dranoff G, Jacks T, Clapp DW, Shannon KM (2000) Nf1 and Gmcsf interact in myeloid leukemogenesis. *Mol Cell* 5: 189-95

Bond J, Labis E, Marceau-Renaut A, Duployez N, Labopin M, Hypolite G, Michel G, Ducassou S, Boutroux H, Nelken B, Bertrand Y, Baruchel A, Petit A, Asnafi V, Leverger G, Preudhomme C, Macintyre E, Lapillonne H (2018) Polycomb repressive complex 2 haploinsufficiency identifies a high-risk subgroup of pediatric acute myeloid leukemia. *Leukemia* 32: 1878-1882

Bonnet D, Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 3: 730-7

Boultwood J, Fidler C, Strickson AJ, Watkins F, Gama S, Kearney L, Tosi S, Kasprzyk A, Cheng JF, Jaju RJ, Wainscoat JS (2002) Narrowing and genomic annotation of the commonly deleted region of the 5q- syndrome. *Blood* 99: 4638-41

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: e33

Busque L, Mio R, Mattioli J, Brais E, Blais N, Lalonde Y, Maragh M, Gilliland DG (1996) Nonrandom X-inactivation patterns in normal females: lyonization ratios vary with age. *Blood* 88: 59-65

Busque L, Patel JP, Figueroa ME, Vasanthakumar A, Provost S, Hamilou Z, Mollica L, Li J, Viale A, Heguy A, Hassimi M, Socci N, Bhatt PK, Gonen M, Mason CE, Melnick A, Godley LA, Brennan CW, Abdel-Wahab O, Levine RL (2012) Recurrent somatic

TET2 mutations in normal elderly individuals with clonal hematopoiesis. *Nat Genet* 44: 1179-81

Cabagnols X, Defour JP, Ugo V, Ianotto JC, Mossuz P, Mondet J, Girodon F, Alexandre JH, Mansier O, Viallard JF, Lippert E, Murati A, Mozziconacci MJ, Saussoy P, Vekemans MC, Knoops L, Pasquier F, Ribrag V, Solary E, Plo I et al. (2015) Differential association of calreticulin type 1 and type 2 mutations with myelofibrosis and essential thrombocytemia: relevance for disease evolution. *Leukemia* 29: 249-52

Cabagnols X, Favale F, Pasquier F, Messaoudi K, Defour JP, Ianotto JC, Marzac C, Le Couedic JP, Droin N, Chachoua I, Favier R, Diop MK, Ugo V, Casadevall N, Debili N, Raslova H, Bellanne-Chantelot C, Constantinescu SN, Bluteau O, Plo I et al. (2016) Presence of atypical thrombopoietin receptor (MPL) mutations in triple-negative essential thrombocythemia patients. *Blood* 127: 333-42

Cancer Genome Atlas Research N, Ley TJ, Miller C, Ding L, Raphael BJ, Mungall AJ, Robertson A, Hoadley K, Triche TJ, Jr., Laird PW, Baty JD, Fulton LL, Fulton R, Heath SE, Kalicki-Veizer J, Kandoth C, Klco JM, Koboldt DC, Kanchi KL, Kulkarni S et al. (2013) Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med* 368: 2059-74

Cao R, Zhang Y (2004) SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. *Mol Cell* 15: 57-67

Caron C, Pivot-Pajot C, van Grunsven LA, Col E, Lestrat C, Rousseaux S, Khochbin S (2003) Cdyl: a new transcriptional co-repressor. *EMBO Rep* 4: 877-82

Caye A, Strullu M, Guidez F, Cassinat B, Gazal S, Fenneteau O, Lainey E, Nouri K, Nakhaei-Rad S, Dvorsky R, Lachenaud J, Pereira S, Vivent J, Verger E, Vidaud D, Galambrun C, Picard C, Petit A, Contet A, Poiree M et al. (2015) Juvenile myelomonocytic leukemia displays mutations in components of the RAS pathway and the PRC2 network. *Nat Genet* 47: 1334-40

Celik H, Koh WK, Kramer AC, Ostrander EL, Mallaney C, Fisher DAC, Xiang J, Wilson WC, Martens A, Kothari A, Fishberger G, Tycksen E, Karpova D, Duncavage EJ, Lee Y, Oh ST, Challen GA (2018) JARID2 Functions as a Tumor Suppressor in Myeloid Neoplasms by Repressing Self-Renewal in Hematopoietic Progenitor Cells. *Cancer Cell* 34: 741-756 e8

Cerquozzi S, Tefferi A (2015) Blast transformation and fibrotic progression in polycythemia vera and essential thrombocythemia: a literature review of incidence and risk factors. *Blood Cancer J* 5: e366

Chachoua I, Pecquet C, El-Khoury M, Nivarthi H, Albu RI, Marty C, Gryshkova V, Defour JP, Vertenoeil G, Ngo A, Koay A, Raslova H, Courtoy PJ, Choong ML, Plo I, Vainchenker W, Kralovics R, Constantinescu SN (2016) Thrombopoietin receptor activation by myeloproliferative neoplasm associated calreticulin mutants. *Blood* 127: 1325-35

Challen GA, Sun D, Mayle A, Jeong M, Luo M, Rodriguez B, Mallaney C, Celik H, Yang L, Xia Z, Cullen S, Berg J, Zheng Y, Darlington GJ, Li W, Goodell MA (2014) Dnmt3a and Dnmt3b have overlapping and distinct functions in hematopoietic stem cells. *Cell Stem Cell* 15: 350-364

Champion KM, Gilbert JG, Asimakopoulos FA, Hinshelwood S, Green AR (1997) Clonal haemopoiesis in normal elderly women: implications for the myeloproliferative disorders and myelodysplastic syndromes. *Br J Haematol* 97: 920-6

Chan CH, Lee SW, Li CF, Wang J, Yang WL, Wu CY, Wu J, Nakayama KI, Kang HY, Huang HY, Hung MC, Pandolfi PP, Lin HK (2010) Deciphering the transcriptional complex critical for RhoA gene expression and cancer metastasis. *Nat Cell Biol* 12: 457-67

Chase A, Leung W, Tapper W, Jones AV, Knoops L, Rasi C, Forsberg LA, Guglielmelli P, Zoi K, Hall V, Chiecchio L, Eder-Azanza L, Bryant C, Lannfelt L, Docherty L, White HE, Score J, Mackay DJ, Vannucchi AM, Dumanski JP et al. (2015) Profound parental bias associated with chromosome 14 acquired uniparental disomy indicates targeting of an imprinted locus. *Leukemia* 29: 2069-74

Chen C, Liu Y, Rappaport AR, Kitzing T, Schultz N, Zhao Z, Shroff AS, Dickins RA, Vakoc CR, Bradner JE, Stock W, LeBeau MM, Shannon KM, Kogan S, Zuber J, Lowe SW (2014) MLL3 is a haploinsufficient 7q tumor suppressor in acute myeloid leukemia. *Cancer Cell* 25: 652-65

Chen D, Pacal M, Wenzel P, Knoepfler PS, Leone G, Bremner R (2009) Division and apoptosis of E2f-deficient retinal progenitors. *Nature* 462: 925-9

Chen E, Beer PA, Godfrey AL, Ortmann CA, Li J, Costa-Pereira AP, Ingle CE, Dermitzakis ET, Campbell PJ, Green AR (2010) Distinct clinical phenotypes associated with JAK2V617F reflect differential STAT1 signaling. *Cancer Cell* 18: 524-35

Chong JL, Wenzel PL, Saenz-Robles MT, Nair V, Ferrey A, Hagan JP, Gomez YM, Sharma N, Chen HZ, Ouseph M, Wang SH, Trikha P, Culp B, Mezache L, Winton DJ, Sansom OJ, Chen D, Bremner R, Cantalupo PG, Robinson ML et al. (2009) E2f1-3 switch from activators in progenitor cells to repressors in differentiating cells. *Nature* 462: 930-4

Cichowski K, Jacks T (2001) NF1 tumor suppressor gene function: narrowing the GAP. *Cell* 104: 593-604

Cimmino L, Abdel-Wahab O, Levine RL, Aifantis I (2011) TET family proteins and their role in stem cell differentiation and transformation. *Cell Stem Cell* 9: 193-204

Coombs CC, Zehir A, Devlin SM, Kishtagari A, Syed A, Jonsson P, Hyman DM, Solit DB, Robson ME, Baselga J, Arcila ME, Ladanyi M, Tallman MS, Levine RL, Berger MF (2017) Therapy-Related Clonal Hematopoiesis in Patients with Non-hematologic Cancers Is Common and Associated with Adverse Clinical Outcomes. *Cell Stem Cell* 21: 374-382 e4

Cooper S, Grijzenhout A, Underwood E, Ancelin K, Zhang T, Nesterova TB, Anil-Kirmizitas B, Bassett A, Kooistra SM, Agger K, Helin K, Heard E, Brockdorff N (2016) Jarid2 binds mono-ubiquitylated H2A lysine 119 to mediate crosstalk between Polycomb complexes PRC1 and PRC2. *Nat Commun* 7: 13661

Courtier F, Carbuccia N, Garnier S, Guille A, Adelaide J, Cervera N, Gelsi-Boyer V, Mozziconacci MJ, Rey J, Vey N, Chaffanet M, Birnbaum D, Murati A (2017) Genomic analysis of myeloproliferative neoplasms in chronic and acute phases. *Haematologica* 102: e11-e14

Cutts BA, Sjogren AK, Andersson KM, Wahlstrom AM, Karlsson C, Swolin B, Bergo MO (2009) Nf1 deficiency cooperates with oncogenic K-RAS to induce acute myeloid leukemia in mice. *Blood* 114: 3629-32

Dameshek W (1951) Some speculations on the myeloproliferative syndromes. *Blood* 6: 372-5

Damm F, Chesnais V, Nagata Y, Yoshida K, Scourzic L, Okuno Y, Itzykson R, Sanada M, Shiraishi Y, Gelsi-Boyer V, Renneville A, Miyano S, Mori H, Shih LY, Park S, Dreyfus F, Guerci-Bresler A, Solary E, Rose C, Cheze S et al. (2013) BCOR and BCORL1 mutations in myelodysplastic syndromes and related disorders. *Blood* 122: 3169-77

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: 819-22

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: 945-61

De Kouchkovsky I, Abdul-Hay M (2016) 'Acute myeloid leukemia: a comprehensive review and 2016 update'. *Blood Cancer J* 6: e441

De Raedt T, Beert E, Pasmant E, Luscan A, Brems H, Ortonne N, Helin K, Hornick JL, Mautner V, Kehrer-Sawatzki H, Clapp W, Bradner J, Vidaud M, Upadhyaya M, Legius E, Cichowski K (2014) PRC2 loss amplifies Ras-driven transcription and confers sensitivity to BRD4-based therapies. *Nature* 514: 247-51

Defour JP, Chachoua I, Pecquet C, Constantinescu SN (2016) Oncogenic activation of MPL/thrombopoietin receptor by 17 mutations at W515: implications for myeloproliferative neoplasms. *Leukemia* 30: 1214-6

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 et al. (2009) Mutation in TET2 in myeloid cancers. *N Engl J Med* 360: 2289-301

Ding J, Komatsu H, Iida S, Yano H, Kusumoto S, Inagaki A, Mori F, Ri M, Ito A, Wakita A, Ishida T, Nitta M, Ueda R (2009) The Asn505 mutation of the c-MPL gene, which causes familial essential thrombocythemia, induces autonomous homodimerization of the c-Mpl protein due to strong amino acid polarity. *Blood* 114: 3325-8

Ding J, Komatsu H, Wakita A, Kato-Uranishi M, Ito M, Satoh A, Tsuboi K, Nitta M, Miyazaki H, Iida S, Ueda R (2004) Familial essential thrombocythemia associated with a dominant-positive activating mutation of the c-MPL gene, which encodes for the receptor for thrombopoietin. *Blood* 103: 4198-200

Dohner H, Estey E, Grimwade D, Amadori S, Appelbaum FR, Buchner T, Dombret H, Ebert BL, Fenaux P, Larson RA, Levine RL, Lo-Coco F, Naoe T, Niederwieser D, Ossenkoppele GJ, Sanz M, Sierra J, Tallman MS, Tien HF, Wei AH et al. (2017) Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood* 129: 424-447

Dopeso H, Rodrigues P, Bilic J, Bazzocco S, Carton-Garcia F, Macaya I, de Marcondes PG, Anguita E, Masanas M, Jimenez-Flores LM, Martinez-Barriocanal A, Nieto R, Segura MF, Schwartz S, Jr., Mariadason JM, Arango D (2018) Mechanisms of inactivation of the tumour suppressor gene RHOA in colorectal cancer. *Br J Cancer* 118: 106-116

Doulatov S, Notta F, Laurenti E, Dick JE (2012) Hematopoiesis: a human perspective. *Cell Stem Cell* 10: 120-36

Elf S, Abdelfattah NS, Chen E, Perales-Paton J, Rosen EA, Ko A, Peisker F, Florescu N, Giannini S, Wolach O, Morgan EA, Tothova Z, Losman JA, Schneider RK, Al-Shahrour F, Mullally A (2016) Mutant Calreticulin Requires Both Its Mutant C-terminus and the Thrombopoietin Receptor for Oncogenic Transformation. *Cancer Discov* 6: 368-81

Ellgaard L, Frickel EM (2003) Calnexin, calreticulin, and ERp57: teammates in glycoprotein folding. *Cell Biochem Biophys* 39: 223-47

Engle EK, Fisher DA, Miller CA, McLellan MD, Fulton RS, Moore DM, Wilson RK, Ley TJ, Oh ST (2015) Clonal evolution revealed by whole genome sequencing in a case of primary myelofibrosis transformed to secondary acute myeloid leukemia. *Leukemia* 29: 869-76

Ernst T, Chase AJ, Score J, Hidalgo-Curtis CE, Bryant C, Jones AV, Waghorn K, Zoi K, Ross FM, Reiter A, Hochhaus A, Drexler HG, Duncombe A, Cervantes F, Oscier D, Boultwood J, Grand FH, Cross NC (2010) Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. *Nat Genet* 42: 722-6

Fader CM, Savina A, Sanchez D, Colombo MI (2005) Exosome secretion and red cell maturation: Exploring molecular components involved in the docking and fusion of multivesicular bodies in K562 cells. *Blood Cells Mol Dis* 35: 153-7

Fenaux P, Giagounidis A, Selleslag D, Beyne-Rauzy O, Mufti G, Mittelman M, Muus P, Te Boekhorst P, Sanz G, Del Canizo C, Guerci-Bresler A, Nilsson L, Platzbecker U, Lubbert M, Quesnel B, Cazzola M, Ganser A, Bowen D, Schlegelberger B, Aul C et al. (2011) A randomized phase 3 study of lenalidomide versus placebo in RBC transfusion-dependent patients with Low-/Intermediate-1-risk myelodysplastic syndromes with del5q. *Blood* 118: 3765-76

Ferrando AA, Lopez-Otin C (2017) Clonal evolution in leukemia. *Nat Med* 23: 1135-1145

Feurstein S, Rucker FG, Bullinger L, Hofmann W, Manukjan G, Gohring G, Lehmann U, Heuser M, Ganser A, Dohner K, Schlegelberger B, Steinemann D (2014) Haploinsufficiency of ETV6 and CDKN1B in patients with acute myeloid leukemia and complex karyotype. *BMC Genomics* 15: 784

Finazzi G, Vannucchi AM, Barbui T (2018) Prefibrotic myelofibrosis: treatment algorithm 2018. *Blood Cancer J* 8: 104

Fritz G, Brachetti C, Bahlmann F, Schmidt M, Kaina B (2002) Rho GTPases in human breast tumours: expression and mutation analyses and correlation with clinical parameters. *Br J Cancer* 87: 635-44

Froehlich J, Versapuech M, Megrelis L, Largeteau Q, Meunier S, Tanchot C, Bismuth G, Delon J, Mangeney M (2016) FAM65B controls the proliferation of transformed and primary T cells. *Oncotarget* 7: 63215-63225

Fuster JJ, MacLauchlan S, Zuriaga MA, Polackal MN, Ostriker AC, Chakraborty R, Wu CL, Sano S, Muralidharan S, Rius C, Vuong J, Jacob S, Muralidhar V, Robertson AA, Cooper MA, Andres V, Hirschi KK, Martin KA, Walsh K (2017) Clonal hematopoiesis associated with TET2 deficiency accelerates atherosclerosis development in mice. *Science* 355: 842-847

Gao K, Tang W, Li Y, Zhang P, Wang D, Yu L, Wang C, Wu D (2015) Front-signal-dependent accumulation of the RHOA inhibitor FAM65B at leading edges polarizes neutrophils. *J Cell Sci* 128: 992-1000

Gao Y, Li H, Ma X, Fan Y, Ni D, Zhang Y, Huang Q, Liu K, Li X, Wang L, Yao Y, Ai Q, Zhang X (2017) E2F3 upregulation promotes tumor malignancy through the transcriptional activation of HIF-2alpha in clear cell renal cell carcinoma. *Oncotarget* 8: 54021-54036

Garg R, Faderl S, Garcia-Manero G, Cortes J, Koller C, Huang X, York S, Pierce S, Brandt M, Beran M, Borthakur G, Kantarjian H, Ravandi F (2009) Phase II study of rabbit anti-thymocyte globulin, cyclosporine and granulocyte colony-stimulating factor in patients with aplastic anemia and myelodysplastic syndrome. *Leukemia* 23: 1297-302

Gatto S, Ball G, Onida F, Kantarjian HM, Estey EH, Beran M (2003) Contribution of beta-2 microglobulin levels to the prognostic stratification of survival in patients with myelodysplastic syndrome (MDS). *Blood* 102: 1622-5

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: 788-800

Genovese G, Kahler AK, Handsaker RE, Lindberg J, Rose SA, Bakhoum SF, Chambert K, Mick E, Neale BM, Fromer M, Purcell SM, Svantesson O, Landen M, Hoglund M, Lehmann S, Gabriel SB, Moran JL, Lander ES, Sullivan PF, Sklar P et al. (2014) Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N Engl J Med* 371: 2477-87

Germing U, Strupp C, Giagounidis A, Haas R, Gattermann N, Starke C, Aul C (2012) Evaluation of dysplasia through detailed cytomorphology in 3156 patients from the Dusseldorf Registry on myelodysplastic syndromes. *Leuk Res* 36: 727-34

Geyer HL, Kosiorek H, Dueck AC, Scherber R, Slot S, Zweegman S, Te Boekhorst PA, Senyak Z, Schouten HC, Sackmann F, Fuentes AK, Hernandez-Maraver D, Pahl HL, Griesshammer M, Stegelmann F, Dohner K, Lehmann T, Bonatz K, Reiter A, Boyer

F et al. (2017) Associations between gender, disease features and symptom burden in patients with myeloproliferative neoplasms: an analysis by the MPN QOL International Working Group. *Haematologica* 102: 85-93

Godfrey AL, Chen E, Pagano F, Silber Y, Campbell PJ, Green AR (2013) Clonal analyses reveal associations of JAK2V617F homozygosity with hematologic features, age and gender in polycythemia vera and essential thrombocythemia. *Haematologica* 98: 718-21

Gondek LP, Haddad AS, O'Keefe CL, Tiu R, Wlodarski MW, Sekeres MA, Theil KS, Maciejewski JP (2007) Detection of cryptic chromosomal lesions including acquired segmental uniparental disomy in advanced and low-risk myelodysplastic syndromes. *Exp Hematol* 35: 1728-38

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: 6182-92

Graubert TA, Shen D, Ding L, Okeyo-Owuor T, Lunn CL, Shao J, Krysiak K, Harris CC, Koboldt DC, Larson DE, McLellan MD, Dooling DJ, Abbott RM, Fulton RS, Schmidt H, Kalicki-Veizer J, O'Laughlin M, Grillot M, Baty J, Heath S et al. (2011) Recurrent mutations in the U2AF1 splicing factor in myelodysplastic syndromes. *Nat Genet* 44: 53-7

Green A, Beer P (2010) Somatic mutations of IDH1 and IDH2 in the leukemic transformation of myeloproliferative neoplasms. *N Engl J Med* 362: 369-70

Greenberg PL, Tuechler H, Schanz J, Sanz G, Garcia-Manero G, Sole F, Bennett JM, Bowen D, Fenaux P, Dreyfus F, Kantarjian H, Kuendgen A, Levis A, Malcovati L, Cazzola M, Cermak J, Fonatsch C, Le Beau MM, Slovak ML, Krieger O et al. (2012) Revised international prognostic scoring system for myelodysplastic syndromes. *Blood* 120: 2454-65

Grinfeld J, Nangalia J, Baxter EJ, Wedge DC, Angelopoulos N, Cantrill R, Godfrey AL, Papaemmanuil E, Gundem G, MacLean C, Cook J, O'Neil L, O'Meara S, Teague JW, Butler AP, Massie CE, Williams N, Nice FL, Andersen CL, Hasselbalch HC et al. (2018)

Classification and Personalized Prognosis in Myeloproliferative Neoplasms. *N Engl J Med* 379: 1416-1430

Guglielmelli P, Biamonte F, Score J, Hidalgo-Curtis C, Cervantes F, Maffioli M, Fanelli T, Ernst T, Winkelman N, Jones AV, Zoi K, Reiter A, Duncombe A, Villani L, Bosi A, Barosi G, Cross NC, Vannucchi AM (2011) EZH2 mutational status predicts poor survival in myelofibrosis. *Blood* 118: 5227-34

Guglielmelli P, Lasho TL, Rotunno G, Score J, Mannarelli C, Pancrazzi A, Biamonte F, Pardanani A, Zoi K, Reiter A, Duncombe A, Fanelli T, Pietra D, Rumi E, Finke C, Gangat N, Ketterling RP, Knudson RA, Hanson CA, Bosi A et al. (2014) The number of prognostically detrimental mutations and prognosis in primary myelofibrosis: an international study of 797 patients. *Leukemia* 28: 1804-10

Guglielmelli P, Pacilli A, Rotunno G, Rumi E, Rosti V, Delaini F, Maffioli M, Fanelli T, Pancrazzi A, Pietra D, Salmoiraghi S, Mannarelli C, Franci A, Paoli C, Rambaldi A, Passamonti F, Barosi G, Barbui T, Cazzola M, Vannucchi AM et al. (2017) Presentation and outcome of patients with 2016 WHO diagnosis of prefibrotic and overt primary myelofibrosis. *Blood* 129: 3227-3236

Haase D, Germing U, Schanz J, Pfeilstocker M, Nosslinger T, Hildebrandt B, Kundgen A, Lubbert M, Kunzmann R, Giagounidis AA, Aul C, Trumper L, Krieger O, Stauder R, Muller TH, Wimazal F, Valent P, Fonatsch C, Steidl C (2007) New insights into the prognostic impact of the karyotype in MDS and correlation with subtypes: evidence from a core dataset of 2124 patients. *Blood* 110: 4385-95

Haase D, Stevenson KE, Neuberg D, Maciejewski JP, Nazha A, Sekeres MA, Ebert BL, Garcia-Manero G, Haferlach C, Haferlach T, Kern W, Ogawa S, Nagata Y, Yoshida K, Graubert TA, Walter MJ, List AF, Komrokji RS, Padron E, Sallman D et al. (2019) TP53 mutation status divides myelodysplastic syndromes with complex karyotypes into distinct prognostic subgroups. *Leukemia* 33: 1747-1758

Haferlach T, Nagata Y, Grossmann V, Okuno Y, Bacher U, Nagae G, Schnittger S, Sanada M, Kon A, Alpermann T, Yoshida K, Roller A, Nadarajah N, Shiraishi Y, Shiozawa Y, Chiba K, Tanaka H, Koeffler HP, Klein HU, Dugas M et al. (2014) Landscape of genetic lesions in 944 patients with myelodysplastic syndromes. *Leukemia* 28: 241-7

Hafner A, Bulyk ML, Jambhekar A, Lahav G (2019) The multiple mechanisms that regulate p53 activity and cell fate. *Nat Rev Mol Cell Biol* 20: 199-210

Haga RB, Ridley AJ (2016) Rho GTPases: Regulation and roles in cancer cell biology. *Small GTPases* 7: 207-221

Han L, Schubert C, Kohler J, Schemionek M, Isfort S, Brummendorf TH, Koschmieder S, Chatain N (2016) Calreticulin-mutant proteins induce megakaryocytic signaling to transform hematopoietic cells and undergo accelerated degradation and Golgi-mediated secretion. *J Hematol Oncol* 9: 45

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

Harutyunyan AS, Giambruno R, Krendl C, Stukalov A, Klampfl T, Berg T, Chen D, Milosevic Feenstra JD, Jager R, Gisslinger B, Gisslinger H, Rumi E, Passamonti F, Pietra D, Muller AC, Parapatics K, Breitwieser FP, Herrmann R, Colinge J, Bennett KL et al. (2016) Germline RBBP6 mutations in familial myeloproliferative neoplasms. *Blood* 127: 362-5

Haupt Y, Maya R, Kazaz A, Oren M (1997) Mdm2 promotes the rapid degradation of p53. *Nature* 387: 296-9

Hayette S, Thomas X, Jallades L, Chabane K, Charlot C, Tigaud I, Gazzo S, Morisset S, Cornillet-Lefebvre P, Plesa A, Huet S, Renneville A, Salles G, Nicolini FE, Magaud JP, Michallet M (2012) High DNA methyltransferase DNMT3B levels: a poor prognostic marker in acute myeloid leukemia. *PLoS One* 7: e51527

Hinds DA, Barnholt KE, Mesa RA, Kiefer AK, Do CB, Eriksson N, Mountain JL, Francke U, Tung JY, Nguyen HM, Zhang H, Gojenola L, Zehnder JL, Gotlib J (2016) Germ line variants predispose to both JAK2 V617F clonal hematopoiesis and myeloproliferative neoplasms. *Blood* 128: 1121-8

Hock H, Meade E, Medeiros S, Schindler JW, Valk PJ, Fujiwara Y, Orkin SH (2004) Tel/Etv6 is an essential and selective regulator of adult hematopoietic stem cell survival. *Genes Dev* 18: 2336-41

Hollenhorst PC, McIntosh LP, Graves BJ (2011) Genomic and biochemical insights into the specificity of ETS transcription factors. *Annu Rev Biochem* 80: 437-71

Honda R, Tanaka H, Yasuda H (1997) Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett* 420: 25-7

Hookham MB, Elliott J, Suessmuth Y, Staerk J, Ward AC, Vainchenker W, Percy MJ, McMullin MF, Constantinescu SN, Johnston JA (2007) The myeloproliferative disorder-associated JAK2 V617F mutant escapes negative regulation by suppressor of cytokine signaling 3. *Blood* 109: 4924-9

Hosono N, Makishima H, Mahfouz R, Przychodzen B, Yoshida K, Jerez A, LaFramboise T, Polprasert C, Clemente MJ, Shiraishi Y, Chiba K, Tanaka H, Miyano S, Sanada M, Cui E, Verma AK, McDevitt MA, List AF, Sauntharajah Y, Sekeres MA et al. (2017) Recurrent genetic defects on chromosome 5q in myeloid neoplasms. *Oncotarget* 8: 6483-6495

Hou HA, Tsai CH, Lin CC, Chou WC, Kuo YY, Liu CY, Tseng MH, Peng YL, Liu MC, Liu CW, Liao XW, Lin LI, Yao M, Tang JL, Tien HF (2018) Incorporation of mutations in five genes in the revised International Prognostic Scoring System can improve risk stratification in the patients with myelodysplastic syndrome. *Blood Cancer J* 8: 39

Hsu JI, Dayaram T, Tovy A, De Braekeleer E, Jeong M, Wang F, Zhang J, Heffernan TP, Gera S, Kovacs JJ, Marszalek JR, Bristow C, Yan Y, Garcia-Manero G, Kantarjian H, Vassiliou G, Futreal PA, Donehower LA, Takahashi K, Goodell MA (2018) PPM1D Mutations Drive Clonal Hematopoiesis in Response to Cytotoxic Chemotherapy. *Cell Stem Cell* 23: 700-713 e6

Huang H, Kong D, Byun KH, Ye C, Koda S, Lee DH, Oh BC, Lee SW, Lee B, Zabolotny JM, Kim MS, Bjorbaek C, Lowell BB, Kim YB (2012) Rho-kinase regulates energy balance by targeting hypothalamic leptin receptor signaling. *Nat Neurosci* 15: 1391-8

Huether R, Dong L, Chen X, Wu G, Parker M, Wei L, Ma J, Edmonson MN, Hedlund EK, Rusch MC, Shurtleff SA, Mulder HL, Boggs K, Vadordaria B, Cheng J, Yergeau D, Song G, Becksfort J, Lemmon G, Weber C et al. (2014) The landscape of somatic mutations in epigenetic regulators across 1,000 paediatric cancer genomes. *Nat Commun* 5: 3630

Hulegardh E, Nilsson C, Lazarevic V, Garelius H, Antunovic P, Rangert Derolf A, Mollgard L, Uggla B, Wennstrom L, Wahlin A, Hoglund M, Juliusson G, Stockelberg D, Lehmann S (2015) Characterization and prognostic features of secondary acute myeloid leukemia in a population-based setting: a report from the Swedish Acute Leukemia Registry. *Am J Hematol* 90: 208-14

Hurtado C, Erquiaga I, Aranaz P, Migueliz I, Garcia-Delgado M, Novo FJ, Vizmanos JL (2011) LNK can also be mutated outside PH and SH2 domains in myeloproliferative neoplasms with and without V617FJAK2 mutation. *Leuk Res* 35: 1537-9

Ianotto JC, Chauveau A, Boyer-Perrard F, Gyan E, Laribi K, Cony-Makhoul P, Demory JL, de Renzis B, Dosquet C, Rey J, Roy L, Dupriez B, Knoops L, Legros L, Malou M, Hutin P, Ranta D, Benbrahim O, Ugo V, Lippert E et al. (2018) Benefits and pitfalls of pegylated interferon-alpha2a therapy in patients with myeloproliferative neoplasm-associated myelofibrosis: a French Intergroup of Myeloproliferative neoplasms (FIM) study. *Haematologica* 103: 438-446

Imi T, Katagiri T, Hosomichi K, Zaimoku Y, Hoang Nguyen V, Nakagawa N, Tajima A, Yoshizato T, Ogawa S, Nakao S (2018) Sustained clonal hematopoiesis by HLA-lacking hematopoietic stem cells without driver mutations in aplastic anemia. *Blood Adv* 2: 1000-1012

Inoue D, Bradley RK, Abdel-Wahab O (2016) Spliceosomal gene mutations in myelodysplasia: molecular links to clonal abnormalities of hematopoiesis. *Genes Dev* 30: 989-1001

Itzykson R, Kosmider O, Cluzeau T, Mansat-De Mas V, Dreyfus F, Beyne-Rauzy O, Quesnel B, Vey N, Gelsi-Boyer V, Raynaud S, Preudhomme C, Ades L, Fenaux P, Fontenay M, Groupe Francophone des M (2011) Impact of TET2 mutations on response rate to azacitidine in myelodysplastic syndromes and low blast count acute myeloid leukemias. *Leukemia* 25: 1147-52

Jabbour E, Kantarjian H (2018) Chronic myeloid leukemia: 2018 update on diagnosis, therapy and monitoring. *Am J Hematol* 93: 442-459

Jacobs KB, Yeager M, Zhou W, Wacholder S, Wang Z, Rodriguez-Santiago B, Hutchinson A, Deng X, Liu C, Horner MJ, Cullen M, Epstein CG, Burdett L, Dean MC, Chatterjee N, Sampson J, Chung CC, Kovaks J, Gapstur SM, Stevens VL et al. (2012)

Detectable clonal mosaicism and its relationship to aging and cancer. *Nat Genet* 44: 651-8

Jadersten M, Saft L, Pellagatti A, Gohring G, Wainscoat JS, Boultwood J, Porwit A, Schlegelberger B, Hellstrom-Lindberg E (2009) Clonal heterogeneity in the 5q- syndrome: p53 expressing progenitors prevail during lenalidomide treatment and expand at disease progression. *Haematologica* 94: 1762-6

Jadersten M, Saft L, Smith A, Kulasekararaj A, Pomplun S, Gohring G, Hedlund A, Hast R, Schlegelberger B, Porwit A, Hellstrom-Lindberg E, Mufti GJ (2011) TP53 mutations in low-risk myelodysplastic syndromes with del(5q) predict disease progression. *J Clin Oncol* 29: 1971-9

Jaiswal S, Fontanillas P, Flannick J, Manning A, Grauman PV, Mar BG, Lindsley RC, Mermel CH, Burtt N, Chavez A, Higgins JM, Moltchanov V, Kuo FC, Kluk MJ, Henderson B, Kinnunen L, Koistinen HA, Ladenvall C, Getz G, Correa A et al. (2014) Age-related clonal hematopoiesis associated with adverse outcomes. *N Engl J Med* 371: 2488-98

Jaiswal S, Natarajan P, Silver AJ, Gibson CJ, Bick AG, Shvartz E, McConkey M, Gupta N, Gabriel S, Ardissino D, Baber U, Mehran R, Fuster V, Danesh J, Frossard P, Saleheen D, Melander O, Sukhova GK, Neuberg D, Libby P et al. (2017) Clonal Hematopoiesis and Risk of Atherosclerotic Cardiovascular Disease. *N Engl J Med* 377: 111-121

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: 1144-8

Jamieson CH, Gotlib J, Durocher JA, Chao MP, Mariappan MR, Lay M, Jones C, Zehnder JL, Lilleberg SL, Weissman IL (2006) The JAK2 V617F mutation occurs in hematopoietic stem cells in polycythemia vera and predisposes toward erythroid differentiation. *Proc Natl Acad Sci U S A* 103: 6224-9

Jan M, Snyder TM, Corces-Zimmerman MR, Vyas P, Weissman IL, Quake SR, Majeti R (2012) Clonal evolution of preleukemic hematopoietic stem cells precedes human acute myeloid leukemia. *Sci Transl Med* 4: 149ra118

Jankowska AM, Szpurka H, Tiu RV, Makishima H, Afable M, Huh J, O'Keefe CL, Ganetzky R, McDevitt MA, Maciejewski JP (2009) Loss of heterozygosity 4q24 and TET2 mutations associated with myelodysplastic/myeloproliferative neoplasms. *Blood* 113: 6403-10

Jeong M, Park HJ, Celik H, Ostrander EL, Reyes JM, Guzman A, Rodriguez B, Lei Y, Lee Y, Ding L, Guryanova OA, Li W, Goodell MA, Challen GA (2018) Loss of Dnmt3a Immortalizes Hematopoietic Stem Cells In Vivo. *Cell Rep* 23: 1-10

Jerez A, Gondek LP, Jankowska AM, Makishima H, Przychodzen B, Tiu RV, O'Keefe CL, Mohamedali AM, Batista D, Sekeres MA, McDevitt MA, Mufti GJ, Maciejewski JP (2012) Topography, clinical, and genomic correlates of 5q myeloid malignancies revisited. *J Clin Oncol* 30: 1343-9

Jiao L, Liu X (2015) Structural basis of histone H3K27 trimethylation by an active polycomb repressive complex 2. *Science* 350: aac4383

Jost E, do ON, Dahl E, Maintz CE, Jousten P, Habets L, Wilop S, Herman JG, Osieka R, Galm O (2007) Epigenetic alterations complement mutation of JAK2 tyrosine kinase in patients with BCR/ABL-negative myeloproliferative disorders. *Leukemia* 21: 505-10

Kagey MH, Newman JJ, Bilodeau S, Zhan Y, Orlando DA, van Berkum NL, Ebmeier CC, Goossens J, Rahl PB, Levine SS, Taatjes DJ, Dekker J, Young RA (2010) Mediator and cohesin connect gene expression and chromatin architecture. *Nature* 467: 430-5

Kandoth C, McLellan MD, Vandin F, Ye K, Niu B, Lu C, Xie M, Zhang Q, McMichael JF, Wyczalkowski MA, Leiserson MDM, Miller CA, Welch JS, Walter MJ, Wendl MC, Ley TJ, Wilson RK, Raphael BJ, Ding L (2013) Mutational landscape and significance across 12 major cancer types. *Nature* 502: 333-339

Kasinath V, Faini M, Poepsel S, Reif D, Feng XA, Stjepanovic G, Aebersold R, Nogales E (2018) Structures of human PRC2 with its cofactors AEBP2 and JARID2. *Science* 359: 940-944

Katagiri T, Sato-Otsubo A, Kashiwase K, Morishima S, Sato Y, Mori Y, Kato M, Sanada M, Morishima Y, Hosokawa K, Sasaki Y, Ohtake S, Ogawa S, Nakao S, Japan Marrow

Donor P (2011) Frequent loss of HLA alleles associated with copy number-neutral 6pLOH in acquired aplastic anemia. *Blood* 118: 6601-9

Khan SN, Jankowska AM, Mahfouz R, Dunbar AJ, Sugimoto Y, Hosono N, Hu Z, Cheriyath V, Vatolin S, Przychodzen B, Reu FJ, Saunthararajah Y, O'Keefe C, Sekeres MA, List AF, Moliterno AR, McDevitt MA, Maciejewski JP, Makishima H (2013) Multiple mechanisms deregulate EZH2 and histone H3 lysine 27 epigenetic changes in myeloid malignancies. *Leukemia* 27: 1301-9

Kim E, Ilagan JO, Liang Y, Daubner GM, Lee SC, Ramakrishnan A, Li Y, Chung YR, Micol JB, Murphy ME, Cho H, Kim MK, Zebari AS, Aumann S, Park CY, Buonamici S, Smith PG, Deeg HJ, Lobry C, Aifantis I et al. (2015) SRSF2 Mutations Contribute to Myelodysplasia by Mutant-Specific Effects on Exon Recognition. *Cancer Cell* 27: 617-30

Klampfl T, Gisslinger H, Harutyunyan AS, Nivarthi H, Rumi E, Milosevic JD, Them NC, Berg T, Gisslinger B, Pietra D, Chen D, Vladimer GI, Bagienski K, Milanesi C, Casetti IC, Sant'Antonio E, Ferretti V, Elena C, Schischlik F, Cleary C et al. (2013a) Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med* 369: 2379-90

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

Klampfl T, Milosevic JD, Puda A, Schonegger A, Bagienski K, Berg T, Harutyunyan AS, Gisslinger B, Rumi E, Malcovati L, Pietra D, Elena C, Della Porta MG, Pieri L, Guglielmelli P, Bock C, Doubek M, Dvorakova D, Suvajdzic N, Tomin D et al. (2013b) Complex patterns of chromosome 11 aberrations in myeloid malignancies target CBL, MLL, DDB1 and LMO2. *PLoS One* 8: e77819

Klein AM, Simons BD (2011) Universal patterns of stem cell fate in cycling adult tissues. *Development* 138: 3103-11

Koenigsmann J, Rudolph C, Sander S, Kershaw O, Gruber AD, Bullinger L, Schlegelberger B, Carstanjen D (2009) Nf1 haploinsufficiency and Icsbp deficiency synergize in the development of leukemias. *Blood* 113: 4690-701

Koya J, Kataoka K, Sato T, Bando M, Kato Y, Tsuruta-Kishino T, Kobayashi H, Narukawa K, Miyoshi H, Shirahige K, Kurokawa M (2016) DNMT3A R882 mutants interact with polycomb proteins to block haematopoietic stem and leukaemic cell differentiation. *Nat Commun* 7: 10924

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: 229-36

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: 1779-90

Kronke J, Fink EC, Hollenbach PW, MacBeth KJ, Hurst SN, Udeshi ND, Chamberlain PP, Mani DR, Man HW, Gandhi AK, Svinkina T, Schneider RK, McConkey M, Jaras M, Griffiths E, Wetzler M, Bullinger L, Cathers BE, Carr SA, Chopra R et al. (2015) Lenalidomide induces ubiquitination and degradation of CK1alpha in del(5q) MDS. *Nature* 523: 183-188

Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D, Jones SJ, Marra MA (2009) Circos: an information aesthetic for comparative genomics. *Genome Res* 19: 1639-45

Kubesova B, Pavlova S, Malcikova J, Kabathova J, Radova L, Tom N, Tichy B, Plevova K, Kantorova B, Fiedorova K, Slavikova M, Bystry V, Kissova J, Gisslinger B, Gisslinger H, Penka M, Mayer J, Kralovics R, Pospisilova S, Doubek M (2018) Low-burden TP53 mutations in chronic phase of myeloproliferative neoplasms: association with age, hydroxyurea administration, disease type and JAK2 mutational status. *Leukemia* 32: 450-461

Kwok B, Hall JM, Witte JS, Xu Y, Reddy P, Lin K, Flamholz R, Dabbas B, Yung A, Al-Hafidh J, Balmert E, Vaupel C, El Hader C, McGinniss MJ, Nahas SA, Kines J, Bejar R (2015) MDS-associated somatic mutations and clonal hematopoiesis are common in idiopathic cytopenias of undetermined significance. *Blood* 126: 2355-61

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: 1652-60

Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, Minden M, Paterson B, Caligiuri MA, Dick JE (1994) A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 367: 645-8

Largaespada DA, Brannan CI, Jenkins NA, Copeland NG (1996) Nf1 deficiency causes Ras-mediated granulocyte/macrophage colony stimulating factor hypersensitivity and chronic myeloid leukaemia. *Nat Genet* 12: 137-43

Lasho TL, Mudireddy M, Finke CM, Hanson CA, Ketterling RP, Szuber N, Begna KH, Patnaik MM, Gangat N, Pardanani A, Tefferi A (2018) Targeted next-generation sequencing in blast phase myeloproliferative neoplasms. *Blood Adv* 2: 370-380

Lasho TL, Pardanani A, Tefferi A (2010) LNK mutations in JAK2 mutation-negative erythrocytosis. *N Engl J Med* 363: 1189-90

Laurie CC, Laurie CA, Rice K, Doheny KF, Zelnick LR, McHugh CP, Ling H, Hetrick KN, Pugh EW, Amos C, Wei Q, Wang LE, Lee JE, Barnes KC, Hansel NN, Mathias R, Daley D, Beaty TH, Scott AF, Ruczinski I et al. (2012) Detectable clonal mosaicism from birth to old age and its relationship to cancer. *Nat Genet* 44: 642-50

Lawrence MS, Stojanov P, Mermel CH, Robinson JT, Garraway LA, Golub TR, Meyerson M, Gabriel SB, Lander ES, Getz G (2014) Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature* 505: 495-501

Le DT, Kong N, Zhu Y, Lauchle JO, Aiyigari A, Braun BS, Wang E, Kogan SC, Le Beau MM, Parada L, Shannon KM (2004) Somatic inactivation of Nf1 in hematopoietic cells results in a progressive myeloproliferative disorder. *Blood* 103: 4243-50

Lee CH, Yu JR, Kumar S, Jin Y, LeRoy G, Bhanu N, Kaneko S, Garcia BA, Hamilton AD, Reinberg D (2018a) Allosteric Activation Dictates PRC2 Activity Independent of Its Recruitment to Chromatin. *Mol Cell* 70: 422-434 e6

Lee SC, North K, Kim E, Jang E, Obeng E, Lu SX, Liu B, Inoue D, Yoshimi A, Ki M, Yeo M, Zhang XJ, Kim MK, Cho H, Chung YR, Taylor J, Durham BH, Kim YJ, Pastore A, Monette S et al. (2018b) Synthetic Lethal and Convergent Biological Effects of Cancer-Associated Spliceosomal Gene Mutations. *Cancer Cell* 34: 225-241 e8

Lee TS, Ma W, Zhang X, Kantarjian H, Albitar M (2009) Structural effects of clinically observed mutations in JAK2 exons 13-15: comparison with V617F and exon 12 mutations. *BMC Struct Biol* 9: 58

Lee W, Teckie S, Wiesner T, Ran L, Prieto Granada CN, Lin M, Zhu S, Cao Z, Liang Y, Sboner A, Tap WD, Fletcher JA, Huberman KH, Qin LX, Viale A, Singer S, Zheng D, Berger MF, Chen Y, Antonescu CR et al. (2014) PRC2 is recurrently inactivated through EED or SUZ12 loss in malignant peripheral nerve sheath tumors. *Nat Genet* 46: 1227-32

Lessard J, Schumacher A, Thorsteinsdottir U, van Lohuizen M, Magnuson T, Sauvageau G (1999) Functional antagonism of the Polycomb-Group genes eed and Bmi1 in hemopoietic cell proliferation. *Genes Dev* 13: 2691-703

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 et al. (2005) Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell* 7: 387-97

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 et al. (2010) DNMT3A mutations in acute myeloid leukemia. *N Engl J Med* 363: 2424-33

Li J, Kent DG, Godfrey AL, Manning H, Nangalia J, Aziz A, Chen E, Saeb-Parsy K, Fink J, Sneade R, Hamilton TL, Pask DC, Silber Y, Zhao X, Ghevaert C, Liu P, Green AR (2014) JAK2V617F homozygosity drives a phenotypic switch in myeloproliferative neoplasms, but is insufficient to sustain disease. *Blood* 123: 3139-51

Li J, Prins D, Park HJ, Grinfeld J, Gonzalez-Arias C, Loughran S, Dovey OM, Klampfl T, Bennett C, Hamilton TL, Pask DC, Sneade R, Williams M, Aungier J, Ghevaert C, Vassiliou GS, Kent DG, Green AR (2018) Mutant calreticulin knockin mice develop thrombocytosis and myelofibrosis without a stem cell self-renewal advantage. *Blood* 131: 649-661

Li Z, Cai X, Cai CL, Wang J, Zhang W, Petersen BE, Yang FC, Xu M (2011) Deletion of Tet2 in mice leads to dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies. *Blood* 118: 4509-18

Lindsley RC (2017) Uncoding the genetic heterogeneity of myelodysplastic syndrome. *Hematology Am Soc Hematol Educ Program* 2017: 447-452

Lindsley RC, Mar BG, Mazzola E, Grauman PV, Shareef S, Allen SL, Pigneux A, Wetzler M, Stuart RK, Erba HP, Damon LE, Powell BL, Lindeman N, Steensma DP, Wadleigh M, DeAngelo DJ, Neuberg D, Stone RM, Ebert BL (2015) Acute myeloid leukemia ontogeny is defined by distinct somatic mutations. *Blood* 125: 1367-76

Ling L, Lobie PE (2004) RhoA/ROCK activation by growth hormone abrogates p300/histone deacetylase 6 repression of Stat5-mediated transcription. *J Biol Chem* 279: 32737-50

List A, Dewald G, Bennett J, Giagounidis A, Raza A, Feldman E, Powell B, Greenberg P, Thomas D, Stone R, Reeder C, Wride K, Patin J, Schmidt M, Zeldis J, Knight R (2006) Lenalidomide in the Myelodysplastic Syndrome with Chromosome 5q Deletion. *New England Journal of Medicine* 355: 1456-1465

Liu Y, Liu S, Yuan S, Yu H, Zhang Y, Yang X, Xie G, Chen Z, Li W, Xu B, Sun L, Shang Y, Liang J (2017) Chromodomain protein CDYL is required for transmission/restoration of repressive histone marks. *J Mol Cell Biol* 9: 178-194

Loberg MA, Bell RK, Goodwin LO, Eudy E, Miles LA, SanMiguel JM, Young K, Bergstrom DE, Levine RL, Schneider RK, Trowbridge JJ (2019) Sequentially inducible mouse models reveal that Npm1 mutation causes malignant transformation of Dnmt3a-mutant clonal hematopoiesis. *Leukemia* 33: 1635-1649

Loh ML (2011) Recent advances in the pathogenesis and treatment of juvenile myelomonocytic leukaemia. *Br J Haematol* 152: 677-87

Loh PR, Genovese G, Handsaker RE, Finucane HK, Reshef YA, Palamara PF, Birmann BM, Talkowski ME, Bakhoum SF, McCarroll SA, Price AL (2018) Insights into clonal haematopoiesis from 8,342 mosaic chromosomal alterations. *Nature* 559: 350-355

Lu X, Huang LJ, Lodish HF (2008) Dimerization by a cytokine receptor is necessary for constitutive activation of JAK2V617F. *J Biol Chem* 283: 5258-66

Lu X, Levine R, Tong W, Wernig G, Pikman Y, Zarnegar S, Gilliland DG, Lodish H (2005) Expression of a homodimeric type I cytokine receptor is required for JAK2V617F-mediated transformation. *Proc Natl Acad Sci U S A* 102: 18962-7

Lubeck DP, Danese M, Jennifer D, Miller K, Richhariya A, Garfin PM (2016) Systematic Literature Review of the Global Incidence and Prevalence of Myelodysplastic Syndrome and Acute Myeloid Leukemia. *Blood* 128: 5930-5930

Lundberg P, Karow A, Nienhold R, Looser R, Hao-Shen H, Nissen I, Girsberger S, Lehmann T, Passweg J, Stern M, Beisel C, Kralovics R, Skoda RC (2014) Clonal evolution and clinical correlates of somatic mutations in myeloproliferative neoplasms. *Blood* 123: 2220-8

Makishima H, Yoshizato T, Yoshida K, Sekeres MA, Radivoyevitch T, Suzuki H, Przychodzen B, Nagata Y, Meggendorfer M, Sanada M, Okuno Y, Hirsch C, Kuzmanovic T, Sato Y, Sato-Otsubo A, LaFramboise T, Hosono N, Shiraishi Y, Chiba K, Haferlach C et al. (2017) Dynamics of clonal evolution in myelodysplastic syndromes. *Nat Genet* 49: 204-212

Malcovati L, Karimi M, Papaemmanuil E, Ambaglio I, Jadersten M, Jansson M, Elena C, Galli A, Walldin G, Della Porta MG, Raaschou-Jensen K, Travaglino E, Kallenbach K, Pietra D, Ljungstrom V, Conte S, Boveri E, Invernizzi R, Rosenquist R, Campbell PJ et al. (2015) SF3B1 mutation identifies a distinct subset of myelodysplastic syndrome with ring sideroblasts. *Blood* 126: 233-41

Mali RS, Ramdas B, Ma P, Shi J, Munugalavadla V, Sims E, Wei L, Vemula S, Nabinger SC, Goodwin CB, Chan RJ, Traina F, Visconte V, Tiu RV, Lewis TA, Stern AM, Wen Q, Crispino JD, Boswell HS, Kapur R (2011) Rho kinase regulates the survival and transformation of cells bearing oncogenic forms of KIT, FLT3, and BCR-ABL. *Cancer Cell* 20: 357-69

Margueron R, Reinberg D (2011) The Polycomb complex PRC2 and its mark in life. *Nature* 469: 343-9

Martignoles JA, Delhommeau F, Hirsch P (2018) Genetic Hierarchy of Acute Myeloid Leukemia: From Clonal Hematopoiesis to Molecular Residual Disease. *Int J Mol Sci* 19

Marty C, Pecquet C, Nivarthi H, El-Khoury M, Chachoua I, Tulliez M, Villeval JL, Raslova H, Kralovics R, Constantinescu SN, Plo I, Vainchenker W (2016) Calreticulin mutants in mice induce an MPL-dependent thrombocytosis with frequent progression to myelofibrosis. *Blood* 127: 1317-24

Masarova L, Patel KP, Newberry KJ, Cortes J, Borthakur G, Konopleva M, Estrov Z, Kantarjian H, Verstovsek S (2017) Pegylated interferon alfa-2a in patients with essential thrombocythaemia or polycythaemia vera: a post-hoc, median 83 month follow-up of an open-label, phase 2 trial. *Lancet Haematol* 4: e165-e175

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: 975-83

Mead AJ, Mullally A (2017) Myeloproliferative neoplasm stem cells. *Blood* 129: 1607-1616

Megrelis L, El Ghoul E, Moalli F, Versapuech M, Cassim S, Ruef N, Stein JV, Mangeney M, Delon J (2018) Fam65b Phosphorylation Relieves Tonic RhoA Inhibition During T Cell Migration. *Front Immunol* 9: 2001

Merlinsky TR, Levine RL, Pronier E (2019) Unfolding the Role of Calreticulin in Myeloproliferative Neoplasm Pathogenesis. *Clin Cancer Res* 25: 2956-2962

Michaelis C, Ciosk R, Nasmyth K (1997) Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. *Cell* 91: 35-45

Milosevic Feenstra JD, Nivarthi H, Gisslinger H, Leroy E, Rumi E, Chachoua I, Bagienski K, Kubesova B, Pietra D, Gisslinger B, Milanesi C, Jager R, Chen D, Berg T, Schalling M, Schuster M, Bock C, Constantinescu SN, Cazzola M, Kralovics R (2016) Whole-exome sequencing identifies novel MPL and JAK2 mutations in triple-negative myeloproliferative neoplasms. *Blood* 127: 325-32

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 et al. (2012) Clinical significance of genetic aberrations in secondary acute myeloid leukemia. *Am J Hematol* 87: 1010-6

Montalban-Bravo G, Garcia-Manero G (2018) Myelodysplastic syndromes: 2018 update on diagnosis, risk-stratification and management. *Am J Hematol* 93: 129-147

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 et al. (2011) Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. *Cancer Cell* 20: 11-24

Mossner M, Jann JC, Nowak D, Platzbecker U, Giagounidis A, Gotze K, Letsch A, Haase D, Shirneshan K, Braulke F, Schlenk RF, Haferlach T, Schafhausen P, Bug G, Lubbert M, Ganser A, Busche G, Schuler E, Nowak V, Pressler J et al. (2016) Prevalence, clonal dynamics and clinical impact of TP53 mutations in patients with myelodysplastic syndrome with isolated deletion (5q) treated with lenalidomide: results from a prospective multicenter study of the german MDS study group (GMDS). *Leukemia* 30: 1956-9

Moulard O, Mehta J, Fryzek J, Olivares R, Iqbal U, Mesa RA (2014) Epidemiology of myelofibrosis, essential thrombocythemia, and polycythemia vera in the European Union. *Eur J Haematol* 92: 289-97

Mrozek K, Heerema NA, Bloomfield CD (2004) Cytogenetics in acute leukemia. *Blood Rev* 18: 115-36

Mulligan P, Westbrook TF, Ottinger M, Pavlova N, Chang B, Macia E, Shi YJ, Barretina J, Liu J, Howley PM, Elledge SJ, Shi Y (2008) CDYL bridges REST and histone methyltransferases for gene repression and suppression of cellular transformation. *Mol Cell* 32: 718-26

Mungall AJ, Palmer SA, Sims SK, Edwards CA, Ashurst JL, Wilming L, Jones MC, Horton R, Hunt SE, Scott CE, Gilbert JG, Clamp ME, Bethel G, Milne S, Ainscough R,

Almeida JP, Ambrose KD, Andrews TD, Ashwell RI, Babbage AK et al. (2003) The DNA sequence and analysis of human chromosome 6. *Nature* 425: 805-11

Muto T, Sashida G, Oshima M, Wendt GR, Mochizuki-Kashio M, Nagata Y, Sanada M, Miyagi S, Saraya A, Kamio A, Nagae G, Nakaseko C, Yokote K, Shimoda K, Koseki H, Suzuki Y, Sugano S, Aburatani H, Ogawa S, Iwama A (2013) Concurrent loss of Ezh2 and Tet2 cooperates in the pathogenesis of myelodysplastic disorders. *J Exp Med* 210: 2627-39

Myant K, Stancheva I (2008) LSH cooperates with DNA methyltransferases to repress transcription. *Mol Cell Biol* 28: 215-26

Nagamachi A, Matsui H, Asou H, Ozaki Y, Aki D, Kanai A, Takubo K, Suda T, Nakamura T, Wolff L, Honda H, Inaba T (2013) Haploinsufficiency of SAMD9L, an endosome fusion facilitator, causes myeloid malignancies in mice mimicking human diseases with monosomy 7. *Cancer Cell* 24: 305-17

Nangalia J, Massie CE, Baxter EJ, Nice FL, Gundem G, Wedge DC, Avezov E, Li J, Kollmann K, Kent DG, Aziz A, Godfrey AL, Hinton J, Martincorena I, Van Loo P, Jones AV, Guglielmelli P, Tarpey P, Harding HP, Fitzpatrick JD et al. (2013) Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. *N Engl J Med* 369: 2391-2405

Nangalia J, Nice FL, Wedge DC, Godfrey AL, Grinfeld J, Thakker C, Massie CE, Baxter J, Sewell D, Silber Y, Campbell PJ, Green AR (2015) DNMT3A mutations occur early or late in patients with myeloproliferative neoplasms and mutation order influences phenotype. *Haematologica* 100: e438-42

Naqvi K, Garcia-Manero G, Sardesai S, Oh J, Vigil CE, Pierce S, Lei X, Shan J, Kantarjian HM, Suarez-Almazor ME (2011) Association of comorbidities with overall survival in myelodysplastic syndrome: development of a prognostic model. *J Clin Oncol* 29: 2240-6

Neukirchen J, Lauseker M, Hildebrandt B, Nolting AC, Kaivers J, Kobbe G, Gattermann N, Haas R, Germing U (2017) Cytogenetic clonal evolution in myelodysplastic syndromes is associated with inferior prognosis. *Cancer* 123: 4608-4616

Nikoloski G, Langemeijer SM, Kuiper RP, Knops R, Massop M, Tonnissen ER, van der Heijden A, Scheele TN, Vandenberghe P, de Witte T, van der Reijden BA, Jansen JH (2010) Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. *Nat Genet* 42: 665-7

Nivarthi H, Chen D, Cleary C, Kubesova B, Jager R, Bogner E, Marty C, Pecquet C, Vainchenker W, Constantinescu SN, Kralovics R (2016) Thrombopoietin receptor is required for the oncogenic function of CALR mutants. *Leukemia* 30: 1759-63

O'Sullivan J, Mead AJ (2019) Heterogeneity in myeloproliferative neoplasms: Causes and consequences. *Adv Biol Regul* 71: 55-68

Ogawa S (2019) Genetics of MDS. *Blood* 133: 1049-1059

Oguchi ME, Noguchi K, Fukuda M (2017) TBC1D12 is a novel Rab11-binding protein that modulates neurite outgrowth of PC12 cells. *PLoS One* 12: e0174883

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: 988-92

Ok CY, Patel KP, Garcia-Manero G, Routbort MJ, Peng J, Tang G, Goswami M, Young KH, Singh R, Medeiros LJ, Kantarjian HM, Luthra R, Wang SA (2015) TP53 mutation characteristics in therapy-related myelodysplastic syndromes and acute myeloid leukemia is similar to de novo diseases. *J Hematol Oncol* 8: 45

Olcaydu D, Harutyunyan A, Jager R, Berg T, Gisslinger B, Pabinger I, Gisslinger H, Kralovics R (2009) A common JAK2 haplotype confers susceptibility to myeloproliferative neoplasms. *Nat Genet* 41: 450-4

Olsson L, Zettermark S, Biloglav A, Castor A, Behrendtz M, Forestier E, Paulsson K, Johansson B (2016) The genetic landscape of paediatric de novo acute myeloid leukaemia as defined by single nucleotide polymorphism array and exon sequencing of 100 candidate genes. *Br J Haematol* 174: 292-301

Ortmann CA, Kent DG, Nangalia J, Silber Y, Wedge DC, Grinfeld J, Baxter EJ, Massie CE, Papaemmanuil E, Menon S, Godfrey AL, Dimitropoulou D, Guglielmelli P,

Bellosillo B, Besses C, Dohner K, Harrison CN, Vassiliou GS, Vannucchi A, Campbell PJ et al. (2015) Effect of mutation order on myeloproliferative neoplasms. *N Engl J Med* 372: 601-612

Palomero T, Couronne L, Khiabanian H, Kim MY, Ambesi-Impiombato A, Perez-Garcia A, Carpenter Z, Abate F, Allegretta M, Haydu JE, Jiang X, Lossos IS, Nicolas C, Balbin M, Bastard C, Bhagat G, Piris MA, Campo E, Bernard OA, Rabadan R et al. (2014) Recurrent mutations in epigenetic regulators, RHOA and FYN kinase in peripheral T cell lymphomas. *Nat Genet* 46: 166-70

Pan F, Wingo TS, Zhao Z, Gao R, Makishima H, Qu G, Lin L, Yu M, Ortega JR, Wang J, Nazha A, Chen L, Yao B, Liu C, Chen S, Weeks O, Ni H, Phillips BL, Huang S, Wang J et al. (2017) Tet2 loss leads to hypermutagenicity in haematopoietic stem/progenitor cells. *Nat Commun* 8: 15102

Papaemmanuil E, Cazzola M, Boultwood J, Malcovati L, Vyas P, Bowen D, Pellagatti A, Wainscoat JS, Hellstrom-Lindberg E, Gambacorti-Passerini C, Godfrey AL, Rapado I, Cvejic A, Rance R, McGee C, Ellis P, Mudie LJ, Stephens PJ, McLaren S, Massie CE et al. (2011) Somatic SF3B1 mutation in myelodysplasia with ring sideroblasts. *N Engl J Med* 365: 1384-95

Papaemmanuil E, Gerstung M, Bullinger L, Gaidzik VI, Paschka P, Roberts ND, Potter NE, Heuser M, Thol F, Bolli N, Gundem G, Van Loo P, Martincorena I, Ganly P, Mudie L, McLaren S, O'Meara S, Raine K, Jones DR, Teague JW et al. (2016) Genomic Classification and Prognosis in Acute Myeloid Leukemia. *N Engl J Med* 374: 2209-2221

Papaemmanuil E, Gerstung M, Malcovati L, Tauro S, Gundem G, Van Loo P, Yoon CJ, Ellis P, Wedge DC, Pellagatti A, Shlien A, Groves MJ, Forbes SA, Raine K, Hinton J, Mudie LJ, McLaren S, Hardy C, Latimer C, Della Porta MG et al. (2013) Clinical and biological implications of driver mutations in myelodysplastic syndromes. *Blood* 122: 3616-27; quiz 3699

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: 1713-8

Park SJ, Bejar R (2018) Clonal Hematopoiesis in Aging. *Curr Stem Cell Rep* 4: 209-219

Passamonti F, Rumi E, Pietra D, Elena C, Boveri E, Arcaini L, Roncoroni E, Astori C, Merli M, Boggi S, Pascutto C, Lazzarino M, Cazzola M (2010) A prospective study of 338 patients with polycythemia vera: the impact of JAK2 (V617F) allele burden and leukocytosis on fibrotic or leukemic disease transformation and vascular complications. *Leukemia* 24: 1574-9

Passamonti F, Thiele J, Girodon F, Rumi E, Carobbio A, Gisslinger H, Kvasnicka HM, Ruggeri M, Randi ML, Gangat N, Vannucchi AM, Gianatti A, Gisslinger B, Mullauer L, Rodeghiero F, d'Amore ES, Bertozzi I, Hanson CA, Boveri E, Marino F et al. (2012) A prognostic model to predict survival in 867 World Health Organization-defined essential thrombocythemia at diagnosis: a study by the International Working Group on Myelofibrosis Research and Treatment. *Blood* 120: 1197-201

Patnaik MM, Belachew A, Finke C, Lasho TL, Hanson CA, Tefferi A (2014) CALR mutations are infrequent in WHO-defined refractory anemia with ring sideroblasts. *Leukemia* 28: 1370-1

Pecquet C, Staerk J, Chaligne R, Goss V, Lee KA, Zhang X, Rush J, Van Hees J, Poirel HA, Scheiff JM, Vainchenker W, Giraudier S, Polakiewicz RD, Constantinescu SN (2010) Induction of myeloproliferative disorder and myelofibrosis by thrombopoietin receptor W515 mutants is mediated by cytosolic tyrosine 112 of the receptor. *Blood* 115: 1037-48

Peeken JC, Jutzi JS, Wehrle J, Koellerer C, Staehle HF, Becker H, Schoenwandt E, Seeger TS, Schanne DH, Gothwal M, Ott CJ, Grunder A, Pahl HL (2018) Epigenetic regulation of NFE2 overexpression in myeloproliferative neoplasms. *Blood* 131: 2065-2073

Pei YQ, Wu Y, Wang F, Cui W (2016) Prognostic value of CALR vs. JAK2V617F mutations on splenomegaly, leukemic transformation, thrombosis, and overall survival in patients with primary fibrosis: a meta-analysis. *Ann Hematol* 95: 1391-8

Pietra D, Li S, Brisci A, Passamonti F, Rumi E, Theocharides A, Ferrari M, Gisslinger H, Kralovics R, Cremonesi L, Skoda R, Cazzola M (2008) Somatic mutations of JAK2

exon 12 in patients with JAK2 (V617F)-negative myeloproliferative disorders. *Blood* 111: 1686-9

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: e270

Poluben L, Puligandla M, Neuberg D, Bryke CR, Hsu Y, Shumeiko O, Yuan X, Voznesensky O, Pihan G, Adam M, Fraenkel E, Rasnic R, Linial M, Klymenko S, Balk SP, Fraenkel PG (2019) Characteristics of myeloproliferative neoplasms in patients exposed to ionizing radiation following the Chernobyl nuclear accident. *Am J Hematol* 94: 62-73

Potter N, Miraki-Moud F, Ermini L, Titley I, Vijayaraghavan G, Papaemmanuil E, Campbell P, Gribben J, Taussig D, Greaves M (2019) Single cell analysis of clonal architecture in acute myeloid leukaemia. *Leukemia* 33: 1113-1123

Prasad P, Ronnerblad M, Arner E, Itoh M, Kawaji H, Lassmann T, Daub CO, Forrest AR, Lennartsson A, Ekwall K, consortium F (2014) High-throughput transcription profiling identifies putative epigenetic regulators of hematopoiesis. *Blood* 123: e46-57

Pronier E, Cifani P, Merlinsky TR, Berman KB, Somasundara AVH, Rampal RK, LaCava J, Wei KE, Pastore F, Maag JL, Park J, Koche R, Kentsis A, Levine RL (2018) Targeting the CALR interactome in myeloproliferative neoplasms. *JCI Insight* 3

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: 245-50

Pulikkan JA, Dengler V, Peramangalam PS, Peer Zada AA, Muller-Tidow C, Bohlander SK, Tenen DG, Behre G (2010) Cell-cycle regulator E2F1 and microRNA-223 comprise an autoregulatory negative feedback loop in acute myeloid leukemia. *Blood* 115: 1768-78

Rampal R, Ahn J, Abdel-Wahab O, Nahas M, Wang K, Lipson D, Otto GA, Yelensky R, Hricik T, McKenney AS, Chiosis G, Chung YR, Pandey S, van den Brink MR, Armstrong SA, Dogan A, Intlekofer A, Manshouri T, Park CY, Verstovsek S et al. (2014) Genomic and functional analysis of leukemic transformation of myeloproliferative neoplasms. *Proc Natl Acad Sci U S A* 111: E5401-10

Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F (2013) Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 8: 2281-2308

Rasmussen KD, Jia G, Johansen JV, Pedersen MT, Rapin N, Bagger FO, Porse BT, Bernard OA, Christensen J, Helin K (2015) Loss of TET2 in hematopoietic cells leads to DNA hypermethylation of active enhancers and induction of leukemogenesis. *Genes Dev* 29: 910-22

Rheinbay E, Parasuraman P, Grimsby J, Tiao G, Engreitz JM, Kim J, Lawrence MS, Taylor-Weiner A, Rodriguez-Cuevas S, Rosenberg M, Hess J, Stewart C, Maruvka YE, Stojanov P, Cortes ML, Seepo S, Cibulskis C, Tracy A, Pugh TJ, Lee J et al. (2017) Recurrent and functional regulatory mutations in breast cancer. *Nature* 547: 55-60

Ribezzo F, Snoeren IAM, Ziegler S, Stoelben J, Olofsen PA, Henic A, Ferreira MV, Chen S, Stalmann USA, Buesche G, Hoogenboezem RM, Kramann R, Platzbecker U, Raaijmakers M, Ebert BL, Schneider RK (2019) Rps14, Csnk1a1 and miRNA145/miRNA146a deficiency cooperate in the clinical phenotype and activation of the innate immune system in the 5q- syndrome. *Leukemia* 33: 1759-1772

Rinaldi CR, Rinaldi P, Alagia A, Gemei M, Esposito N, Formiggini F, Martinelli V, Senyuk V, Nucifora G, Pane F (2010) Preferential nuclear accumulation of JAK2V617F in CD34+ but not in granulocytic, megakaryocytic, or erythroid cells of patients with Philadelphia-negative myeloproliferative neoplasia. *Blood* 116: 6023-6

Rinke J, Muller JP, Blaess MF, Chase A, Meggendorfer M, Schafer V, Winkelmann N, Haferlach C, Cross NCP, Hochhaus A, Ernst T (2017) Molecular characterization of EZH2 mutant patients with myelodysplastic/myeloproliferative neoplasms. *Leukemia* 31: 1936-1943

Rougerie P, Largeteau Q, Megrelis L, Carrette F, Lejeune T, Toffali L, Rossi B, Zeghouf M, Cherfils J, Constantin G, Laudanna C, Bismuth G, Mangeney M, Delon J (2013)

Fam65b is a new transcriptional target of FOXO1 that regulates RhoA signaling for T lymphocyte migration. *J Immunol* 190: 748-55

Rumi E, Cazzola M (2017a) Advances in understanding the pathogenesis of familial myeloproliferative neoplasms. *Br J Haematol* 178: 689-698

Rumi E, Cazzola M (2017b) Diagnosis, risk stratification, and response evaluation in classical myeloproliferative neoplasms. *Blood* 129: 680-692

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: 974-9

Rumi E, Harutyunyan AS, Pietra D, Feenstra JD, Cavalloni C, Roncoroni E, Casetti I, Bellini M, Milanesi C, Renna MC, Gotti M, Astori C, Kralovics R, Cazzola M (2016) LNK mutations in familial myeloproliferative neoplasms. *Blood* 128: 144-5

Rumi E, Pietra D, Ferretti V, Klampfl T, Harutyunyan AS, Milosevic JD, Them NC, Berg T, Elena C, Casetti IC, Milanesi C, Sant'antonio E, Bellini M, Fugazza E, Renna MC, Boveri E, Astori C, Pascutto C, Kralovics R, Cazzola M et al. (2014a) JAK2 or CALR mutation status defines subtypes of essential thrombocythemia with substantially different clinical course and outcomes. *Blood* 123: 1544-51

Rumi E, Pietra D, Guglielmelli P, Bordoni R, Casetti I, Milanesi C, Sant'Antonio E, Ferretti V, Pancrazzi A, Rotunno G, Severgnini M, Pietrelli A, Astori C, Fugazza E, Pascutto C, Boveri E, Passamonti F, De Bellis G, Vannucchi A, Cazzola M et al. (2013) Acquired copy-neutral loss of heterozygosity of chromosome 1p as a molecular event associated with marrow fibrosis in MPL-mutated myeloproliferative neoplasms. *Blood* 121: 4388-95

Rumi E, Pietra D, Pascutto C, Guglielmelli P, Martinez-Trillos A, Casetti I, Colomer D, Pieri L, Pratcorona M, Rotunno G, Sant'Antonio E, Bellini M, Cavalloni C, Mannarelli C, Milanesi C, Boveri E, Ferretti V, Astori C, Rosti V, Cervantes F et al. (2014b) Clinical effect of driver mutations of JAK2, CALR, or MPL in primary myelofibrosis. *Blood* 124: 1062-9

Sachs Z, Been RA, DeCoursin KJ, Nguyen HT, Mohd Hassan NA, Noble-Orcutt KE, Eckfeldt CE, Pomeroy EJ, Diaz-Flores E, Geurts JL, Diers MD, Hasz DE, Morgan KJ, MacMillan ML, Shannon KM, Largaespada DA, Wiesner SM (2016) Stat5 is critical for the development and maintenance of myeloproliferative neoplasm initiated by Nf1 deficiency. *Haematologica* 101: 1190-1199

Sakata-Yanagimoto M, Enami T, Yoshida K, Shiraishi Y, Ishii R, Miyake Y, Muto H, Tsuyama N, Sato-Otsubo A, Okuno Y, Sakata S, Kamada Y, Nakamoto-Matsubara R, Tran NB, Izutsu K, Sato Y, Ohta Y, Furuta J, Shimizu S, Komeno T et al. (2014) Somatic RHOA mutation in angioimmunoblastic T cell lymphoma. *Nat Genet* 46: 171-5

Salati S, Prudente Z, Genovese E, Pennucci V, Rontauroli S, Bartalucci N, Mannarelli C, Ruberti S, Zini R, Rossi C, Bianchi E, Guglielmelli P, Tagliafico E, Vannucchi AM, Manfredini R (2017) Calreticulin affects hematopoietic stem/progenitor cell fate by impacting erythroid and megakaryocytic differentiation. *Stem Cells Dev*

Saliba J, Saint-Martin C, Di Stefano A, Lenglet G, Marty C, Keren B, Pasquier F, Valle VD, Secardin L, Leroy G, Mahfoudhi E, Grosjean S, Droin N, Diop M, Dessen P, Charrier S, Palazzo A, Merlevede J, Meniane JC, Delaunay-Darivon C et al. (2015) Germline duplication of ATG2B and GSKIP predisposes to familial myeloid malignancies. *Nat Genet* 47: 1131-40

Sanchez R, Zhou MM (2011) The PHD finger: a versatile epigenome reader. *Trends Biochem Sci* 36: 364-72

Sangkhae V, Etheridge SL, Kaushansky K, Hitchcock IS (2014) The thrombopoietin receptor, MPL, is critical for development of a JAK2V617F-induced myeloproliferative neoplasm. *Blood* 124: 3956-63

Sanulli S, Justin N, Teissandier A, Ancelin K, Portoso M, Caron M, Michaud A, Lombard B, da Rocha ST, Offer J, Loew D, Servant N, Wassef M, Burlina F, Gamblin SJ, Heard E, Margueron R (2015) Jarid2 Methylation via the PRC2 Complex Regulates H3K27me3 Deposition during Cell Differentiation. *Mol Cell* 57: 769-783

Sashida G, Wang C, Tomioka T, Oshima M, Aoyama K, Kanai A, Mochizuki-Kashio M, Harada H, Shimoda K, Iwama A (2016) The loss of Ezh2 drives the pathogenesis

of myelofibrosis and sensitizes tumor-initiating cells to bromodomain inhibition. *J Exp Med* 213: 1459-77

Sato Y, Suto Y, Pietenpol J, Golub TR, Gilliland DG, Davis EM, Le Beau MM, Roberts JM, Vogelstein B, Rowley JD, et al. (1995) TEL and KIP1 define the smallest region of deletions on 12p13 in hematopoietic malignancies. *Blood* 86: 1525-33

Savina A, Furlan M, Vidal M, Colombo MI (2003) Exosome release is regulated by a calcium-dependent mechanism in K562 cells. *J Biol Chem* 278: 20083-90

Schaaf CA, Kwak H, Koenig A, Misulovin Z, Gohara DW, Watson A, Zhou Y, Lis JT, Dorsett D (2013) Genome-wide control of RNA polymerase II activity by cohesin. *PLoS Genet* 9: e1003382

Schaaf CA, Misulovin Z, Gause M, Koenig A, Gohara DW, Watson A, Dorsett D (2013) Cohesin and polycomb proteins functionally interact to control transcription at silenced and active genes. *PLoS Genet* 9: e1003560

Schanz J, Sole F, Mallo M, Luno E, Cervera J, Granada I, Hildebrandt B, Slovak ML, Ohyashiki K, Fonatsch C, Pfeilstocker M, Nosslinger T, Valent P, Giagounidis A, Aul C, Lubbert M, Stauder R, Krieger O, Le Beau MM, Bennett JM et al. (2018) Clonal architecture in patients with myelodysplastic syndromes and double or minor complex abnormalities: Detailed analysis of clonal composition, involved abnormalities, and prognostic significance. *Genes Chromosomes Cancer* 57: 547-556

Schischlik F, Jager R, Rosebrock F, Hug E, Schuster M, Holly R, Fuchs E, Milosevic Feenstra JD, Bogner E, Gisslinger B, Schalling M, Rumi E, Pietra D, Fischer G, Fae I, Vulliard L, Menche J, Haferlach T, Meggendorfer M, Stengel A et al. (2019) Mutational landscape of the transcriptome offers putative targets for immunotherapy of myeloproliferative neoplasms. *Blood* 134: 199-210

Schneider RK, Adema V, Heckl D, Jaras M, Mallo M, Lord AM, Chu LP, McConkey ME, Kramann R, Mullally A, Bejar R, Sole F, Ebert BL (2014) Role of casein kinase 1A1 in the biology and targeted therapy of del(5q) MDS. *Cancer Cell* 26: 509-20

Schulze I, Rohde C, Scheller-Wendorff M, Baumer N, Krause A, Herbst F, Riemke P, Hebestreit K, Tschanter P, Lin Q, Linhart H, Godley LA, Glimm H, Dugas M, Wagner

W, Berdel WE, Rosenbauer F, Muller-Tidow C (2016) Increased DNA methylation of Dnmt3b targets impairs leukemogenesis. *Blood* 127: 1575-86

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: 1208-13

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: 459-68

Shallis RM, Ahmad R, Zeidan AM (2018) Aplastic anemia: Etiology, molecular pathogenesis, and emerging concepts. *Eur J Haematol* 101: 711-720

Shi Y, Sawada J, Sui G, Affar el B, Whetstine JR, Lan F, Ogawa H, Luke MP, Nakatani Y, Shi Y (2003) Coordinated histone modifications mediated by a CtBP co-repressor complex. *Nature* 422: 735-8

Shide K, Kameda T, Yamaji T, Sekine M, Inada N, Kamiunten A, Akizuki K, Nakamura K, Hidaka T, Kubuki Y, Shimoda H, Kitanaka A, Honda A, Sawaguchi A, Abe H, Miike T, Iwakiri H, Tahara Y, Sueta M, Hasuike S et al. (2017) Calreticulin mutant mice develop essential thrombocythemia that is ameliorated by the JAK inhibitor ruxolitinib. *Leukemia* 31: 1136-1144

Shieh SY, Ikeda M, Taya Y, Prives C (1997) DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell* 91: 325-34

Shimizu T, Kubovcakova L, Nienhold R, Zmajkovic J, Meyer SC, Hao-Shen H, Geier F, Dirnhofer S, Guglielmelli P, Vannucchi AM, Feenstra JD, Kralovics R, Orkin SH, Skoda RC (2016) Loss of Ezh2 synergizes with JAK2-V617F in initiating myeloproliferative neoplasms and promoting myelofibrosis. *J Exp Med* 213: 1479-96

Shiozawa Y, Malcovati L, Galli A, Sato-Otsubo A, Kataoka K, Sato Y, Watatani Y, Suzuki H, Yoshizato T, Yoshida K, Sanada M, Makishima H, Shiraishi Y, Chiba K, Hellstrom-Lindberg E, Miyano S, Ogawa S, Cazzola M (2018) Aberrant splicing and

defective mRNA production induced by somatic spliceosome mutations in myelodysplasia. *Nat Commun* 9: 3649

Shlush LI, Zandi S, Mitchell A, Chen WC, Brandwein JM, Gupta V, Kennedy JA, Schimmer AD, Schuh AC, Yee KW, McLeod JL, Doedens M, Medeiros JJ, Marke R, Kim HJ, Lee K, McPherson JD, Hudson TJ, Consortium HP-LGP, Brown AM et al. (2014) Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature* 506: 328-33

Siegel RL, Miller KD, Jemal A (2015) Cancer statistics, 2015. *CA Cancer J Clin* 65: 5-29

Sloand EM, Mainwaring L, Fuhrer M, Ramkissoon S, Risitano AM, Keyvanafar K, Lu J, Basu A, Barrett AJ, Young NS (2005) Preferential suppression of trisomy 8 compared with normal hematopoietic cell growth by autologous lymphocytes in patients with trisomy 8 myelodysplastic syndrome. *Blood* 106: 841-51

Sloand EM, Melenhorst JJ, Tucker ZC, Pfannes L, Brenchley JM, Yong A, Visconte V, Wu C, Gostick E, Scheinberg P, Olnes MJ, Douek DC, Price DA, Barrett AJ, Young NS (2011) T-cell immune responses to Wilms tumor 1 protein in myelodysplasia responsive to immunosuppressive therapy. *Blood* 117: 2691-9

Smith AE, Mohamedali AM, Kulasekararaj A, Lim Z, Gaken J, Lea NC, Przychodzen B, Mian SA, Nasser EE, Shooter C, Westwood NB, Strupp C, Gattermann N, Maciejewski JP, Germing U, Mufti GJ (2010) Next-generation sequencing of the TET2 gene in 355 MDS and CMML patients reveals low-abundance mutant clones with early origins, but indicates no definite prognostic value. *Blood* 116: 3923-32

Sole F, Espinet B, Sanz GF, Cervera J, Calasanz MJ, Luno E, Prieto F, Granada I, Hernandez JM, Cigudosa JC, Diez JL, Bureo E, Marques ML, Arranz E, Rios R, Martinez Climent JA, Vallespi T, Florensa L, Woessner S (2000) Incidence, characterization and prognostic significance of chromosomal abnormalities in 640 patients with primary myelodysplastic syndromes. Grupo Cooperativo Espanol de Citogenetica Hematologica. *Br J Haematol* 108: 346-56

Son HJ, Kim MS, Yoo NJ, Lee SH (2019) Absence of Promoter Mutation in TBC1D12 Gene in Solid and Hematologic Neoplasia. *Pathol Oncol Res* 25: 1675-1676

Sperling AS, Gibson CJ, Ebert BL (2017) The genetics of myelodysplastic syndrome: from clonal haematopoiesis to secondary leukaemia. *Nat Rev Cancer* 17: 5-19

Steensma DP (2015) Myelodysplastic Syndromes: Diagnosis and Treatment. *Mayo Clin Proc* 90: 969-83

Steensma DP, Bejar R, Jaiswal S, Lindsley RC, Sekeres MA, Hasserjian RP, Ebert BL (2015) Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood* 126: 9-16

Stegelmann F, Bullinger L, Griesshammer M, Holzmann K, Habdank M, Kuhn S, Maile C, Schauer S, Dohner H, Dohner K (2010) High-resolution single-nucleotide polymorphism array-profiling in myeloproliferative neoplasms identifies novel genomic aberrations. *Haematologica* 95: 666-9

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: 1217-9

Suessmuth Y, Elliott J, Percy MJ, Inami M, Attal H, Harrison CN, Inokuchi K, McMullin MF, Johnston JA (2009) A new polycythaemia vera-associated SOCS3 SH2 mutant (SOCS3F136L) cannot regulate erythropoietin responses. *Br J Haematol* 147: 450-8

Sun A, Li F, Liu Z, Jiang Y, Zhang J, Wu J, Shi Y (2018) Structural and biochemical insights into human zinc finger protein AEBP2 reveals interactions with RBBP4. *Protein Cell* 9: 738-742

Suresh K, Kliot T, Piunti A, Kliot M (2016) Epigenetic mechanisms drive the progression of neurofibromas to malignant peripheral nerve sheath tumors. *Surg Neurol Int* 7: S797-S800

Suzuki A, Shin JW, Wang Y, Min SH, Poncz M, Choi JK, Discher DE, Carpenter CL, Lian L, Zhao L, Wang Y, Abrams CS (2013) RhoA is essential for maintaining normal megakaryocyte ploidy and platelet generation. *PLoS One* 8: e69315

Swerdlow SH CE, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J (2016) *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues.* Lyon: International Agency for Research on Cancer

Tam CS, Abruzzo LV, Lin KI, Cortes J, Lynn A, Keating MJ, Thomas DA, Pierce S, Kantarjian H, Verstovsek S (2009) The role of cytogenetic abnormalities as a prognostic marker in primary myelofibrosis: applicability at the time of diagnosis and later during disease course. *Blood* 113: 4171-8

Tapper W, Jones AV, Kralovics R, Harutyunyan AS, Zoi K, Leung W, Godfrey AL, Guglielmelli P, Callaway A, Ward D, Aranaz P, White HE, Waghorn K, Lin F, Chase A, Baxter EJ, Maclean C, Nangalia J, Chen E, Evans P et al. (2015) Genetic variation at MECOM, TERT, JAK2 and HBS1L-MYB predisposes to myeloproliferative neoplasms. *Nat Commun* 6: 6691

Tefferi A, Guglielmelli P, Larson DR, Finke C, Wassie EA, Pieri L, Gangat N, Fjerza R, Belachew AA, Lasho TL, Ketterling RP, Hanson CA, Rambaldi A, Finazzi G, Thiele J, Barbui T, Pardanani A, Vannucchi AM (2014a) Long-term survival and blast transformation in molecularly annotated essential thrombocythemia, polycythemia vera, and myelofibrosis. *Blood* 124: 2507-13; quiz 2615

Tefferi A, Guglielmelli P, Lasho TL, Gangat N, Ketterling RP, Pardanani A, Vannucchi AM (2018a) MIPSS70+ Version 2.0: Mutation and Karyotype-Enhanced International Prognostic Scoring System for Primary Myelofibrosis. *J Clin Oncol* 36: 1769-1770

Tefferi A, Guglielmelli P, Pardanani A, Vannucchi AM (2018b) Myelofibrosis Treatment Algorithm 2018. *Blood Cancer J* 8: 72

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: 1302-9

Tefferi A, Lasho TL, Finke CM, Elala Y, Hanson CA, Ketterling RP, Gangat N, Pardanani A (2016a) Targeted deep sequencing in primary myelofibrosis. *Blood Adv* 1: 105-111

Tefferi A, Lasho TL, Finke CM, Knudson RA, Ketterling R, Hanson CH, Maffioli M, Caramazza D, Passamonti F, Pardanani A (2014b) CALR vs JAK2 vs MPL-mutated

or triple-negative myelofibrosis: clinical, cytogenetic and molecular comparisons. *Leukemia* 28: 1472-7

Tefferi A, Lasho TL, Guglielmelli P, Finke CM, Rotunno G, Elala Y, Pacilli A, Hanson CA, Pancrazzi A, Ketterling RP, Mannarelli C, Barraco D, Fanelli T, Pardanani A, Gangat N, Vannucchi AM (2016b) Targeted deep sequencing in polycythemia vera and essential thrombocythemia. *Blood Adv* 1: 21-30

Tefferi A, Mudireddy M, Mannelli F, Begna KH, Patnaik MM, Hanson CA, Ketterling RP, Gangat N, Yogarajah M, De Stefano V, Passamonti F, Rosti V, Finazzi MC, Rambaldi A, Bosi A, Guglielmelli P, Pardanani A, Vannucchi AM (2018c) Blast phase myeloproliferative neoplasm: Mayo-AGIMM study of 410 patients from two separate cohorts. *Leukemia* 32: 1200-1210

Tefferi A, Rumi E, Finazzi G, Gisslinger H, Vannucchi AM, Rodeghiero F, Randi ML, Vaidya R, Cazzola M, Rambaldi A, Gisslinger B, Pieri L, Ruggeri M, Bertozzi I, Sulai NH, Casetti I, Carobbio A, Jeryczynski G, Larson DR, Mullauer L et al. (2013) Survival and prognosis among 1545 patients with contemporary polycythemia vera: an international study. *Leukemia* 27: 1874-81

Tefferi A, Vannucchi AM (2017) Genetic Risk Assessment in Myeloproliferative Neoplasms. *Mayo Clin Proc* 92: 1283-1290

Tefferi A, Vannucchi AM, Barbui T (2018d) Essential thrombocythemia treatment algorithm 2018. *Blood Cancer J* 8: 2

Tefferi A, Wassie EA, Guglielmelli P, Gangat N, Belachew AA, Lasho TL, Finke C, Ketterling RP, Hanson CA, Pardanani A, Wolanskyj AP, Maffioli M, Casalone R, Pacilli A, Vannucchi AM, Passamonti F (2014c) Type 1 versus Type 2 calreticulin mutations in essential thrombocythemia: a collaborative study of 1027 patients. *Am J Hematol* 89: E121-4

Theocharides AP, Lundberg P, Lakkaraju AK, Lysenko V, Myburgh R, Aguzzi A, Skoda RC, Manz MG (2016) Homozygous calreticulin mutations in patients with myelofibrosis lead to acquired myeloperoxidase deficiency. *Blood* 127: 3253-9

Thoennissen NH, Krug UO, Lee DH, Kawamata N, Iwanski GB, Lasho T, Weiss T, Nowak D, Koren-Michowitz M, Kato M, Sanada M, Shih LY, Nagler A, Raynaud SD,

Muller-Tidow C, Mesa R, Haferlach T, Gilliland DG, Tefferi A, Ogawa S et al. (2010) Prevalence and prognostic impact of allelic imbalances associated with leukemic transformation of Philadelphia chromosome-negative myeloproliferative neoplasms. *Blood* 115: 2882-90

Thol F, Kade S, Schlarmann C, Loffeld P, Morgan M, Krauter J, Wlodarski MW, Kolking B, Wichmann M, Gorlich K, Gohring G, Bug G, Ottmann O, Niemeyer CM, Hofmann WK, Schlegelberger B, Ganser A, Heuser M (2012) Frequency and prognostic impact of mutations in SRSF2, U2AF1, and ZRSR2 in patients with myelodysplastic syndromes. *Blood* 119: 3578-84

Thota S, Viny AD, Makishima H, Spitzer B, Radivoyevitch T, Przychodzen B, Sekeres MA, Levine RL, Maciejewski JP (2014) Genetic alterations of the cohesin complex genes in myeloid malignancies. *Blood* 124: 1790-8

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: 3931-40

Tiong IS, Casolari DA, Nguyen T, Van Velzen MJ, Ambler K, D'Andrea RJ, Ross DM (2016) Masked polycythaemia vera is genetically intermediate between JAK2V617F mutated essential thrombocythaemia and overt polycythaemia vera. *Blood Cancer J* 6: e459

Titmarsh GJ, Duncombe AS, McMullin MF, O'Rorke M, Mesa R, De Vocht F, Horan S, Fritschi L, Clarke M, Anderson LA (2014) How common are myeloproliferative neoplasms? A systematic review and meta-analysis. *Am J Hematol* 89: 581-7

Tobiasson M, Kittang AO (2019) Treatment of myelodysplastic syndrome in the era of next-generation sequencing. *J Intern Med* 286: 41-62

Toffalini F, Demoulin JB (2010) New insights into the mechanisms of hematopoietic cell transformation by activated receptor tyrosine kinases. *Blood* 116: 2429-37

Tong W, Lodish HF (2004) Lnk inhibits Tpo-mpl signaling and Tpo-mediated megakaryocytopoiesis. *J Exp Med* 200: 569-80

Tong W, Zhang J, Lodish HF (2005) Lnk inhibits erythropoiesis and Epo-dependent JAK2 activation and downstream signaling pathways. *Blood* 105: 4604-12

Trikha P, Sharma N, Opavsky R, Reyes A, Pena C, Ostrowski MC, Roussel MF, Leone G (2011) E2f1-3 are critical for myeloid development. *J Biol Chem* 286: 4783-95

Trikha P, Sharma N, Pena C, Reyes A, Pecot T, Khurshid S, Rawahneh M, Moffitt J, Stephens JA, Fernandez SA, Ostrowski MC, Leone G (2016) E2f3 in tumor macrophages promotes lung metastasis. *Oncogene* 35: 3636-46

Triviai I, Zeschke S, Rentel J, Spanakis M, Scherer T, Gabdoulline R, Panagiota V, Thol F, Heuser M, Stocking C, Kroger N (2019) ASXL1/EZH2 mutations promote clonal expansion of neoplastic HSC and impair erythropoiesis in PMF. *Leukemia* 33: 99-109

Tuna M, Knuutila S, Mills GB (2009) Uniparental disomy in cancer. *Trends Mol Med* 15: 120-8

Ueda T, Sanada M, Matsui H, Yamasaki N, Honda ZI, Shih LY, Mori H, Inaba T, Ogawa S, Honda H (2012) EED mutants impair polycomb repressive complex 2 in myelodysplastic syndrome and related neoplasms. *Leukemia* 26: 2557-60

Vago L, Perna SK, Zanussi M, Mazzi B, Barlassina C, Stanghellini MT, Perrelli NF, Cosentino C, Torri F, Angius A, Forno B, Casucci M, Bernardi M, Peccatori J, Corti C, Bondanza A, Ferrari M, Rossini S, Roncarolo MG, Bordignon C et al. (2009) Loss of mismatched HLA in leukemia after stem-cell transplantation. *N Engl J Med* 361: 478-88

Vainchenker W, Constantinescu SN (2013) JAK/STAT signaling in hematological malignancies. *Oncogene* 32: 2601-13

Vainchenker W, Kralovics R (2017) Genetic basis and molecular pathophysiology of classical myeloproliferative neoplasms. *Blood* 129: 667-679

Vainchenker W, Plo I, Marty C, Varghese LN, Constantinescu SN (2019) The role of the thrombopoietin receptor MPL in myeloproliferative neoplasms: recent findings and potential therapeutic applications. *Expert Rev Hematol* 12: 437-448

Valent P, Kern W, Hoermann G, Milosevic Feenstra JD, Sotlar K, Pfeilstocker M, Germing U, Sperr WR, Reiter A, Wolf D, Arock M, Haferlach T, Horny HP (2019) Clonal Hematopoiesis with Oncogenic Potential (CHOP): Separation from CHIP and Roads to AML. *Int J Mol Sci* 20

Valent P, Orazi A, Steensma DP, Ebert BL, Haase D, Malcovati L, van de Loosdrecht AA, Haferlach T, Westers TM, Wells DA, Giagounidis A, Loken M, Orfao A, Lubbert M, Ganser A, Hofmann WK, Ogata K, Schanz J, Bene MC, Hoermann G et al. (2017) Proposed minimal diagnostic criteria for myelodysplastic syndromes (MDS) and potential pre-MDS conditions. *Oncotarget* 8: 73483-73500

Vallapureddy RR, Mudireddy M, Penna D, Lasho TL, Finke CM, Hanson CA, Ketterling RP, Begna KH, Gangat N, Pardanani A, Tefferi A (2019) Leukemic transformation among 1306 patients with primary myelofibrosis: risk factors and development of a predictive model. *Blood Cancer J* 9: 12

Vallois D, Dobay MP, Morin RD, Lemonnier F, Missiaglia E, Juilland M, Iwaszkiewicz J, Fataccioli V, Bisig B, Roberti A, Grewal J, Bruneau J, Fabiani B, Martin A, Bonnet C, Michielin O, Jais JP, Figeac M, Bernard OA, Delorenzi M et al. (2016) Activating mutations in genes related to TCR signaling in angioimmunoblastic and other follicular helper T-cell-derived lymphomas. *Blood* 128: 1490-502

Vannucchi AM, Antonioli E, Guglielmelli P, Rambaldi A, Barosi G, Marchioli R, Marfisi RM, Finazzi G, Guerini V, Fabris F, Randi ML, De Stefano V, Caberlon S, Tafuri A, Ruggeri M, Specchia G, Liso V, Rossi E, Pogliani E, Gugliotta L et al. (2007) Clinical profile of homozygous JAK2 617V>F mutation in patients with polycythemia vera or essential thrombocythemia. *Blood* 110: 840-6

Vannucchi AM, Barbui T, Cervantes F, Harrison C, Kiladjian JJ, Kroger N, Thiele J, Buske C, Committee EG (2015) Philadelphia chromosome-negative chronic myeloproliferative neoplasms: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 26 Suppl 5: v85-99

Vannucchi AM, Lasho TL, Guglielmelli P, Biamonte F, Pardanani A, Pereira A, Finke C, Score J, Gangat N, Mannarelli C, Ketterling RP, Rotunno G, Knudson RA, Susini MC, Laborde RR, Spolverini A, Pancrazzi A, Pieri L, Manfredini R, Tagliafico E et al. (2013) Mutations and prognosis in primary myelofibrosis. *Leukemia* 27: 1861-9

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: 1599-611

Venton G, Courtier F, Charbonnier A, D'Incan E, Saillard C, Mohty B, Mozziconacci MJ, Birnbaum D, Murati A, Vey N, Rey J (2018) Impact of gene mutations on treatment response and prognosis of acute myeloid leukemia secondary to myeloproliferative neoplasms. *Am J Hematol* 93: 330-338

Verburgh E, Achten R, Louw VJ, Brusselmans C, Delforge M, Boogaerts M, Hagemeijer A, Vandenberghe P, Verhoef G (2007) A new disease categorization of low-grade myelodysplastic syndromes based on the expression of cytopenia and dysplasia in one versus more than one lineage improves on the WHO classification. *Leukemia* 21: 668-77

Villalobos IB, Takahashi Y, Akatsuka Y, Muramatsu H, Nishio N, Hama A, Yagasaki H, Saji H, Kato M, Ogawa S, Kojima S (2010) Relapse of leukemia with loss of mismatched HLA resulting from uniparental disomy after haploidentical hematopoietic stem cell transplantation. *Blood* 115: 3158-61

von Eyss B, Maaskola J, Memczak S, Mollmann K, Schuetz A, Loddenkemper C, Tanh MD, Otto A, Muegge K, Heinemann U, Rajewsky N, Ziebold U (2012) The SNF2-like helicase HELLS mediates E2F3-dependent transcription and cellular transformation. *EMBO J* 31: 972-85

Walter MJ, Ding L, Shen D, Shao J, Grillot M, McLellan M, Fulton R, Schmidt H, Kalicki-Veizer J, O'Laughlin M, Kandoth C, Baty J, Westervelt P, DiPersio JF, Mardis ER, Wilson RK, Ley TJ, Graubert TA (2011) Recurrent DNMT3A mutations in patients with myelodysplastic syndromes. *Leukemia* 25: 1153-8

Walter MJ, Shen D, Ding L, Shao J, Koboldt DC, Chen K, Larson DE, McLellan MD, Dooling D, Abbott R, Fulton R, Magrini V, Schmidt H, Kalicki-Veizer J, O'Laughlin M, Fan X, Grillot M, Witowski S, Heath S, Frater JL et al. (2012) Clonal architecture of secondary acute myeloid leukemia. *N Engl J Med* 366: 1090-8

Walter MJ, Shen D, Shao J, Ding L, White BS, Kandoth C, Miller CA, Niu B, McLellan MD, Dees ND, Fulton R, Elliot K, Heath S, Grillot M, Westervelt P, Link DC, DiPersio

JF, Mardis E, Ley TJ, Wilson RK et al. (2013) Clonal diversity of recurrently mutated genes in myelodysplastic syndromes. *Leukemia* 27: 1275-82

Walz C, Ahmed W, Lazarides K, Betancur M, Patel N, Hennighausen L, Zaleskas VM, Van Etten RA (2012) Essential role for Stat5a/b in myeloproliferative neoplasms induced by BCR-ABL1 and JAK2(V617F) in mice. *Blood* 119: 3550-60

Wang H, Wang XQ, Xu XP, Lin GW (2010) Cytogenetic evolution correlates with poor prognosis in myelodysplastic syndrome. *Cancer Genet Cytogenet* 196: 159-66

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

Wang L, Wheeler DA, Prchal JT (2016) Acquired uniparental disomy of chromosome 9p in hematologic malignancies. *Exp Hematol* 44: 644-52

Wang Q, Dong S, Yao H, Wen L, Qiu H, Qin L, Ma L, Chen S (2014) ETV6 mutation in a cohort of 970 patients with hematologic malignancies. *Haematologica* 99: e176-8

Wang X, Paucek RD, Gooding AR, Brown ZZ, Ge EJ, Muir TW, Cech TR (2017) Molecular analysis of PRC2 recruitment to DNA in chromatin and its inhibition by RNA. *Nat Struct Mol Biol* 24: 1028-1038

Weinberg OK, Hasserjian RP (2019) The current approach to the diagnosis of myelodysplastic syndromes(). *Semin Hematol* 56: 15-21

Weissmann S, Alpermann T, Grossmann V, Kowarsch A, Nadarajah N, Eder C, Dicker F, Fasan A, Haferlach C, Haferlach T, Kern W, Schnittger S, Kohlmann A (2012) Landscape of TET2 mutations in acute myeloid leukemia. *Leukemia* 26: 934-42

Welch JS, Ley TJ, Link DC, Miller CA, Larson DE, Koboldt DC, Wartman LD, Lamprecht TL, Liu F, Xia J, Kandoth C, Fulton RS, McLellan MD, Dooling DJ, Wallis JW, Chen K, Harris CC, Schmidt HK, Kalicki-Veizer JM, Lu C et al. (2012) The origin and evolution of mutations in acute myeloid leukemia. *Cell* 150: 264-78

Wimazal F, Sperr WR, Kundi M, Meidlinger P, Fonatsch C, Jordan JH, Thalhammer-Scherrer R, Schwarzinger I, Geissler K, Lechner K, Valent P (2001) Prognostic value of lactate dehydrogenase activity in myelodysplastic syndromes. *Leuk Res* 25: 287-94

Wimazal F, Sperr WR, Kundi M, Vales A, Fonatsch C, Thalhammer-Scherrer R, Schwarzinger I, Valent P (2008) Prognostic significance of serial determinations of lactate dehydrogenase (LDH) in the follow-up of patients with myelodysplastic syndromes. *Ann Oncol* 19: 970-6

Woll PS, Kjallquist U, Chowdhury O, Doolittle H, Wedge DC, Thongjuea S, Erlandsson R, Ngara M, Anderson K, Deng Q, Mead AJ, Stenson L, Giustacchini A, Duarte S, Giannoulatou E, Taylor S, Karimi M, Scharenberg C, Mortera-Blanco T, Macaulay IC et al. (2014) Myelodysplastic syndromes are propagated by rare and distinct human cancer stem cells in vivo. *Cancer Cell* 25: 794-808

Wong TN, Ramsingh G, Young AL, Miller CA, Touma W, Welch JS, Lamprecht TL, Shen D, Hundal J, Fulton RS, Heath S, Baty JD, Klco JM, Ding L, Mardis ER, Westervelt P, DiPersio JF, Walter MJ, Graubert TA, Ley TJ et al. (2015) Role of TP53 mutations in the origin and evolution of therapy-related acute myeloid leukaemia. *Nature* 518: 552-555

Xie M, Lu C, Wang J, McLellan MD, Johnson KJ, Wendl MC, McMichael JF, Schmidt HK, Yellapantula V, Miller CA, Ozenberger BA, Welch JS, Link DC, Walter MJ, Mardis ER, Dipersio JF, Chen F, Wilson RK, Ley TJ, Ding L (2014) Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nat Med* 20: 1472-8

Xiong C, Wen Z, Yu J, Chen J, Liu CP, Zhang X, Chen P, Xu RM, Li G (2018) UBN1/2 of HIRA complex is responsible for recognition and deposition of H3.3 at cis-regulatory elements of genes in mouse ES cells. *BMC Biol* 16: 110

Yan D, Hutchison RE, Mohi G (2012) Critical requirement for Stat5 in a mouse model of polycythemia vera. *Blood* 119: 3539-49

Yang Y, Akada H, Nath D, Hutchison RE, Mohi G (2016) Loss of Ezh2 cooperates with Jak2V617F in the development of myelofibrosis in a mouse model of myeloproliferative neoplasm. *Blood* 127: 3410-23

Yao H, Ma Y, Hong Z, Zhao L, Monaghan SA, Hu MC, Huang LJ (2017) Activating JAK2 mutants reveal cytokine receptor coupling differences that impact outcomes in myeloproliferative neoplasm. *Leukemia* 31: 2122-2131

Ye D, Xiong Y, Guan KL (2012) The mechanisms of IDH mutations in tumorigenesis. *Cell Res* 22: 1102-4

Yonal-Hindilerden I, Daglar-Aday A, Akadam-Teker B, Yilmaz C, Nalcaci M, Yavuz AS, Sargin D (2015) Prognostic significance of ASXL1, JAK2V617F mutations and JAK2V617F allele burden in Philadelphia-negative myeloproliferative neoplasms. *J Blood Med* 6: 157-75

Yoo HY, Sung MK, Lee SH, Kim S, Lee H, Park S, Kim SC, Lee B, Rho K, Lee JE, Cho KH, Kim W, Ju H, Kim J, Kim SJ, Kim WS, Lee S, Ko YH (2014) A recurrent inactivating mutation in RHOA GTPase in angioimmunoblastic T cell lymphoma. *Nat Genet* 46: 371-5

Yoshida K, Sanada M, Shiraishi Y, Nowak D, Nagata Y, Yamamoto R, Sato Y, Sato-Otsubo A, Kon A, Nagasaki M, Chalkidis G, Suzuki Y, Shiosaka M, Kawahata R, Yamaguchi T, Otsu M, Obara N, Sakata-Yanagimoto M, Ishiyama K, Mori H et al. (2011) Frequent pathway mutations of splicing machinery in myelodysplasia. *Nature* 478: 64-9

Yoshizato T, Dumitriu B, Hosokawa K, Makishima H, Yoshida K, Townsley D, Sato-Otsubo A, Sato Y, Liu D, Suzuki H, Wu CO, Shiraishi Y, Clemente MJ, Kataoka K, Shiozawa Y, Okuno Y, Chiba K, Tanaka H, Nagata Y, Katagiri T et al. (2015) Somatic Mutations and Clonal Hematopoiesis in Aplastic Anemia. *N Engl J Med* 373: 35-47

Yoshizato T, Nannya Y, Atsuta Y, Shiozawa Y, Iijima-Yamashita Y, Yoshida K, Shiraishi Y, Suzuki H, Nagata Y, Sato Y, Kakiuchi N, Matsuo K, Onizuka M, Kataoka K, Chiba K, Tanaka H, Ueno H, Nakagawa MM, Przychodzen B, Haferlach C et al. (2017) Genetic abnormalities in myelodysplasia and secondary acute myeloid leukemia: impact on outcome of stem cell transplantation. *Blood* 129: 2347-2358

Young AL, Challen GA, Birmann BM, Druley TE (2016) Clonal haematopoiesis harbouring AML-associated mutations is ubiquitous in healthy adults. *Nat Commun* 7: 12484

Zaimoku Y, Takamatsu H, Hosomichi K, Ozawa T, Nakagawa N, Imi T, Maruyama H, Katagiri T, Kishi H, Tajima A, Muraguchi A, Kashiwase K, Nakao S (2017) Identification of an HLA class I allele closely involved in the autoantigen presentation in acquired aplastic anemia. *Blood* 129: 2908-2916

Zhang M, Wang Y, Jones S, Sausen M, McMahon K, Sharma R, Wang Q, Belzberg AJ, Chaichana K, Gallia GL, Gokaslan ZL, Riggins GJ, Wolinksy JP, Wood LD, Montgomery EA, Hruban RH, Kinzler KW, Papadopoulos N, Vogelstein B, Bettegowda C (2014) Somatic mutations of SUZ12 in malignant peripheral nerve sheath tumors. *Nat Genet* 46: 1170-2

Zhang R, Kim YM, Wang X, Li Y, Lu X, Sternenberger AR, Li S, Lee JY (2015) Genomic Copy Number Variations in the Myelodysplastic Syndrome and Acute Myeloid Leukemia Patients with del(5q) and/or -7/del(7q). *Int J Med Sci* 12: 719-26

Zhang X, Su J, Jeong M, Ko M, Huang Y, Park HJ, Guzman A, Lei Y, Huang YH, Rao A, Li W, Goodell MA (2016) DNMT3A and TET2 compete and cooperate to repress lineage-specific transcription factors in hematopoietic stem cells. *Nat Genet* 48: 1014-23

Zhang Y, Yang X, Gui B, Xie G, Zhang D, Shang Y, Liang J (2011) Corepressor protein CDYL functions as a molecular bridge between polycomb repressor complex 2 and repressive chromatin mark trimethylated histone lysine 27. *J Biol Chem* 286: 42414-25

Zhao YL, Zhong SR, Zhang SH, Bi JX, Xiao ZY, Wang SY, Jiao HL, Zhang D, Qiu JF, Zhang LJ, Huang CM, Chen XL, Ding YQ, Ye YP, Liang L, Liao WT (2019) UBN2 promotes tumor progression via the Ras/MAPK pathway and predicts poor prognosis in colorectal cancer. *Cancer Cell Int* 19: 126

Zink F, Stacey SN, Norddahl GL, Frigge ML, Magnusson OT, Jonsdottir I, Thorgeirsson TE, Sigurdsson A, Gudjonsson SA, Gudmundsson J, Jonasson JG, Tryggvadottir L, Jonsson T, Helgason A, Gylfason A, Sulem P, Rafnar T, Thorsteinsdottir U, Gudbjartsson DF, Masson G et al. (2017) Clonal hematopoiesis, with and without candidate driver mutations, is common in the elderly. *Blood* 130: 742-752

7. ABBREVIATIONS

5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
A	Alanine
AEBP2	AE binding protein 2
AKT	Alpha serine/threonine-protein kinase
allHSCT	Allogenic hematoietic stem cell transpantation
AML	Acute myeloid leukemia
AP	Accelerated phase
APOBEC2	Apolipoprotein B MRNA Editing Enzyme Catalytic Subunit 2
ASXL1	Additional sex combs like 1
ATM	Ataxia telangiectasia mutated
ATP2B1	ATPase Plasma Membrane Ca2+ Transporting 1
BaF3	Murine pro-B cell line
Bap1	BRCA1 associated protein 1
BCOR	BCL6 Corepressor
BCR-ABL1	Breakpoint cluster region - Abelson murine leukemia viral oncogene homolog 1
BM	Bone marrow
bp	Base pair
BRCA1	BRCA1 DNA Repair Associated
C	Cysteine
C6orf132	Chromosome 6 Open Reading Frame 132
C6orf195	Chromosome 6 Open Reading Frame 195
$Ca2^{+}$	Calcium ion
CALR	Calreticulin
CAR	Commonly affected region
CASP8	Caspase 8
CBL	Casitas B-lineage lymphoma proto-oncogene
CCUS	Clonal cytopenias of undetermined significance
CD34	Hematopoietic progenitor antigen CD34
CDK10	Cyclin Dependent Kinase 10
cDNA	Complementary Deoxyribonucleic acid
CDR	Commonly deleted region
CDYL	Chromodomain Y Like
CEBPA	CCAAT/enhancer binding protein alpha
CH	Clonal hematopoiesis
CHEK	Checkpoint kinase
CHIP	Clonal hematopoiesis of indeterminate potential
CHOP	Clonal hematopoiesis of oncogenic potential
chr	Chromosome
CK1α	Casein Kinase 1 Alpha 1
c-Kit	KIT Proto-Oncogene, Receptor Tyrosine Kinase
CLVS2	Clavesin 2
CML	Chronic myeloid leukemia
CMML	Chronic myelomonocytic leukemia
CNL	Chronic neutrophilic leukemia
CSNK1A1	Casein Kinase 1 Alpha 1

CTL	Cytotoxic T-cells
CUX1	Cut Like Homeobox 1
CYP2C18	Cytochrome P450 Family 2 Subfamily C Member 18
CYP2C19	Cytochrome P450 Family 2 Subfamily C Member 19
CYP39A1	Cytochrome P450 Family 39 Subfamily A Member 1
D	Aspartic acid
ddCT	Delta-delat cycle treshold
DDX41	DEAD-Box Helicase 41
del	Deletion
DNA	Deoxyribonucleic acid
DNAH8	Dynein Axonemal Heavy Chain 8
dnAML	De novo acute myeloid leukemia
DNMT3A	DNA (cytosine-5)-methyltransferase 3 alpha
DUSP6	Dual Specificity Phosphatase 6
E	Glutamic acid
E2F3	E2F Transcription Factor 3
EED	Embryonic ectoderm development
EMH	Extramedullary hematopoiesis
EPO	Erythropoietin
EPOR	Erythropoietin receptor
ER	Endoplasmatic reticulum
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2
ERGIC	Endoplasmatic reticulum - Golgi intermediate compartment
ERK	Mitogen-activated protein kinase
ERP57	Endoplasmatic reticulum resident protein 57
ET	Essential thrombocythemia
ETV6	Ets variant 6
EZH1	Enhancer of zeste homolog 1
EZH2	Enhancer of zeste homolog 2
F	Phenylalanine
FAM217A	Family With Sequence Similarity 217 Member A
FAM65B	Family With Sequence Similarity 65 Member B
FERM	Band 4.1, ezrin, radixin, and moesin domain
FLI1	Friend leukemia intergarion 1 transcription factor
FLT3	Fms-related tyrosine kinase 3
FOXP1	Forkhead Box P1
FRA10B	Fragile Site, BrdU Type, Rare, Fra(10)(Q25.2)
fs	Frameshift
G	Glycine
G3BP1	G3BP Stress Granule Assembly Factor 1
G-CSFR	Granulocyte colony-stimulating factor receptor
GFI1B	Growth Factor Independent 1B Transcriptional Repressor
H	Histidine
H2A	Histone 2A
H2AK119u1	Mono-ubiquitination of Lysine 119 of Histone 2A
H3	Histone 3
H3K27me3	Thrimethilation of Lysine 27 of Histone H3
HBS1L	HBS1 Like Translational GTPase

HELLS	Helicase, Lymphoid Specific
hg	Human genome
HIRA	Histone Cell Cycle Regulator
HLA	Major Histocompatibility Complex, Class I
Hmga2	High mobility group AT-hook 2
Hox	Homeobox protein
HP1α	Heterochromatin protein 1a
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
HSC	Hematopoietic stem cell
HU	Hydroxyurea
I	Isoleucine
ICUS	Idiopathic cytopenias of undetermined significance
IDH1	Isocitrate dehydrogenase 1
IDH2	Isocitrate dehydrogenase 2
IDUS	Idiopathic dysplasia of unknown significance
IKZF1	IKAROS Family Zinc Finger 1
IL-1	Interleukin 1
IL-8	Interleukin 8
ins	Insertion
inv	Inversion
IPSET	International Prognostic Score for Thrombosis in Essential Thrombocythemia
IPSS	International Prognostic Scoring System for myelodysplastic syndromes
IPSS-R	Revised International Prognostic Scoring System for myelodysplastic syndromes
IRF8	Interferon Regulatory Factor 8
JAK	Janus kinase
Jak1	Janus kinase 1
JAK2	Janus kinase 2
JARID2	Jumonji AT rich interactive domain 2
JH1	Jak homology domain 1
JH2	Jak homology domain 2
JMML	Juvenile myelomonocytic leukemia
K	Lysine
kb	Kilo base pair
KIT	KIT Proto-Oncogene, Receptor Tyrosine Kinase
KITLG	KIT Ligand
KLHL31	Kelch Like Family Member 31
KMT2C	Lysine Methyltransferase 2C (MLL)
KO	Knock out
L	Leucine
LDH	Lactate dehydrogenase
Lin28b	Lin-28 homolog B
LMO2	LIM domain only 2
LNK	See SH2B3
LOH	Loss of heterozygosity
LT	Leukemic transformation
LUC7L2	LUC7 Like 2, Pre-MRNA Splicing Factor
MAP3K4	Mitogen-Activated Protein Kinase Kinase Kinase 4

MAPK	Mitogen-activated protein kinase
Mb	Mega base pair
MDM4	Mouse double minute 4 p53 binding protein homolog
MDS	Myelodysplastic syndrome
MDS-RS	Myelodiscplastic syndrome with ring sideroblasts
MECOM	MDS1 And EVI1 Complex Locus
MEK	Mitogen-activated protein kinase kinase 1
MIPSS70+	Mutation and Karyotype-Enhanced International Prognostic Scoring System for Primary Myelofibrosis
miR	Micro ribonucleic acid
MLL	Lysine Methyltransferase 2C
MPL	Myeloproliferative leukemia virus oncogene (Thrombopoietin receptor)
MPN	Myeloproliferative neoplasm
MPNST	Malignant peripheral nerve sheath tumor
MPN-U	Myeloproliferative neoplasm, unclassifiable
mPV	Masked polycythemia vera
mRNA	Messenger rybonucleic acid
MRPS18A	Mitochondrial Ribosomal Protein S18A
mTOR	Mammalian target of rapamycin
MYB	MYB Proto-Oncogene, Transcription Factor
N	Number
N	Asparagine
NCR3	Natural Cytotoxicity Triggering Receptor 3
NF1	Neurofibromin 1
NFE2	Nuclear factor, erythroid 2
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NLR Family Pyrin Domain Containing Protein 3
NOS	Not otherwise specified
NRAS	Neuroblastoma RAS viral oncogene homolog
OR2B3	Olfactory Receptor Family 2 Subfamily B Member 3
OS	Overall survival
p	Short chromosomal arm
P	Proline
P^{32}	Phosphorus-32
p53	Tumor protein p53
PACRG	Parkin Coregulated
PARK2	Parkin RBR E3 Ubiquitin Protein Ligase
PARP	Poly (ADP-ribose) polymerase
PB	Peripheral blood
PCR	Polymerase Chain Reaction
PDGFR	Platelet Derived Growth Factor Receptor Beta
Ph-negative	Philadelphia chromosome negative
PI3K	Phosphatidylinositol 3-kinase
PINT	Long Intergenic Non-Protein Coding RNA, P53 Induced Transcript
PMF	Primary myelofibrosis
PRC1	Polycomb repressive complex 1
PRC2	Polycomb repressive complex 2

PRMT5	Protein arginine methyltransferase 5
PRPF8	Pre-MRNA Processing Factor 8
PSORS1C1	Psoriasis Susceptibility 1 Candidate 1
PTPN11	Protein Tyrosine Phosphatase Non-Receptor Type 11
PV	Polycythemia vera
q	Long chromosomal arm
Q	Glutamine
qPCR	Quantitative Polymerase Chain Reaction
R	Arginine
Rad21	RAD21 Cohesin Complex Component
RAEB	Refractory Anaemia with Excess Blasts
RARS-T	Refractory anemia with ring sideroblasts
RbAp46	Retinoblastoma binding protein 7
RbAp48	Retinoblastoma binding protein 4
RBBP6	Retinoblastoma-Binding Protein 6
rIFNα	Recombinant interpheron alpha
RNA	Ribonucleic acid
ROCK	Rho Associated Coiled-Coil Containing Protein Kinase
RPS14	Ribosomal Protein S14
RREB1	Ras Responsive Element Binding Protein 1
RSPH9	Radial Spoke Head 9 Homolog
RUNX1	Runt-related transcription factor 1
S	Serine
SAMD9L	Sterile Alpha Motif Domain Containing 9 Like
sAML	Secondary acute myeloid leukemia
SF3B1	Spicing factor 3b subunit 1
SH2	Src homology 2
SH2B3	SH2B adaptor protein 3 = Lymphocyte adaptor protein (LNK)
Smc1A	Structural Maintenance Of Chromosomes 1A
Smc3	Structural Maintenance Of Chromosomes 3
sMF	Secondary myelofibrosis
SNP	Single nucleotide polymorphism
SOCS3	Suppressor of cytokine signaling 3
SPL	Splenomegaly
SRSF2	Serine/arginine-rich splicing factor 2
Stag1	Stromal Antigen 1
Stag2	Stromal Antigen 2
STAT	Signal transducer and activator of transcription
STX11	Syntaxin 11
SUZ12	Suppressor of zeste 12 homolog
t	Translocation
T	Threonine
TARSL2	Threonyl-TRNA Synthetase 3
TBC1D12	TBC1 Domain Family Member 12
TERT	Telomerase Reverse Transcriptase
TET2	Ten-eleven translocation oncogene family member 2

THBS2	Thrombospondin 2
TJAP1	Tight Junction Associated Protein 1
TM2D3	TM2 Domain-Containing Protein 3
t-MDS	Therapy-related myeldysplastic syndrome
TMEM200A	Transmembrane Protein 200A
TP53	Tumor protein p53
TPO	Thrombopoietin
TPOR	Thrombopoietin receptor (MPL)
TRERF1	Transcriptional Regulating Factor 1
U2AF1	U2 small nuclear RNA auxiliary factor 1
UBN2	Ubinuclein 2
UHRF1BP1	UHRF1 Binding Protein 1
UPD	Uniparental disomy
UPN	Unique patient number
V	Valine
W	Tryptophan
WHO	World health organization
wt	Wild type
WT1	WT1 Transcription Factor
X	Stop codon
Y	Tyrosine
ZBTB9	Zinc Finger And BTB Domain Containing 9
ZNF165	Zinc Finger Protein 165
ZNF292	Zinc Finger Protein 292
ZRSR2	Zinc Finger CCCH-Type, RNA Binding Motif And Serine/Arginine Rich 2

APPENDIX

Table 20. All 57 detected chromosomal aberrations of chr6

UPN	Diagnosis	Type of aberration	Chr	Start position	End position	Aberration length (Mb)
Pateint 27	dnAML	deletion	chr6	38200000	38800000	0,60
Patient 01	ET	UPD	chr6	1	29450000	29,45
Patient 02	PMF	deletion	chr6	135780000	137450000	1,67
Patient 03	PMF	gain	chr6	34850000	35020000	0,17
Patient 04	PMF	deletion	chr6	1	25900000	25,90
Patient 05	PMF	UPD	chr6	1	33600000	33,60
Patient 06	PV	deletion	chr6	18220000	29900000	11,68
Patient 07	PV	gain	chr6	75750000	76090000	0,34
Patient 08	PV	deletion	chr6	167040000	167780000	0,74
Patient 09	PV	gain	chr6	49200000	50100000	0,90
Patient 10	PV	UPD	chr6	1	26200000	26,20
Patient 10	PV	deletion	chr6	14100000	15700000	1,60
Patient 11	sMF	gain	chr6	1	170899992	170,90
Patient 12	post-MPN AML	deletion	chr6	1	30450000	30,45
Patient 12	post-MPN AML	gain	chr6	30450000	37920000	7,47
Patient 12	post-MPN AML	deletion	chr6	37920000	42790000	4,87
Patient 12	post-MPN AML	gain	chr6	42790000	44050000	1,26
Patient 12	post-MPN AML	deletion	chr6	44050000	57400000	13,35
Patient 13	post-MPN AML	deletion	chr6	1	28855000	28,85
Patient 14	accelerated phase PV	UPD	chr6	1	36450000	36,45
Patient 14	accelerated phase PV	deletion	chr6	14500000	15600000	1,10
Patient 15	post-MPN AML	gain	chr6	12660000	40800000	28,14
Patient 16	post-MPN AML	deletion	chr6	64460000	80160000	15,70
Patient 17	post-MPN AML	gain	chr6	1	170899992	170,90
Patient 18	post-MPN AML	deletion	chr6	67200000	86840000	19,64
Patient 19	post-MPN AML	deletion	chr6	1	29000000	29,00
Patient 19	post-MPN AML	gain	chr6	29050000	45500000	16,45
Patient 20	post-MPN AML	deletion	chr6	14130000	15800000	1,67
Patient 20	post-MPN AML	deletion	chr6	19955000	20820000	0,87
Patient 21	post-MPN AML	deletion	chr6	1	26800000	26,80
Patient 22	MDS	deletion	chr6	150680000	151150000	0,47
Patient 23	post-MDS AML	UPD	chr6	1	26480000	26,48
Patient 25	post-MDS AML	deletion	chr6	5150000	5460000	0,31
Patient 26	dnAML	UPD	chr6	1	38800000	38,80
Patient 26	dnAML	UPD	chr6	1	57500000	57,50
Patient 28	dnAML	deletion	chr6	135300000	137450000	2,15
Patient 29	dnAML	deletion	chr6	31200000	31900000	0,70
Patient 30	dnAML	deletion	chr6	73600000	149100000	75,50
Patient 30	dnAML	deletion	chr6	149100000	170899992	21,80
Patient 32	dnAML	gain	chr6	125800000	170899992	45,10
Patient 33	dnAML	deletion	chr6	1	11530000	11,53
Patient 33	dnAML	gain	chr6	11530000	14540000	3,01
Patient 33	dnAML	deletion	chr6	14540000	16410000	1,87
Patient 33	dnAML	gain	chr6	16410000	19150000	2,74
Patient 33	dnAML	deletion	chr6	19150000	28070000	8,92
Patient 33	dnAML	gain	chr6	28070000	47200000	19,13
Patient 34	dnAML	deletion	chr6	1	22270000	22,27
Patient 34	dnAML	gain	chr6	22270000	41300000	19,03
Patient 35	dnAML	gain	chr6	92620000	93300000	0,68
Patient 36	dnAML	gain	chr6	168000000	168400000	0,40
Patient 37	CML	deletion	chr6	32200000	33300000	1,10
Patient 38	post-MPN AML	gain	chr6	12700000	40800000	28,10
Patient 47	post-MDS AML	deletion	chr6	160500000	163300000	2,80
Patient 83	dnAML	deletion	chr6	1	26160000	26,16
Patient 83	dnAML	gain	chr6	26160000	34400000	8,24
Patient 83	dnAML	deletion	chr6	43800000	71000000	27,20
Patient 83	dnAML	deletion	chr6	158400000	170899992	12,50

The reference genome used for defining genomic positions of the lesions is human reference genome hg18. UPN, unique patient number; ET, essential thrombocythemia; PMF, primary myelofibrosis; PV, polycythemia vera; sMF; secondary myelofibrosis; post-MPN AML, post-myeloproliferative neoplasm acute myeloid leukemia; post-MDS AML, post-myelodysplastic syndrome acute myeloid leukemia; dnAML, *de novo* acute myeloid leukemia; CML, chronic myeloid leukemia; UPD, uniparental disomy;

Table 21. All detected deletions covering genes encoding PRC2 members

UPN	Diagnosis	Type of aberration	Chr	Start position	End position	Aberration length (Mb)	Gene
Patient 04	PMF	deletion	chr6	1	25900000	25,90	JARID2
Patient 04	PMF	deletion	chr17	2955000	30550000	27,60	SUZ12
Patient 10	PV	deletion	chr6	14100000	15700000	1,60	JARID2
Patient 12	post-MPN AML	deletion	chr6	1	30450000	30,45	JARID2
Patient 12	post-MPN AML	deletion	chr7	75700000	158821424	83,12	EZH2
Patient 13	post-MPN AML	deletion	chr12	10000000	20400000	10,40	AEBP2
Patient 13	post-MPN AML	deletion	chr6	1	28855000	28,85	JARID2
Patient 14	sMF	deletion	chr6	14500000	15600000	1,10	JARID2
Patient 15	post-MPN AML	deletion	chr7	125650000	158821424	33,17	EZH2
Patient 15	post-MPN AML	deletion	chr17	7080000	62000000	54,92	EZH1, SUZ12
Patient 19	post-MPN AML	deletion	chr6	1	29000000	29,00	JARID2
Patient 20	post-MPN AML	deletion	chr6	14130000	15800000	1,67	JARID2
Patient 21	post-MPN AML	deletion	chr6	1	26800000	26,80	JARID2
Patient 23	post-MDS AML	deletion	chr7	64500000	158821424	94,32	EZH2
Patient 24	sMF	deletion	chr7	59000000	158821424	99,82	EZH2
Patient 28	dnAML	deletion	chr7	141600000	158821424	17,22	EZH2
Patient 28	dnAML	deletion	chr17	6580000	27700000	21,12	SUZ12
Patient 28	dnAML	deletion	chr12	1	22800000	22,80	AEBP2
Patient 30	dnAML	deletion	chr7	1	158821424	158,82	EZH2
Patient 33	dnAML	deletion	chr6	14540000	16410000	1,87	JARID2
Patient 33	dnAML	deletion	chr7	72800000	158821424	86,02	EZH2
Patient 34	dnAML	deletion	chr17	23650000	35400000	11,75	SUZ12
Patient 34	dnAML	deletion	chr6	1	22270000	22,27	JARID2
Patient 39	CML	deletion	chr7	1	158821424	158,82	EZH2
Patient 40	CML	deletion	chr7	92000000	158821424	66,82	EZH2
Patient 41	dnAML	deletion	chr17	25750000	27500000	1,75	SUZ12
Patient 42	dnAML	deletion	chr17	26000000	27400000	1,40	SUZ12
Patient 43	dnAML	deletion	chr17	27200000	31400000	4,20	SUZ12
Patient 44	dnAML	deletion	chr17	19300000	28450000	9,15	SUZ12
Patient 44	dnAML	deletion	chr7	99400000	158821424	59,42	EZH2
Patient 45	dnAML	deletion	chr7	1	158821424	158,82	EZH2
Patient 46	dnAML	deletion	chr7	147900000	148250000	0,35	EZH2
Patient 47	post-MDS AML	deletion	chr12	19200000	21000000	1,80	AEBP2
Patient 47	post-MDS AML	deletion	chr12	11500000	34200000	22,70	AEBP2
Patient 47	post-MDS AML	deletion	chr7	126680000	158821424	32,14	EZH2
Patient 48	dnAML	deletion	chr11	80400000	116660000	36,26	EED
Patient 49	dnAML	deletion	chr12	19000000	22350000	3,35	AEBP2
Patient 49	dnAML	deletion	chr17	26300000	31340000	5,04	SUZ12
Patient 49	dnAML	deletion	chr7	152390000	158821424	6,43	EZH2
Patient 50	dnAML	deletion	chr17	23180000	49100000	25,92	EZH1, SUZ12
Patient 51	dnAML	deletion	chr7	105500000	158821424	53,32	EZH2
Patient 52	dnAML	deletion	chr17	37650000	41550000	3,90	EZH1
Patient 52	dnAML	deletion	chr17	14700000	27380000	12,68	SUZ12
Patient 52	dnAML	deletion	chr12	11000000	25100000	14,10	AEBP2
Patient 52	dnAML	deletion	chr7	83440000	158821424	75,38	EZH2
Patient 53	dnAML	deletion	chr7	1	158821424	158,82	EZH2
Patient 54	dnAML	deletion	chr17	1	39800000	39,80	EZH1, SUZ12
Patient 55	dnAML	deletion	chr12	9550000	25850000	16,30	AEBP2
Patient 55	dnAML	deletion	chr7	79800000	158821424	79,02	EZH2
Patient 56	dnAML	deletion	chr12	8500000	29500000	21,00	AEBP2
Patient 56	dnAML	deletion	chr17	23800000	45600000	21,80	EZH1, SUZ12
Patient 57	dnAML	deletion	chr17	1	78774742	78,77	EZH1, SUZ12
Patient 58	dnAML	deletion	chr12	14450000	20600000	6,15	AEBP2
Patient 58	dnAML	deletion	chr7	77200000	158821424	81,62	EZH2
Patient 59	dnAML	deletion	chr12	7200000	20680000	13,48	AEBP2
Patient 59	dnAML	deletion	chr7	1	158821424	158,82	EZH2
Patient 60	dnAML	deletion	chr7	124700000	158821424	34,12	EZH2

Patient 61	ET	deletion	chr11	85000000	125740000	40,74	EED
Patient 62	ET	deletion	chr7	81250000	158821424	77,57	EZH2
Patient 63	ET	deletion	chr11	84940000	125470000	40,53	EED
Patient 64	post-MPN AML	deletion	chr12	14000000	25180000	11,18	AEBP2
Patient 65	post-MDS AML	deletion	chr12	10400000	27600000	17,20	AEBP2
Patient 65	post-MDS AML	deletion	chr7	116600000	150400000	33,80	EZH2
Patient 66	post-MDS AML	deletion	chr7	1	158821424	158,82	EZH2
Patient 67	post-MDS AML	deletion	chr7	1	158821424	158,82	EZH2
Patient 68	post-MPN AML	deletion	chr17	1	78774742	78,77	EZH1, SUZ12
Patient 69	post-MDS AML	deletion	chr17	25600000	27400000	1,80	SUZ12
Patient 69	post-MDS AML	deletion	chr7	1	158821424	158,82	EZH2
Patient 70	post-MDS AML	deletion	chr12	9000000	27500000	18,50	AEBP2
Patient 70	post-MDS AML	deletion	chr7	61600000	155000000	93,40	EZH2
Patient 71	post-MDS AML	deletion	chr17	25950000	27420000	1,47	SUZ12
Patient 72	post-MPN AML	deletion	chr17	24850000	30950000	6,10	SUZ12
Patient 73	post-MPN AML	deletion	chr7	81860000	158821424	76,96	EZH2
Patient 74	post-MPN AML	deletion	chr7	81900000	158821424	76,92	EZH2
Patient 75	post-MPN AML	deletion	chr7	1	158821424	158,82	EZH2
Patient 76	post-MPN AML	deletion	chr7	1	158821424	158,82	EZH2
Patient 77	post-MPN AML	deletion	chr7	1	158821424	158,82	EZH2
Patient 78	post-MPN AML	deletion	chr7	59100000	158821424	99,72	EZH2
Patient 79	post-MPN AML	deletion	chr17	25795000	27480000	1,69	SUZ12
Patient 80	post-MPN AML	deletion	chr7	92206000	158821424	66,62	EZH2
Patient 81	post-MDS AML	deletion	chr7	97100000	158821424	61,72	EZH2
Patient 82	post-MPN AML	deletion	chr17	24450000	29700000	5,25	SUZ12
Patient 82	post-MPN AML	deletion	chr7	101350000	158821424	57,47	EZH2
Patient 83	dnAML	deletion	chr6	1	26160000	26,16	JARID2
Patient 83	dnAML	deletion	chr17	1	78774742	78,77	EZH1, SUZ12

The reference genome used for defining genomic positions of the lesions is human reference genome hg18. UPN, unique patient number; ET, essential thrombocythemia; PMF, primary myelofibrosis; PV, polycythemia vera; sMF; secondary myelofibrosis; post-MPN AML, post-myeloproliferative neoplasm acute myeloid leukemia; post-MDS AML, post-myelodysplastic syndrome acute myeloid leukemia; dnAML, *de novo* acute myeloid leukemia; CML, chronic myeloid leukemia; UPD, uniparental disomy;

CURRICULUM VITAE

Ana Puda

E-mail: ana.pudja@yahoo.com
Citizenship: Serbian
City of residence: Vienna, Austria
Visa type in Austria: Daueraufenthalt – EU
Driving license: B
Date of birth: 14.12.1984.
Family status: Married, 3 children (2015, 2015, 2012)

EXPERIENCE AND COMPETENCE PROFILE

- International research experience in hematology, genetics and structural biology
- Project Management certification and know-how
- Experience in the NGO sector and EU projects
- Prominent analytical, strategic, conceptual and organizational skills
- Result and quality orientation, combined with creative, networked thinking and the ability to prioritize
- Excellent time and resource management
- Confident communicator and presenter
- Highly motivated team player with drive and charisma
- High sense of responsibility, reliability, fairness and integrity
- Open, communicative and positive personality

RESEARCH AND EXPERIENCE

09/2019- **Project Manager,** St. Anna Children Cancer Research Institute, Vienna

2018 **Project Management University Course,** Uni for Life / Karl-Franzens-Universität Graz

2015-2018 **Family Management**

2010-2015 **PhD Student, CeMM - Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria.**
Group of Prof. Dr. Robert Kralovics

2009-2010 **Postgraduate student in training, Institute for Medical Research, Military Medical Academy (VMA), Belgrade, Serbia**.
Group of Prof. Dr. Zvonko Magic

2009 **Master student, EMBL (European Molecular Biology Laboratory), Grenoble, France**.
Group of Prof. Dr. Imre Berger

2004-2009 **Project assistant at NGO "Environmental ambassadors", Belgrade, Serbia**

CERTIFICATES

2019 **International Project Management Association Certificate – Level D**

EDUCATION

2018	**Modern Management: Project Management University Course**, Uni for Life / Karl-Franzens-Universität Graz
2010-	**PhD, Molecular Signal Transduction**, Medical University of Vienna, Austria PhD thesis project**: Role of PRC2 in leukemic transformation of chronic myeloid malignancies**
2003-2009	**B.Sc. and M.Sc, Molecular Biology and Physiology**, University of Belgrade, Serbia Master thesis project: **New expression tools for eukaryotic multiprotein complex production** (EMBL, Grenoble)

SKILLS AND COMPETENCIES

Computer Skills

- Microsoft Office package
- MAC OS programs
- SPSS statistical software
- Genotyping Console (Affymetrix) software
- Sequencher (Gene Codes Corporation) software

Languages

- Serbian: native
- English: native
- German: fluent
- French: advanced
- Spanish: basic

Organizational and person skills

- Time management
- Prioritizing
- Strong communication skills
- Strong presentation skills
- Interpersonal communication
- Motivator
- Structured work style
- Adaptability
- Reliability
- Team player
- Positive attitude
- Proactive
- Empathy
- Punctuality
- Attention to detail
- Creative problem solving
- Patience and stress-resistant
- Customer orientation
- Explaining complex topics in simple words

Experimental Skills

- DNA sequencing (capillary and NGS)
- Genome-Wide Human SNP arrays
- DNA library preparation
- DNA and RNA isolation
- PCR
- DNA cloning
- Cell culture
- Drug screens
- FACS
- Transfection/Infection
- Protein Expression

PUBLICATIONS / CONFERENCES

2015 EHA (European Hematology Association) Congress, Vienna, Austria. Poster presentation: High resolution cytogenetic mapping and whole exome sequencing reveal a complex pattern of chromosome 6p aberrations in patients with myeloid malignancies. **Pudja A**, Milosevic JD, Klampfl T et al. EHA Learning Center. Pudja A. Jun 12, 2015; 100547

2013 Complex Patterns of Chromosome 11 Aberrations in Myeloid Malignancies Target CBL, MLL, DDB1 and LMO2. Klampfl T, Milosevic JD, **Puda A**, et al. PLoS One. 2013 Oct 16;8(10):e77819. doi: 10.1371/journal.pone.0077819.

2012 Clinical significance of genetic aberrations in secondary acute myeloid leukemia. Milosevic JD, **Puda A**, Malcovati L, et al. Am J Hematol. 2012 Jul 9. doi:10.1002/ajh.23309

2012 Frequent deletions of JARID2 in leukemic transformation of chronic myeloid malignancies. **Puda A**, Milosevic JD, Berg T, et al. Am J Hematol. 2012 Mar;87(3):245-50. doi: 10.1002/ajh.22257.

2011 ASH (American Society of Hematology) Annual Meeting and Exposition, San Diego, CA, USA. Poster presentation: Distinct Genetic Lesions Drive Leukemogenesis in Secondary Acute Myeloid Leukemia. **Puda A**, Milosevic JD, Berg T et al. Blood 2011 118:3559

2011 Reporting by selected Analytical Tools for Environmental Management: Hazardous waste management in South Eastern Europe. Mihajlov A, **Puda A**, Jovanovic F, Stevanovic-Carapina H. Journal of Environmental Protection and Ecology, 2011 12(2):565-569.