universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„Analysis of *CUX1* and *SH2B2* in the pathogenesis of myeloproliferative neoplasms“

Verfasserin

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angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag.rer.nat.)

Wien, 2011

Studienkennzahl lt. Studienblatt:	A 490
Studienrichtung lt. Studienblatt:	Diplomstudium Molekulare Biologie
Betreuer:	Ao. Univ.-Prof. Mag. Dr. Ernst Müllner

ZUSAMMENFASSUNG

Myeloproliferative Neoplasien (MPN) sind klonale hämatologische Erkrankungen, welche zu den myeloischen Malignomen gehören. Laut der WHO Klassifizierung von 2008, gehören Polycythämia vera (PV), Essentielle Thrombozythämie (ET), Primäre Myelofibrose (PMF), Chronische myeloische Leukämie (CML), Chronische Neutrophilenleukämie (CNL), Chronische Eosinophilenleukämie (CEL), Systemische Mastozytose (SM) und unklassifizierbare Myeloproliferative Neoplasien zu den MPN. Das Hauptmerkmal von MPN ist die übermäßige Bildung von terminal differenzierten Blutzellen, die zu der myeloischen Reihe gehören. Die individuellen klinischen Merkmale der drei klassischen *BCR-ABL1*-negativen MPN sind Erythrozytose in PV, Thrombozythämie in ET und Fibrosierung des Knochenmarkgewebes in PMF. Eine Transformation zur akuten Leukämie ist, neben Thrombose, die größte Komplikation von MPN aufgrund von einer schlechten Prognose und einer voraussichtlichen Lebensdauer von 5 Monaten. Diese Studie konzentriert sich auf den drei klassischen *BCR-ABL1*-negativen MPN, PV, ET und PMF und deren Transformation zu AML.

In 29 Patienten unserer Kohorte wurden Deletionen und uniparentale Disomien (UPD) von Chromosom 7q gefunden und in einer der Deletionen befand sich nur *CUX1*. Es wurde berichtet, dass Deletionen von Chromosom 7q mit der Transformation assoziiert sind, deswegen wurde eine Sequenzanalyse von *CUX1* und *SH2B2*, das 1kb von *CUX1* entfernt liegt, in Patienten, die akute Leukämie entwickelt haben, durchgeführt.

Als Methode zur Aufklärung der Konsequenzen von *CUX1* und *SH2B2* Deletionen wurden shRNAs, die gegen die spezifischen Gene gerichtet waren, in der Baf3/EpoR Zelllinie getestet. Die Wachstumskinetik der transduzierten Zelllinien hat keinen Effekt von den shRNAs gezeigt.

TP53 wurde in 24 Patienten, die eine Krankheitsprogression zu sMF oder AML erfahren haben, sequenziert. Es wurde berichtet, dass auch dieses Gen mit der Tranformation von MPN Patienten assoziiert ist. In unserer Kohorte wurden 5 somatische Mutationen (21%) gefunden.

Diese Resultate liefern Beweise zu der Komplexität von MPN. Die Identifikation dieser neuen genetischen Veränderungen in MPN Patienten könnte Auswirkungen auf bessere Möglichkeiten der Diagnose und Therapie haben.

ABSTRACT

Myeloproliferative neoplasms (MPN) are clonal hematologic diseases that belong to the myeloid malignancies. According to the 2008 WHO classification, diseases that are considered MPN include polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), chronic myelogenous leukemia (CML), chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia (CEL), hypereosinophilic syndrome (HES), mast cell disease, and unclassifiable myeloproliferative neoplasms. The main phenotypic feature of MPN is the excessive production of terminally differentiated blood cells, belonging to the myeloid lineage. The individual clinical features of the classical *BCR-ABL1*-negative MPN are erythrocytosis in PV, thrombocythemia in ET, and replacement of the bone marrow by fibrotic tissue in PMF. Transformation into post-MPN AML is, besides thrombosis, the main complication of MPN, because of the poor prognosis and a mean survival of 5 months. This study focuses on the three classical *BCR-ABL1*-negative MPN, PV, ET, and PMF, and their transformation to leukemia.

Deletions and uniparental disomies (UPDs) of chromosome 7q were found in 29 patients from our cohort, and one of them carried a single gene deletion which contained *CUX1*. Deletions of chromosome 7q were reported to be associated with transformation, and because of this, sequence analysis of *CUX1* and *SH2B2*, which is located 1kb away from *CUX1*, was performed in patients that developed post-MPN AML.

As an approach to elucidate the consequences of *CUX1* and *SH2B2* deletion, RNA short hairpins targeting the specific genes were tested in the Baf3/EpoR cell line. Transduced cell lines did not reveal an effect of the hairpins in growth kinetics.

TP53 was sequenced in 24 patients that experienced disease progression either to sMF or post-MPN AML. This gene was also reported to be associated with transformation of MPN patients and in our cohort 5 somatic mutations (21%) were found.

These results provide evidence of the complexity of MPN. The identification of these new genetic alterations in MPN patients could have implications in better diagnosis and treatment possibilities.

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1 INTRODUCTION

1.1 Myeloproliferative neoplasms

Myeloproliferative neoplasms (MPN), according to the 2008 classification of the World Health Organisation (WHO), comprise nine clonal hematologic diseases. They belong to the myeloid malignancies, which include also acute myeloid leukemia (AML), myelodysplastic syndromes (MDS), MDS/MPN, and MPN eosinophilia (MPN-eos) (figure 1). MPN share the main phenotypic feature of excessive production of terminally differentiated blood cells, such as those of the granulocytic (neutrophil, eosinophil, basophil), monocytic/macrophage, erythroid, megakaryocytic and mast cell lineages. The three classical *BCR-ABL1*-negative MPN are polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). Other diseases that are considered MPN under the 2008 WHO classification include chronic myelogenous leukemia (CML), chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia (CEL), hypereosinophilic syndrome (HES), mast cell disease, and unclassifiable myeloproliferative neoplasms (figure 1).[2]

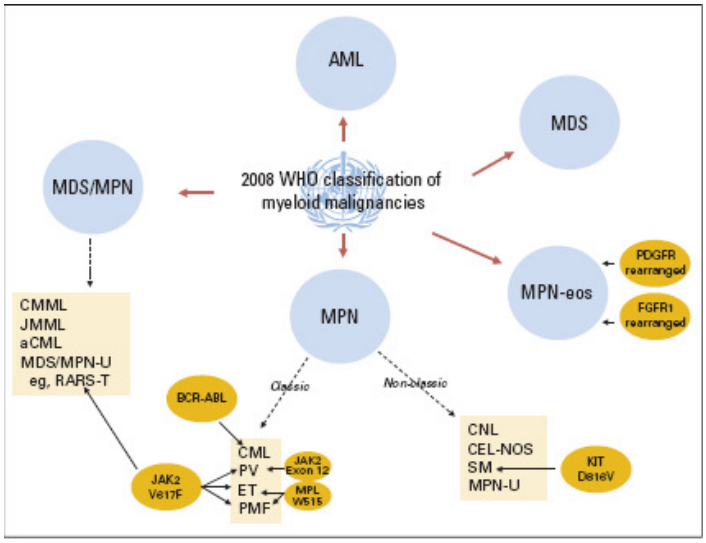


Figure 1 The 2008 WHO classification for myeloid neoplasms[1]

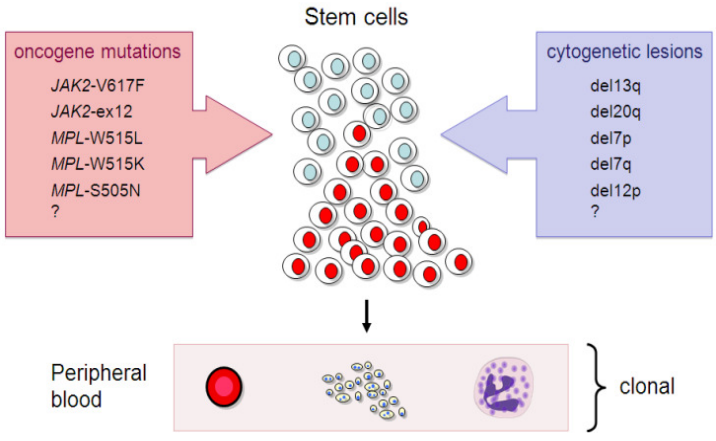


Figure 2 Clonal origin of MPN (kindly provided by Dr. Robert Kralovics)

In 1951 William Dameshek described in the *Blood* journal editorial entitled "Some speculations on the myeloproliferative syndromes" the clinical and pathologic similarities and the common origin of CML, PV, ET, and PMF.[3] Later, analyses of X-chromosome inactivation patterns

in female patients showed that MPN are clonal disorders arising from a transformation of a single hematopoietic stem cell (figure 2).[4] Adamson *et al.* showed that peripheral blood cells from a PV patient expressed only one isoform of the polymorphic X-linked *G6PD* gene, proving that these cells originated from one clone[5] and other studies confirmed this finding also for ET and PMF patients.[6-7] X-chromosome-based clonality assays have the advantage that they can confirm the clonal origin of pathologic cells in disorders in which the reasons for clonality are unknown, but this information is restricted only to women.[4]

MPN display different phenotypes. For example, chronic myeloid leukemia (CML) is characterized by the *BCR-ABL1* fusion gene resulting from a reciprocal translocation between chromosomes 9 and 22 (t(9;22)(q34;q11), Philadelphia chromosome).[8-11] The individual clinical features of the classical Philadelphia chromosome-negative MPN are increased erythrocyte cell counts (erythrocytosis) in PV, elevated platelet levels (thrombocythemia) in ET, and replacement of the bone marrow by fibrotic tissue in PMF (figure 3). Although each of the MPN is recognized as a distinct clinicopathological entity, they share common features, such as hyperproliferation of terminally differentiated blood cells, bone marrow hypercellularity, tendency to thrombosis and hemorrhage, and a risk of secondary leukemic transformation.[12-13] The estimated annual incidence of PV, ET, and PMF is five out of 100,000.[14-15]

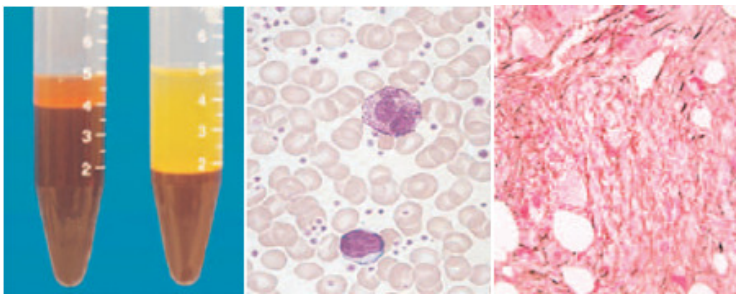


Figure 3 Laboratory features of PV, ET, and PMF[12]

Besides the sporadic MPN form, there are also cases of familial MPN, which is an autosomal dominantly inherited disease with incomplete penetrance. It is also characterized by the fact that the three classical Philadelphia chromosome-negative MPN can be variably presented in a single family, and that its clinical and molecular features cannot be distinguished from sporadic MPN.[16-17] Most probably an unknown germline mutation predisposes carriers to acquire other mutations and develop MPN.[18]

1.2 Transformation

The disease course of MPN is generally chronic, but some patients progress into post-PV or post-ET secondary myelofibrosis (sMF), into an "accelerated phase" (AP) or transform into acute leukemia. The rate of progression to sMF is 10-20% after 15-20 years.[19-20] Transformation into post-MPN AML is, besides thrombosis, the main complication of MPN, because of the poor prognosis and a mean survival of 5 months.[21] The yearly risk for transformation is 0.38% for PV, 0.37% for ET, and 1.09% for PMF.[22]

Disease progression is associated with aberrations of chromosomes 1q and 9p, and transformation with gains of 1q, and 3q, deletions of 7q, 5q, 6p, and 7p, and uniparental disomies of 19q, and 22q (figure 4). This was shown by comparison of the distribution of chromosomal abnormalities between samples in chronic phase, sMF/AP, and post-MPN AML. Commonly affected regions were mapped to target genes on chromosomes 3p (*FOXP1*), 4q (*TET2*), 7p (*IKZF1*), 7q (*CUX1*), 12p (*ETV6*), and 21q (*RUNX1*). It was shown that patients with sMF/AP had significantly more chromosomal aberrations than patients in the chronic phase and less compared to the ones with post-MPN AML. It was also found that there is no association of disease duration with the frequency of chromosomal aberrations which could theoretically accumulate during the evolution of the malignant clone. On the other hand the patients' age at the time of sample associated with the number of defects.[21] One of the chromosomal aberrations that are most significantly associated with sMF/AP and post-MPN AML are gains on chromosome 1q. The common amplified region of 1q contains the *MDM4* gene, which is an inhibitor of p53 that is also associated with post-MPN AML.[21,23] The CDR of chromosome 7q contained the *CUX1* gene (cut-like homeobox 1). This deletion is one of the most significantly associated with post-MPN AML.[21,24] Knowing the modifications leading to MPN disease progression could be important for new therapies which could prevent the transformation to post-MPN AML.[21]

Aberration type		Post-MPN AML (n=29)	Chronic MPN (n=321)	P value	P value*
gain	1q	5 (17.24%)	2 (0.62%)	5.20×10^{-5}	0.0013
deletion	7q	5 (17.24%)	2 (0.62%)	5.20×10^{-5}	0.0013
deletion	5q	4 (13.79%)	1 (0.31%)	1.82×10^{-4}	0.0046
deletion	6p	4 (13.79%)	1 (0.31%)	1.82×10^{-4}	0.0046
deletion	7p	3 (10.34%)	0 (0.00%)	5.16×10^{-4}	0.0129
UPD	19q	3 (10.34%)	0 (0.00%)	5.16×10^{-4}	0.0129
gain	3q	3 (10.34%)	1 (0.31%)	0.0019	0.0487
UPD	22q	3 (10.34%)	1 (0.31%)	0.0019	0.0487

*p values after Bonferroni correction for multiple testing; significant associations are highlighted.

Figure 4 Association of individual chromosomal aberrations with progression to post-MPN AML[21]

1.3 JAK2

Originally, it was thought that the elevated production of blood cells in MPN is due to hypersensitivity of hematopoietic progenitors to cytokines. It was demonstrated in 1974 by Prchal and Axelrad that endogenous erythroid colonies (EEC), which are an indicator of abnormal in vitro growth of hematopoietic progenitors, grow without exogenous erythropoietin.[25] Already early reports were suggesting that JAK-STAT signaling may play a role in the pathogenesis of PV, ET, and PMF. One of the arguments in favor of this hypothesis was that *JAK2*-knockout mice didn't have any erythropoiesis and were embryonic lethal.[26-27]

Further investigations applying different experimental approaches led to the identification of a gain-of-function mutation in the Janus kinase 2 gene (*JAK2*, 9p24)[28-32] that is present in almost all patients with PV (90-95%),[30] and in 50-60% of patients with ET or PMF.[33] Aberrant signaling caused by mutations in tyrosine kinases is a common characteristic for MPN. Some examples for this are the *BCR-ABL* translocation in CML, *FIP1L1-PDGFRA* in CEL, and *PDGFRA/B* translocations in chronic myelomonocytic leukemia and MDS/MPN overlap diseases often associated with eosinophilia.[33] The activating mutation was found by four different approaches. One of it was to make a microsatellite mapping to define the region of 9p containing the *JAK2* gene.[28] Another was to use siRNA against *JAK2* which impaired erythroid-terminal differentiation and blocked EEC formation.[30] Other groups sequenced the tyrosine kinase genome of MPN patients.[29,31]

The *JAK2* gene encodes a cytoplasmic tyrosine kinase that is essential in signal transduction of hematopoietic cytokines, as for example erythropoietin (Epo), thrombopoietin (Tpo), IL-3, granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage CSF (GM-CSF) receptors. The JAK family of proteins contains four kinases, JAK1, JAK2, JAK3, and TYK2, which have essential, non-redundant roles in cytokine signaling.[34] The JAK2 kinase is bound to cytokine receptors without

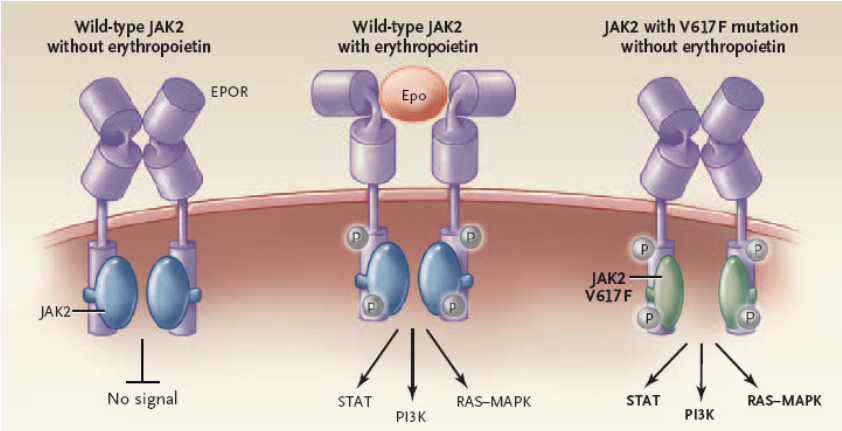


Figure 5 Role of JAK2 in pathway signaling and erythropoietin binding[12]

intrinsic kinase activity (e.g. the erythropoietin receptor) in the endoplasmic reticulum and is required for their cell-surface expression.[35] When erythropoietin binds to the extracellular domain of the erythropoietin receptor, it provokes a conformational change[36-38] and a subsequent phosphorylation and activation of JAK2.[39] The activated JAK2 kinase then phosphorylates the receptor's cytoplasmic domain and thus, promotes docking of downstream effector proteins via recruitment of SH2-domain containing proteins, such as STAT3 and STAT5,[41-42] and initiates intracellular signaling cascades (figure 5).[40-41] STAT proteins get phosphorylated in that way by JAK2, dimerize and translocate to the nucleus, where they activate the transcription of genes involved in many cellular processes.[41-42] The activity of JAK2 is regulated by the requirement to bind to specific domains in receptors, suppression of activation by the pseudokinase domain, and the requirement for phosphorylation within the activation loop.[34]

JAK2 has three specific domains – the typical kinase domain (JH1), a pseudokinase domain (JH2), and regions of homology (JH3–7) located in the N-terminal half of the protein and unique among the JAK family of proteins. The amino terminal domain (JH5-7) which contains the FERM domain and a region of receptor homology (box 1 and box 2 domains) is needed for association with cytokine receptors.[34] The acquired guanine-to-thymidine mutation at position 1849 of the *JAK2* gene, which was identified in MPN patients, causes a phenylalanine for valine substitution at codon 617 (*JAK2*-V617F).[28-31] As the mutation lies within the pseudokinase domain of JAK2, it disrupts its kinase-regulatory activity and causes constitutive activation of the kinase in the absence of ligand (figure 6).[43] Furthermore, it results in cytokine-independent activation of the JAK-STAT, PI3K, and RAS-MAPK signaling pathways (figure 5),[28,30-31,44] involved in erythropoietin-receptor signaling.[39,45-47] It has been shown that overexpression of *JAK2*-V617F in cell lines leads to phosphorylation of JAK2 in the absence of cytokine stimulation and that these cells exhibit cytokine independence or hypersensitivity.[28-30]

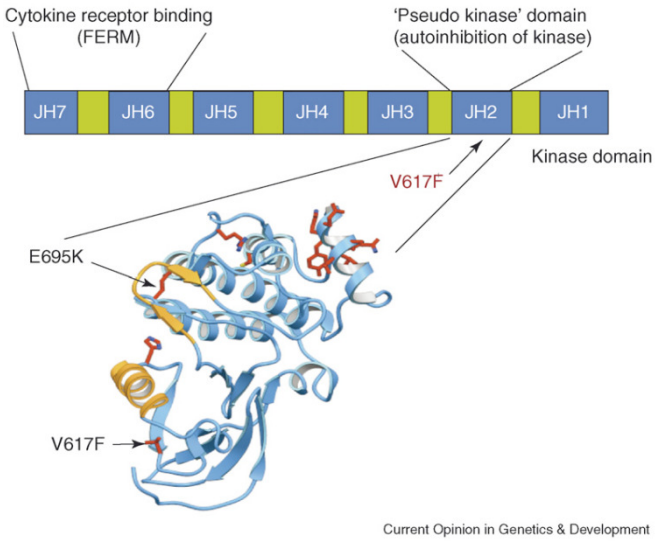


Figure 6 Structure of JAK2 and JAK2-V617F[34]

Expression of *JAK2*-V617F in murine hematopoietic cells by a retroviral vector recapitulates the clinical features of PV, especially erythrocytosis.[48-49] On the other hand, transgenic models with more physiologic levels of *JAK2*-V617F expression resemble ET and PMF.[50-52] *JAK2*-V617F allele burden in MPN patients has been also measured in some studies. It tends to be highest in PV and PMF patients and lower in patients with ET.[52-53] Two studies showed that in PV patients there is an association between higher *JAK2*-V617F allele burden and leukocytosis.[54-55] These observations confirm that there is a causal link between the *JAK2*-V617F mutation and the MPN phenotype and that the pathogenesis of MPN is characterized through its heterogeneity.[12,18] Some studies also suggest that the effects of the mutation can be modified by the individual genetic background. In C57Bl/6 mice, transplantation with *JAK2*-V617F-transduced cells resulted in a PV-like disease,[49,56-57] however, in Balb/C mice, the transplantations resulted not only in erythrocytosis, but also leukocytosis, and a development of myelofibrosis.[48]

Other mutations in *JAK2* have been also identified, lying in the exon 12 region of the gene. A combination of missense, insertion, or deletion mutations was found, all located just 5' of the pseudokinase domain.[58-59] This finding was supported by cell line experiments, a retroviral transplant assay and subsequent studies which were performed on more patients.[60-62] In contrast to *JAK2*-V617F, the exon 12 mutations appear only in PV patients.[58]

Many of the patients with PV or PMF are homozygous for *JAK2*-V617F. The mechanism leading to homozygosity is not loss of the wild-type allele, but uniparental disomy (UPD), resulting from mitotic recombination affecting chromosome 9p, and duplication of this region (figure 7). Mitotic recombination is due to an exchange of chromosomal DNA between non-sister chromatids during mitosis. The observed breakpoints are spread between the *JAK2* locus and the centromere,[18,28] indicating that there is no single fragile site that is susceptible to recombination.[12] Acquired UPD is thought to be one of the genetic mechanisms involved in tumor suppressor inactivation, because it leads to homozygosity of mutated or deleted alleles of tumor suppressors via mitotic recombination.[63-66] UPD can be

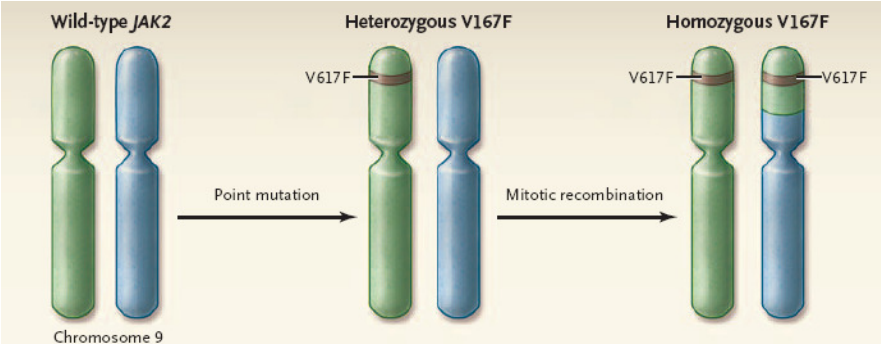


Figure 7 Mitotic recombination as the mechanism of 9pLOH[12]

detected by comparing genotypes of tumor and non-tumor-derived DNA samples and assessing polymorphic DNA markers such as single nucleotide polymorphisms (SNPs), microsatellite markers (simple repeat sequences), insertion-deletion polymorphisms or tandem repeats.[18]

In 2009 a common haplotype that contains the *JAK2* gene and preferentially acquires *JAK2* mutations was identified. Investigations revealed a nonrandom distribution of the somatic *JAK2*-V617F mutation between two parental alleles. Moreover, it was shown that more than 80% of all *JAK2*-V617F mutations occur on this specific haplotype, which is referred to as the *JAK2* GGCC haplotype.[67-68] Disease-associated SNPs and haplotypes which are identified by genome-wide association studies are thought to influence either the expression of genes or the sequence of the proteins they encode. A certain combination of SNPs could make a haplotype differentially susceptible to somatic mutagenesis. [67-68] Two alternative hypotheses were made to explain this observation. One of it is that *JAK2* mutations occur randomly on both haplotypes, but the GGCC haplotype has specific properties necessary to propagate MPN disease phenotype. Alternatively, the GGCC haplotype is more prone to somatic mutagenesis and mutations of the *JAK2* gene occur more frequently on this specific haplotype. However, the mechanism by which this differential mutability of haplotypes might be reached remains to be resolved.[67-68]

1.4 Other mutations

Most MPN patients carry an activating *JAK2* or *MPL* mutation, but some have also *SH2B3*, *CBL*, *TET2*, *ASXL1*, *IDH*, *IKZF1*, or *EZH2* mutations (figure 8).[1]

The main known phenotypic mutations in MPN are *JAK2*-V617F,[28-31] *JAK2* exon 12 mutations (*JAK2*-ex12),[59] and mutations of the thrombopoietin receptor gene *MPL* such as *MPL*-W515L, *MPL*-W515K, *MPL*-S505N, *MPL*-A506T, and *MPL*-A519T.[69-72]

JAK2-ex12 mutations have been found in about 1% of *JAK2*-V617F negative PV patients, but not in other MPN.[58] However, due to the rarity of this mutation it is difficult to predict whether other MPN also harbor *JAK2*-ex12 mutations at lower frequencies.[59,61]

Activating gain-of-function mutations in the thrombopoietin receptor gene *MPL* (myeloproliferative leukemia virus, 1p34) were identified in about 10% of patients with *JAK2*-V617F-negative PMF and in 3% of patients with V617F-negative ET, but not in

PV.[69-71,73] Expression of *MPL*-W515L establishes factor independent growth in hematopoietic cells while activating STAT, MAPK and PI3K–Akt signaling pathways in a similar fashion to *JAK2*-V617F. *JAK2*-V617F and *MPL*-W515L activate similar signaling pathways when expressed *in vitro*, but *MPL*-W515L expression *in vivo* results in marked thrombocytosis and myelofibrosis.[74]

c-CBL (casitas B-lineage lymphoma, 11q23.3) mutations in myeloid malignancies are usually associated with 11q UPD.[75] In a recent study on MPN patients, the mutations were only found in either exon 8 or 9 in 6% of patients with PMF.[76] Cbl proteins are multifunctional adaptor proteins and E3 ubiquitin ligases which are involved in the trafficking and degradation of activated tyrosine kinases.[77]

TET2 (TET oncogene family member 2, 4q24) can be found in *JAK2*-V617F positive and negative MPN. The mutational frequencies are around 16% in PV, 5% in ET, and 17% in PMF.[78-79] Together with *ASXL1*, *TET2* could contribute to the epigenetic regulation of hematopoiesis.[79-80]

ASXL1 (additional sex combs-like 1, 20q11.1) mutations are seen in around 8% of MPN patients. A heterozygous *ASXL1* mutation was identified in five MPN patients who were all *JAK2*-V617F negative.[81]

IDH1 and *IDH2* (isocitrate dehydrogenase, 2q33.3 and 15q26.1) were found in 1,9% of PV, 0,8% of ET, and 4,2% of PMF patients.[82] Functional characterization of *IDH* mutations suggests neoenzymatic activity in converting α-ketoglutarate to the possibly oncogenic 2-hydroxyglutarate.[1]

IKZF1(IKAROS family zinc finger 1, 7p12) mutation frequency is 0,2% in chronic phase PV, ET, and PMF patients, but 21% in blast phase patients, which shows that there is a significant association of *IKZF1* deletions with leukemic transformation.[83] *IKZF1* encodes a transcription factor which has a pleiotropic function in the regulation of hematopoiesis.[84] Functional studies in mouse models suggest that decreased Ikaros function is oncogenic.[84-88]

EZH2 (encodes the catalytic subunit of the polycomb repressive complex 2, 7q36.1) was reported to be a target of 7qUPDs,[89-90] and suggested to be a tumor suppressor in myeloid malignancies.[89]

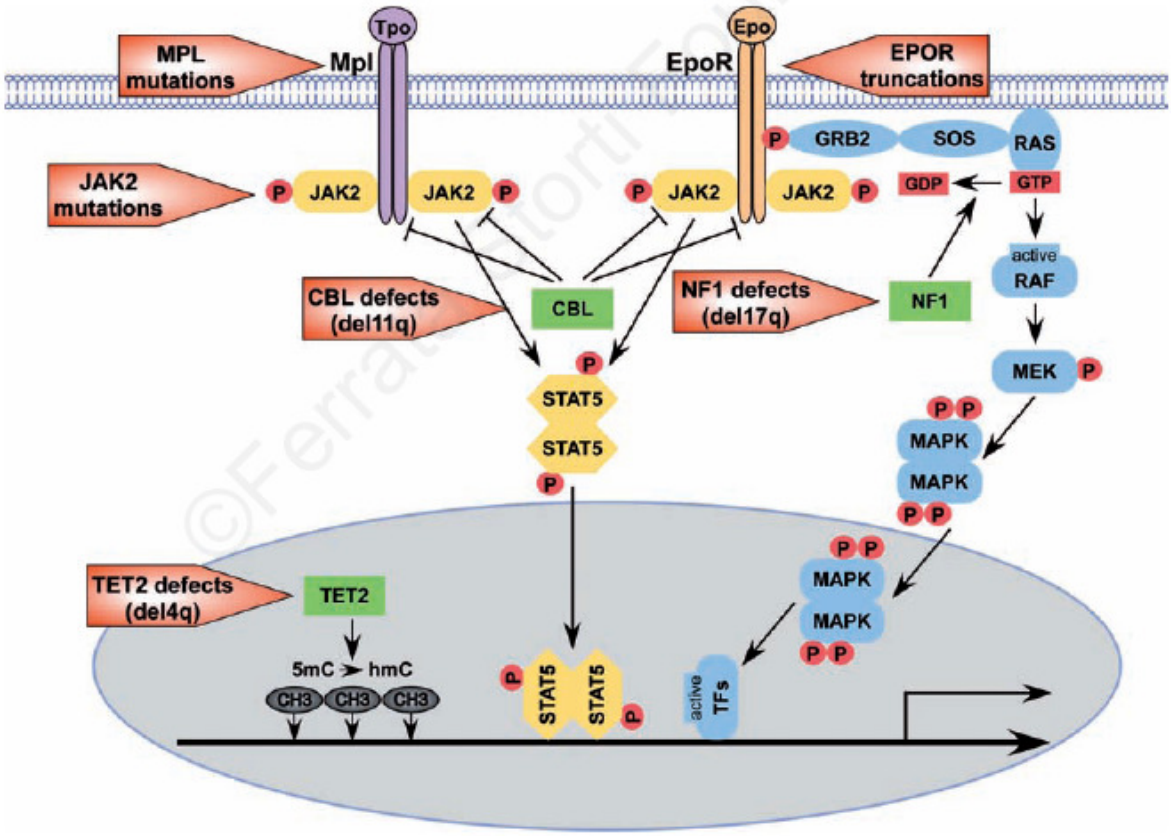


Figure 8 Mechanisms and pathways targeted by mutations in MPN[91]

Host modifiers, previous and subsequent mutations have also been postulated to play a role in MPN pathogenesis.[92-94] In accordance with this hypothesis, murine models of *JAK2*-V617F positive MPN show pronounced strain-specific variation in phenotype, including variability in the degree of leukocytosis and fibrosis.[56]

1.5 Chromosomes 7q and 12q

CUX1 (cut-like homeobox 1, *CUTL1, CDP*) is located on chromosome 7q and encodes a transcription factor that has a role in cell cycle regulation, cell motility, invasion, and hematopoiesis.[95-99] An increase in *CUX1* expression has been observed during cell cycle progression following exit from quiescence[100-101], following TGF-β stimulation[96], in breast tumors and cancer cell lines[102], in malignant plasma cells[103], and in acute lymphoblastic leukemia.[104]

CUX1 is located on 7q22 and contains 33 exons. Resulting from proteolytic processing or transcription initiation at an alternative start site, at least three protein isoforms can be expressed in human (p200, p110, p75). In mice there were additional isoforms identified – p75, p80, p90, p110, p150 and p200 (figure 9).[95,97] They have distinct DNA binding and transcriptional properties.[95] The p200 form of *CUX1* has four conserved DNA-binding domains, three Cut repeats and one Cut homeodomain (HOX). Originally, *CUX1* was shown to function as a transcriptional repressor that down-regulates lineage specific genes in precursor cells that later become expressed in terminally differentiated cells.[95,97,105] The expression and activity of *CUX1* are regulated by alternative transcription initiation, proteolytic processing, phosphorylation and acetylation.[95]

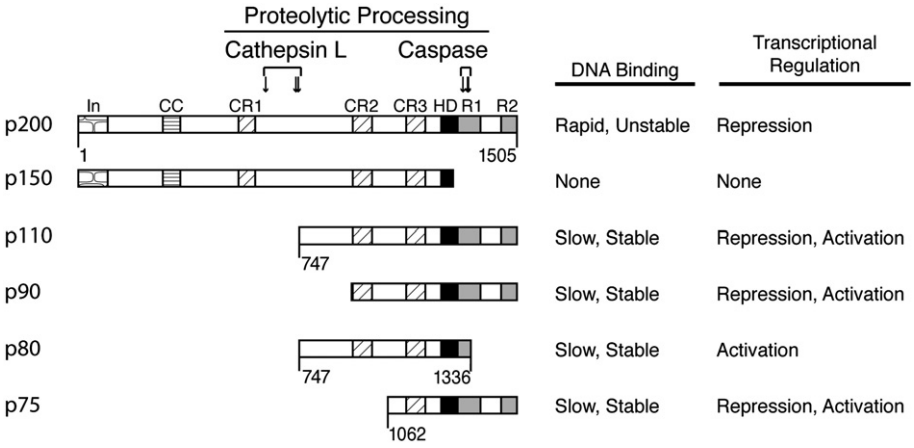


Figure 9 Cux1 isoforms exhibit distinct DNA binding and transcriptional properties[95]

Three knock-out mouse models have been generated for *CUX1*. In the first model only the Cut repeat 1 was lacking and this resulted in mice with wavy hair, curly whiskers from day 2 to day 19, and impaired lactation in homozygous females that resulted in a high percentage (50%) of post-natal lethality in their litters.[97,106] In another model the CUX1 protein was truncated after the Cut repeat 3 and lacked the Cut homeodomain and the carboxy-terminal region. Only few homozygous mice survived to weaning age and these failed to thrive, had a reduced stature, gained little weight, had wavy whiskers and lost fur at 2 or 3 weeks of age. Homozygous mutant mice had also a reduction in the number of B cells in the bone marrow, and the number of T cells was reduced 5-fold in the thymus. On the other hand there was an increase in the number of myeloid cells in the bone marrow, spleen and peripheral blood. This showed that *CUX1* expression is important for homeostasis in the hematopoietic system.[107-108] In the third model exons 22 and 23 (most of the Cut repeat 3 and the entire Cut homeodomain) were replaced with LacZ. 99% of homozygous mice died after birth because of respiratory failure, and the surviving mice displayed growth retardation and an abnormal hair coat.[109]

There were also many *CUX1* transgenic mouse models generated. In one case, the mice developed multiorgan organomegaly, and the higher cell number was due to a higher number of proliferating cells. This indicates that Cux1 expression does not interfere with terminal differentiation.[109-110] Other transgenic mice were designed to compare the different isoforms of *CUX1*. Most of the p75 mice suffered from a MPN-like myeloid leukemia which was characterized by splenomegaly, hepatomegaly, and frequent infiltration of leukocytes into non-hematopoietic organs. It was impossible to transplant the disease into recipient mice which would suggest that expression of the p75 *CUX1* transgene did rather take place in a committed myeloid progenitor than in the hematopoietic stem cell.[99]

Region 7q22 where *CUX1* is located, is a region which is frequently deleted in uterine leiomyomas,[111] acute myeloid leukemia,[112] and myelodysplastic syndromes.[113] In one study there was a patient found which had a 0.88Mb deletion on chromosome 7q which contained only the genes *CUX1* and *SH2B2*.[114] Recently, a missense mutation in the HOX domain of *CUX1* was identified through a previous screen of a large cohort of *BCR-ABL1*-negative MPN patients at the time of leukemic transformation. The complexity of 7q rearrangements suggests that maybe not a mutation in a single gene, but the alteration of different genetic factors could be an important part of the pathogenesis in patients with deletions of chromosome 7q.[105]

The gene located right after *CUX1* on chromosome 7q is *SH2B2*. The SH2B family has three members, *SH2B1* (*SH2-B*), *SH2B2* (*APS*), and *SH2B3* (*LNK*). All of them contain an N-terminal dimerization (DD), a central pleckstrin homology (PH), and a C-terminal SH2 domain (figure 10).[115-117] SH2B2 is an adaptor protein that binds via the SH2 domain to JAK2, and subsequently phosphorylates the insulin receptor. *SH2B2* may regulate by this means energy balance and body weight by enhancing *JAK2*-mediated cytokine signaling.[117] It also increases insulin signaling in cultured cells,[119] but surprisingly a deletion of *SH2B2* results in enhanced insulin sensitivity and cytokine action.[120-121] In mice a novel isoform of *SH2B2*, *SH2B2β*, was also reported, which lacks a SH2 domain, dimerizes with *SH2B1* and *SH2B2α*, and thereby inhibits cellular responses mediated by these genes.[117] Another function of *SH2B2* is the enhancement of

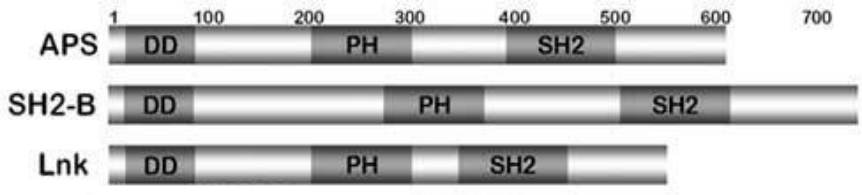


Figure 10 Domain organization of human SH2B1, SH2B2, and SH2B3[118]

neurotrophin signaling by direct modulation of Trk receptor autophosphorylation,[122] and the inhibition of the JAK/STAT pathway by recruitment of c-Cbl into the receptor/JAK complex.[123-125] It was also reported that *SH2B2* is expressed in brain, kidney, muscle, and mature B cells in spleen, and that it plays a role in signaling in B cells.[126-128]

The strongest argument in favor of a role of *SH2B2* in the pathogenesis of MPN is that its family member, *SH2B3*, was recently reported to be a tumor suppressor in MPN. *SH2B3* mutations were found in one patient with PMF and one with ET. These mutations were then transfected into Baf3 cells and were shown to exhibit JAK/STAT activation.[129] In a second study, nine novel *SH2B3* mutations were identified.[130]

It was shown in *SH2B3* knockout mice, that the lack of this gene leads to deregulation of thrombopoietin/thrombopoietin receptor signaling and similar myeloproliferative characteristics to those found in MPN patients. These mice display hypersensitivity to cytokines, increased number of in vitro multilineage (CFU-GEMM), erythroid (CFU-E), and megakaryocytic (CFU-MK) progenitor colonies, high platelet counts, splenomegaly, fibrosis, and extramedullary hematopoiesis.[131-132] *SH2B3* levels correlate with an increase in the *JAK2*-V617F mutant allele burden in MPN patients and its expression is regulated by the TPO-signaling pathway. It was also shown that there is a tighter association of *SH2B3* with the mutated *JAK2*, but it is unclear how *JAK2*-V617F can surmount *SH2B3* inhibition.[133] *SH2B3*$^{-/-}$ mice proofed that the adaptor protein is a negative regulator of cytokine signaling during hematopoiesis. It controls TPO-induced self-renewal, quiescence and proliferation of hematopoietic stem cells (HSC) and myeloid progenitors.[134-137] These animals showed disrupted B lymphopoiesis, and abnormal megakaryopoiesis and erythropoiesis.[138-140] SH2B3 binds through its SH2 domain to JAK2 and by this means negatively modulates MPL and EPOR signaling. It was also shown that it binds to and regulates *MPL*-W515L and *JAK2*-V617F.[134,141-142]

SH2B3 is located near *CUX2* on chromosome 12q, showing that *CUX1* with *SH2B2* on chromosome 7q, and *CUX2* with *SH2B3* on chromosome 12q, could have had an important role during evolution, and that is why the genes got duplicated. *CUX* (Cut homeobox) genes can be found in all metazoans.[143]

CUX2 (*CUTL2*) is a homolog of *CUX1* and a regulator of dendrite branching, spine development, and synapse formation in the cerebral cortex. *Cux2* knockout mice display reduced synaptic function and defects in working memory.[144] It plays also a role in regulating

the formation of dorsal spinal cord interneurons.[145] In one patient a homozygous deletion of *CUX2* was found recently,[146] which could mean that *CUX* family members play a role in MPN pathogenesis.[21]

1.6 TP53

A recent study showed that amplifications of chromosome 1q were significantly associated with transformation to AML. In patients with post-MPN AML 18,18% had a gain of chromosome 1q and it was shown that the minimal amplified region harbored *MDM4*.[23] This gene is an inhibitor of p53 which is often amplified in different cancer types.[147] The observation led to the analysis of the p53 pathway contribution in post-MPN AML. Somatic mutations were found in 27,3% of patients which transformed into leukemia. None of these patients had a gain of chromosome 1q which means that these events are mutual exclusive as has been also observed in some solid tumors.[23,148]

1.7 Heterogeneity in MPN pathogenesis

There are two models of MPN pathogenesis based on a single-hit or a multi-hit concept.[12-13,28,34,149] The first model suggests that the acquisition of *JAK2* mutations is the disease-initiating event that causes the onset of disease phenotype and the clonal hematopoiesis. On the contrary, the second model assumes that there are "pre-*JAK2*" mutations that cause clonal hematopoiesis before the *JAK2* mutation and the onset of the disease phenotype (figure 11).

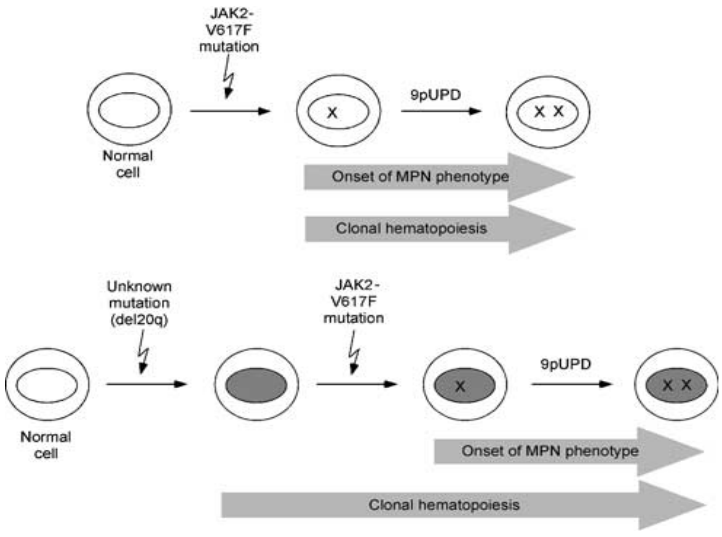


Figure 11 Two possible models of MPN pathogenesis[18]

Neither of the two models can be generally applied to all MPN patients because of the intrinsic genetic heterogeneity. This clonal diversity is due to the variability of chromosomal aberrations and somatic mutations present in MPN.[18]

1.8 Diagnosis

Diagnosis of PV, ET and PMF is based on a combined evaluation of clinical and laboratory features (figure 12).[150] One of the new diagnostic tools is the screening for the *JAK2*-V617F mutation with a sensitivity of (97%).[151-152] The likelihood of thrombotic complications in PV and ET can be also estimated by the age of the patient and his history of thrombosis.[20,153-157] Risk factors for shortened survival are for example history of thrombosis, leukocytosis, advanced age, and anemia.[20,156-158]

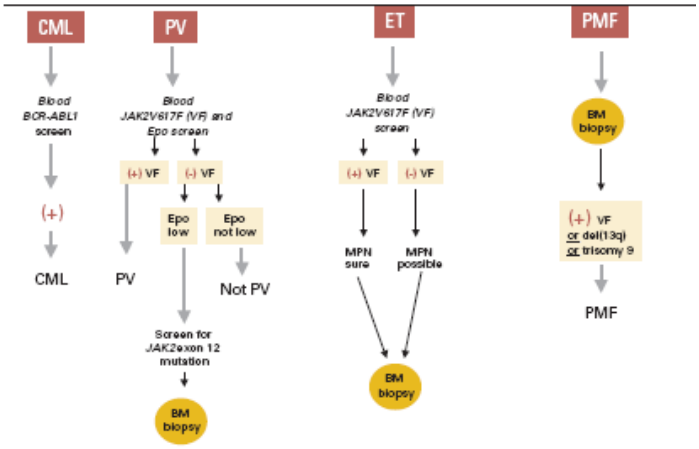


Figure 12 Diagnostic algorithm[1]

1.9 Treatment

The current therapy for PV, ET, and PMF is not curative. In PMF, and post-ET/PV MF allogeneic stem-cell transplantation (alloSCT) is potentially curative. The aim of current treatment in PV and ET is to prevent thrombohemorrhagic complications, and in PMF to reduce anemia, and splenomegaly.[1] In PV patients low-dose aspirin is used, because of its antithrombotic effect,[159] and hydroxyurea for high-risk ET.[160-161] PV and ET patients who are either intolerant or resistant to hydroxyurea are treated with IFN-α[162-163] or busulfan.[164-165] Two recent studies of pegylated INF-α reported around 80% hematologic remissions and decreases in *JAK2*-V617F allele burden.[162-163] For PMF patients, the approach is to observe low-risk patients without any therapeutic intervention,[1,154] and consider high or intermediate-2 risk patients for investigational drug therapy or alloSCT.[1] Due to the genetic heterogeneity of MPN, it can be difficult to cure patients with small molecule inhibitors that target JAK2-V617F. These drugs could cause elimination of myeloid cells positive for this mutation, but it is questionable, if they would restore polyclonal hematopoiesis in patients with high genetic and clonal diversity.[18]

2 AIM OF THE STUDY

Using 6.0 microarrays, we identified chromosomal aberrations associated with transformation to post-MPN AML and disease progression. Gains of chromosome 1q and 3q, deletions of 7q, 5q, 6p and 7p, and UPDs of 19q and 22q showed significant association with post-MPN AML compared to chronic phase. The common deleted region (CDR), which is the minimum overlap of all deletions detected for a particular chromosome in a cohort, contained only the *CUX1* gene.[21] However, the neighboring gene which is only around 1kb away from *CUX1*, is *SH2B2*, a homolog of *SH2B3* previously reported to be associated with MPN pathogenesis.[129-130] It could still be that an enhancer region for *SH2B2* is located in the CDR on 7q.

In order to identify novel mutations in the candidate genes, the granulocytic DNA of 29 patients with post-MPN AML and 3 chronic patients with del7q from three cohorts were sequenced.

For the subsequent functional analysis of *CUX1* and *SH2B2*, it was attempted to knock-down these genes in Baf3/EpoR cells. The growth properties of the cells were followed after the lentiviral delivery of shRNA pins targeting *CUX1* and *SH2B2*. If one of the genes would be important for the pathogenesis of MPN, a knock down would lead to a proliferative advantage of these cells which would have a reduced expression of *CUX1* or *SH2B2*. Other experiments were to identify how hydroxyurea treatment changes the growth dynamics of the transduced Baf3/EpoR cells expressing shRNA pins against *CUX1* and *SH2B2*, and to identify erythropoietin sensitivity changes in these cells.

Another gene that is associates with transformation to post-MPN AML is p53, which was sequenced for 24 paired samples.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Equipment

- 3130xl Genetic Analyzer (Applied Biosystems)
- Real-time PCR machine: 7900HT Fast Real-Time PCR System (Applied Biosystems)
- FACS cell analyzer: BD LSRFortessa™ (BD Biosciences)
- Thermocycler: PeqSTAR 96 Universal (peqlab)
- Centrifuges: Eppendorf Centrifuges 3810, 5418, 5424, 5424R (Eppendorf)
- Microcentrifuge: Galaxy MiniStar (VWR)
- Vacuum pump: Membrane Vacuum Pump MP86 (Biometra)
- Water bath (GFL 1002)
- Incubator: Galaxy 170R (New Brunswick)
- Vortex: Vortex-Genie 2 (Scientific Industries, Inc.)
- Thermoblock: Dry Block Heating Thermostat Bio TDB-100 (A. Hartenstein)
- Gas burner: Fuego SCS (Carl ROTH)
- Electrophoresis power supply: Owl EC-105 Compact Power Supply (Thermo Electron Corporation)
- Gel-imaging:UVsolo TS Imaging System (Biometra, An Analytik Jena Company)

3.1.2 Plastics

- Gel electrophoresis chamber: EasyPhor (Biozym Scientific GmbH)
- PCR plates: Thermo-Fast® 96 non-skirted, skirted, and detection plate (Thermo Scientific)
- Plates for PCR purification: MinElute 96 UF plate (Qiagen), Nunc 96-well conical microplate (Thermo Scientific), Millipore MultiScreen®-HV 96-well plates (Fisher Scientific)
- Real-time PCR plates: MicroAmp Fast Optical 96-well Reaction Plate with Barcode (Applied Biosystems)
- Tips: TipOne® Filter Tips 10µL, 20µL, 100µL, 200µL, 1000µL (Starlab)
- Pipettes: 2mL, 5mL, 10mL, 25mL (Greiner bio-one)

- Tubes: 15mL and 50mL conical tubes (Sarstedt), 15mL and 50mL polypropylene conical tubes (BD Falcon), 14mL polypropylene round bottom tubes (BD Falcon)
- Microcentrifuge tubes: 1.5mL, 2mL, black 1.5mL (Eppendorf)
- Culture plates: 6-well tissue culture plate, flat bottom, with low-evaporation lid (BD Falcon), Microtest™ tissue culture plate 96-well, flat bottom, with low-evaporation lid (BD Falcon)
- Culture dish: Cell Culture Dish 100x20mm (Corning)
- Petri dish: 100x15mm (BD Falcon)
- Cryogenic vials (Corning)
- Syringes: 500µL, 12.5mL (Eppendorf)

3.1.3 Materials for working with DNA/RNA

- PCR mix: AmpliTaq Gold® DNA Polymerase + buffer + $MgCl_2$ (Applied Biosystems), AmpliTaq® 360 master mix + 360 enhancer (Applied Biosystems)
- dNTP mix (Fermentas)
- Tris 5mM: diluted Tris 1M pH 7.5 (Amresco)
- Big Dye: BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems)
- Agarose: StarPure Agarose Low EEO Standard (Starlab)
- DNA ladder: DirectLoad™ Wide Range DNA Marker (Sigma-Aldrich)
- Ethidium bromide (Oncor)
- Gel extraction: peqGOLD Gel Extraction Kit (Peqlab)
- Sephadex: Sephadex™ G-50 Superfine (GE Healthcare)
- Formamide: Hi-Di™ formamide (Applied Biosystems)
- RNA preparation: TRIzol® reagent (Invitrogen)
- Chloroform (Merck Chemicals)
- DNA purification: Wizard® Genomic DNA purification kit (Promega)
- Isopropanol: 2-Propanol für die Molekularbiologie (AppliChem)
- Ethanol: Ethanol absolute for analysis Emsure® (Merck Chemicals)
- Mini prep: QIAprep Spin Miniprep Kit (Qiagen)
- Maxi prep: PureYield™ Plasmid Maxiprep System (Promega)
- Restriction enzymes: XhoI, EcoRI (New England BioLabs)
- Buffer for restriction: NEBuffer 2 (New England BioLabs)
- BSA: 10xBSA (New England BioLabs)

- Ligase: T4 DNA Ligase + 10x Reaction Buffer (New England BioLabs)
- Reverse transcriptase: MultiScribe™ Reverse Transcriptase (Applied Biosystems)
- Real-time PCR: SYBR Green PCR Master Mix (Applied Biosystems), TaqMan® Gene Expression Master Mix (Applied Biosystems)

3.1.4 Buffers and solutions

- 10xTBE pH ~8.0:
  890mM boric acid (Life Technologies)
  890mM Tris (Invitrogen)
  20mM EDTA-$Na_2 \cdot 2H_2O$ (USB)
- DNA loading dye:
  0.25% bromophenol blue (Sigma-Aldrich)
  30% glycerol in H_2O (Sigma-Aldrich)
- 2xHBS buffer pH 7.04:
  280mM NaCl (Sigma-Aldrich)
  10mM KCl (Sigma-Aldrich)
  1.5mM $Na_2HPO_4 \cdot 2H_2O$ (Merck Chemicals)
  12mM D(+)-Glucose (Merck Chemicals)
  50mM HEPES (Roche Applied Science)

3.1.5 Materials for blood preparation

- Histopaque: Histopaque®-1077 (Sigma-Aldrich)
- DPBS: Dulbecco's Phosphate-Buffered Saline (Invitrogen)

3.1.6 Media

- DMEM (Dulbecco's phosphate buffered saline, Invitrogen)
  Supplements: 10% FBS (Gibco® Fetal Bovine Serum, Invitrogen)
  1% penicillin/ streptomycin (Invitrogen)

- IMDM (Iccove's modified Dulbecco's medium, Invitrogen)
  Supplements: 10% FBS (Gibco® Fetal Bovine Serum, Invitrogen)
  1% penicillin/ streptomycin (Invitrogen)
  1U/mL recombinant erythropoietin (ERYPO, Janssen-Cilag Pharma)

- S.O.C. medium (Invitrogen)

3.1.7 Other materials

- $CaCl_2$ (Sigma-Aldrich)
- Competent cells: XL10-Gold Ultracompetent cells + β-mercaptoethanol (Agilent Technologies), DH5α competent cells
- Hygromycin (Invitrogen)
- DMSO: Dimethyl Sulfoxide (Sigma-Aldrich)
- LB Medium (MP Biomedicals)
- Bacto agar (DIFCO)

3.2 METHODS

3.2.1 Patient cohort

In the study 6 patients from Vienna (Austria), 19 from Pavia (Italy), and 7 from Florence (Italy) were included. Blood samples were collected after written informed consent of the patients.

3.2.2 Blood preparation and DNA purification

For blood preparation, two tubes with 7,5mL of patient blood each were used. At the beginning, two tubes with 300µL blood sample were prepared for whole genome DNA purification and storage. The tubes with the remaining blood were centrifuged at 99g for 10 minutes without brake. After the centrifugation, the buffy coat with leucocytes, which lies between the serum and the erythrocytes, was taken up with a pipette with circling movements and then diluted in PBS. To isolate the mononuclear cells (MNCs) and the granulocytes, 5mL of Histopaque®-1077, which allows a density gradient centrifugation, was put into new tubes and on top of it, 5mL of the buffy coat fraction was carefully placed. After centrifugation of the tubes for 30 minutes at 400g with no brake, the erythrocytes went through the gradient and accumulated at the bottom, above which a layer of granulocytes formed, then the

Histopaque®-1077 and above, the MNCs and plasma were concentrated. Afterwards, plasma and MNCs were put in one tube, the Histopaque®-1077 was discarded and granulocytes with the erythrocytes were put in another tube. The MNCs were washed twice with PBS (5-10mL) and centrifuged for 10 minutes at 250g with brake 5, and in the tube with granulocytes and whole genome DNA, the erythrocytes were lysed with cell lysis buffer (~50mL). During cell lysis the content changed after 10 minutes from red and turbid to dark red and clear. Following centrifugation for 10 minutes at 250g (1 minute at 14.000g for whole genome DNA), the supernatant was removed, and the pellet was washed with 10mL (1mL) PBS. After the second washing and centrifugation, the pellet was vortexed to loosen it from the bottom of the tube.

For DNA purification, the nuclei lysis solution from the Promega Wizard® Genomic DNA Purification Kit was added to the probes (2mL, and 300µL for whole genome DNA) and left overnight at room temperature. Then the protein precipitation solution was added (660µL; 100µL), and the samples were vortexed for 20 seconds. Afterwards, the whole genome DNA sample was centrifuged at 14.000rpm for 1 minute and the others at 2000g for 10 minutes. Subsequently, the supernatant was put into a new tube with isopropanol (2mL; 300µL), and the probes were mixed by inversion. Then they were centrifuged as before. After that, 70% ethanol (2mL; 300µL) was added to the pellet for washing, and the probes were again centrifuged. In the end, the ethanol was aspirated and the pellet air-dried. Then the DNA was rehydrated in DNA rehydration solution (300µL; 100µL). After 1h at 65°C or overnight at 4°C, the DNA concentration was measured using Nanodrop.

3.2.3 Microarray genotyping

Microarray genotyping was performed on genomic DNA from peripheral blood granulocytes, based on the fact that the phenotypic effect is mostly seen in the myeloid lineage, to which granulocytes belong to. Microarray genotyping was done using the Genome-Wide Human SNP 6.0 arrays (Affymetrix) according to the manufacturer's protocols. Evaluation of copy number and loss of heterozygosity (LOH) was perfomed by the Genotyping Console version 3.0.2 software (Affymetrix).

3.2.4 Sequencing

PCR reactions were performed with the PeqSTAR thermocycler using 10xAmpliTaq GOLD® buffer, 25mM $MgCl_2$, 2,5mM dNTPs, 10µM forward primer, 10µM reverse primer, 50% DMSO, AmpliTaq GOLD® Polymerase 5U/µL, template, and ddH_2O, or AmpliTaq® 360 master mix, 360 GC enhancer, 10µM forward primer, 10µM reverse primer, template, and ddH_2O for GC-rich DNA sequences.

PCR master mix with AmpliTaq GOLD®	**volume [µL] for 1 reaction**
10xAmpliTaq GOLD® buffer	2,0
25mM $MgCl_2$	1,2
2.5 mM dNTPs	1,6
10µM forward primer	0,7
10µM reverse primer	0,7
AmpliTaq GOLD® polymerase 5U/µL	0,1
10ng/µL DNA	2,0
ddH_2O	11,7
	20,0

PCR master mix with AmpliTaq® 360	**volume [µL] for 1 reaction**
AmpliTaq® 360 master mix	10,0
360 GC enhancer	2,0
10µM forward primer	0,8
10µM reverse primer	0,8
10ng/µL DNA	2,0
ddH_2O	4,4
	20,0

Table 1 PCR master mix

PCR touchdown program		
95°C	5'	
94°C	30''	10 cycles
67°C-57°C	30''	
72°C	30''	
94°C	30''	26 cycles
57°C	30''	
72°C	30''	
72°C	10'	
4°C	∞	

Table 2 PCR cycling conditions

Verification of PCR reactions was performed on 1,5% agarose gels which were run for 25 minutes at 120V. 1µL ethidium bromide per 100mL agarose gel was used (0,01µL/mL).

For removal of unincorporated dNTPs and primers, 50µL ddH_2O were added to the PCR products and then they were transferred carefully into a QIAGEN MinElute 96 UF Plate. The plate was placed on a vacuum manifold and the vacuum was turned on to a maximum of 800mbar until the wells were dry. The wells were washed twice by adding 60µL ddH_2O. After the third washing and drying, the plate was sealed at the bottom with aluminum sealing foil to avoid leaking. 20µL of 5mM Tris pH 7.5 were added to the wells to resuspend the PCR products, and then the plate was sealed. Incubation was made by shaking the plate on a vortex (speed 3 for 10 minutes). Afterwards the probes were transferred to a non-skirted PCR plate.

Subsequently, the BigDye reaction was prepared in order to add fluorescent dNTPs to the purified PCR products.

BigDye® master mix	volume [µL] for 1 reaction
5xBigDye® sequencing buffer	1,0
BigDye® Terminator v3.1	0,5
10µM primer	1,0
DNA	7,0
ddH_2O	0,5
	10,0

96°C	1'	
96°C	10''	25 cycles
50°C	5''	
60°C	4'	
4°C	∞	

Table 3 BigDye master mix for sequencing, and thermal cycling conditions

10µL ddH_2O were added to each BigDye sequencing product. The blue Centrifuge Alignment Frame was placed on the top of a v-bottom collection plate, and then the HV plate was placed on the assembly. 250µL of 6% (w/v) Sephadex G-50 gel were distributed with a stepper pipette per well, and the lid was closed. The plate was centrifuged at 910g for 5 minutes to pack minicolumns and then the flow-through was discarded. This step was repeated one more time. A clean v-bottom collection plate was adapted under the HV plate. 20µL of sequencing reactions were added carefully to the center of each well and centrifuged 910g for 5 minutes.

10µL of Hi-Di Formamide were added per well to a 96-well detection plate for the 3130xl sequencer. 2µL of the purified products were transferred per well. Septa were adapted on the plate and the plate was spun down. The samples were denatured for 2 minutes at 98°C, and then placed on ice for 2 minutes. After that, the plates were placed on the 3130xl plate assembly for their sequencing.

CUX1	Forward	Reverse	Product size
Exon1	CCTCCTGGCGGCTCCTGAAC	GAGGGACTCGGCCCCGACTC	294bp
Exon2	CTGGTCAAATGCAAATAGGA	CCAGCATTCTAAGATCCCTT	241bp
Exon3	AATTAGCACTGACTGCCACG	CACCATCTGGGCAGCAC	258bp
Exon4	TCTAAGAGGCTGAAGCCCAG	GGAAGTTGCCACTGATGGTG	335bp
Exon5	GAGCTGATGTCCCAGGAGC	AGCTGGTCTCAAAAGTATGGG	332bp
Exon6	AACACACCGATCCTACCAGG	TGGGCTTATTTTGCAGATCC	321bp
Exon7	ACAAAGTTGGGGTGTGGAAG	GAAGAGCAACAGGTATCCGC	366bp
Exon8	CTCCTCACAGAAATCTTTGCC	CAGCACAGAGGTCAAGAACC	267bp
Exon9	CGAGCCCTGAGCTAGGAAG	AAAATGGAAGGATAACCGGC	246bp
Exon10	TCATAATCGCTCAATTATTTCTTCC	CAATTGGTCAAAGCAAACCC	464bp
Exon11	CACCATCCACACACTGACATC	CAAGTCCAGAAAGGCAGAGG	396bp
Exon12	CGTTGACTCCATTCGCAAG	ATTTACGGGCATCTGCTGAG	315bp
Exon13	AGAAAATAATCACTCTGGTGGC	TTTTGGTAGTGCCCATCTCC	661bp
Exon14	ACAGATGGAGGGAGGCAGG	TGCATCTGTCCAGACTCACG	286bp
Exon15	CAGACCGTGGGTTGGAGAG	TCACCAGCTGCCTGATACAC	324bp
Exon16	CACTCCTTGCCACACCCAC	GGAAGGGACTACTCTTTGGGG	271bp
Exon17	CTTCTACCCCATGGCATTTG	TCTGTGCCACATCTCTCTCC	320bp
Exon18	ATCCCTGACTTCTGCCTGTG	GGCTCCACTCTGTGAGCTTC	497bp
Exon19	CCAGCCACATTCACATTGTC	TACATTGCACTGAAGCGGTG	489bp
Exon20	GAGTAGACTGTGCACCCAGG	GACCCTGTCCCAGATCACAC	366bp
Exon21	CACCCTAGGGCCCTTTCTG	AGAGATGCAGCTTGGGGTAG	288bp
Exon22-23	ACATGTCTAAGACCCACCCG	AGCGGATAAGGGCAGTTTC	631bp
Exon1a	AGCGGCGCACCCTTAGGGTC	CACCAGGCCGCCCTAGAGCA	222bp
Exon15a_1	TTTGTTTTCCCTTTTGCGG	TCCCAGCACTTTCTGACTGG	511bp
Exon15a_2	CTCCAAGGCTATGCAGGAAG	GCACAAATGTTTCATCACGC	522bp
Exon16a	TCTTTAGTGACAGGCGGCTC	GTTCCAGGCCAGAATCACAC	275bp
Exon17a	ATCTGCCTCCTTGTGTCACC	CGTGCCTAAATGCTTGAGAAC	356bp
Exon18a_1	CCCACACTTTGCAGTAGGTC	TTTCACCTGTCTCAGGACCC	466bp
Exon18a_2	ACCTCTCGCCATCTCCCTG	GTTCTGTGGTGTCTCGCTGC	451bp
Exon18a_3	AGAAGAAATGCCGCCTCCTC	CTTGGGTGAATTGAAATGGG	521bp
Exon19a	CAGAAGTCAGCCCTAGACGC	CTCCCTCTCTGCATAGCCC	369bp
Exon20a	GAAACCTTTCACCTGCTCCC	CACTGTCAGCTCGCTCTCC	439bp
Exon21a	CCGTCTGCTTCTCCTACAGAG	ACTCTGTGGTTGGCTTGGC	513bp
Exon22a	CCCTGAGCCTTTAAACTCCTG	ATGGGTCAATGTCCCTCATC	399bp
Exon23a	TGCTCTATGCAAAGTCCTGC	AAGGAACGGACCAATCACC	516bp
Exon24a_1	TGGAGAATAGGGGAGTGGTG	CTGCTGCTGCTGTTGCTG	690bp
Exon24a_2	GAGGACGCCGCTACCTCAGC	GACCCCGTCCAGGCCCTTGC	324bp

SH2B2	Forward	Reverse	Product size
Exon1	CCACAGCCACTTCCACATC	CCCGAGAGTGGGAGAAAGG	283bp
Exon2_1	CACACAGCCCCAGAGAGTC	CACGCACAGGCTCATGTTG	560bp
Exon2_2	CCAACTTCCTGGACGTCTTC	TGGCACCAAATACTTCACCC	663bp
Exon3	GAGGGCCACTCTAACACCTG	GTCCTGCCTTAGGCTCCTTC	301bp
Exon4	ATCAGCCATTTGAGCCACTC	ATTTCTCAAGCTGAGCTCCC	291bp
Exon5	GAGGTTACAGTCAGCCACCG	GTGATGTGTAGGAGGGACGC	630bp
Exon6	GAGCCTTGGTCCTTCCATC	CTGAGGCTATGGGACAGGAG	376bp
Exon7	TGCAACTCGGAAACCTGAG	GTGGGAGAAAGGACGACAG	553bp

SH2B3	Forward	Reverse	Product size
Exon2_2	GCTCCTTCCAGCACTTTCG	CTGGAAAGCCATCACACCTC	431bp

TP53	Forward	Reverse	Product size
Exon1-2	TCTCAGACACTGGCATGGTG	TGGGTGAAAAGAGCAGTCAG	449bp
Exon3	CGTTCTGGTAAGGACAAGGG	GAAGAGGAATCCCAAAGTTCC	489bp
Exon4-5	GCATGTTTGTTTCTTTGCTGC	CATGGGGTTATAGGGAGGTC	588bp
Exon6	GTGCTGGGCACCTGTAGTC	AGCAGTAAGGAGATTCCCCG	482bp
Exon7-8	TGGTTGGGAGTAGATGGAGC	GCCCCAATTGCAGGTAAAAC	492bp
Exon9	TGCCGTTTTCTTCTTGACTGT	GCAGGCTAGGCTAAGCTATGA	334bp
Exon10	TGCATGTTGCTTTTGTACCG	AGCTGCCTTTGACCATGAAG	316bp
Exon11	ATTTGAATTCCCGTTGTCCC	GCAAGCAAGGGTTCAAAGAC	292bp

Table 4 Primer sequences for *CUX1*, *SH2B2*, *SH2B3*, and *TP53*, used for exon sequencing

3.2.5 Cloning of shRNA pins

Six shRNA constructs targeting *CUX1*, five shRNAs against *SH2B2*, and a scrambled oligonucleotide for each gene as a control were designed for ligation into the SFLV vector (kindly provided by Lenhard Rudolph from the Ulm University). The vector carries resistance genes against ampicillin and hygromycin, and GFP, BFP, or RFP as fluorescent markers.

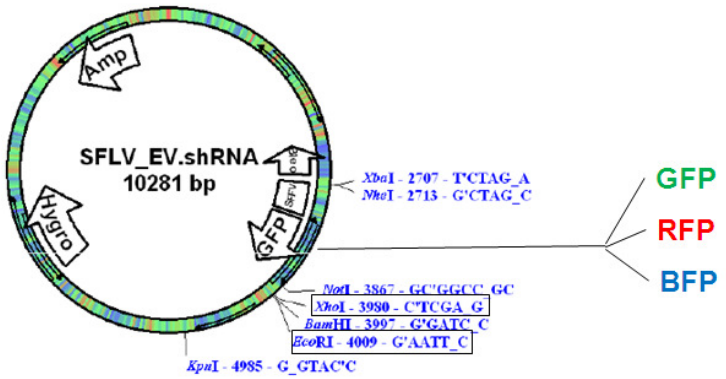


Figure 13 SFLV vector with BFP or RFP marker and XhoI and EcoRI restriction sites

			Sequence 5' to 3'	Overhang	Code
CUX1	shRNA_1	forward	TCGAACCAGCGCATCTTCGGACATTATAGTGAAGCCACAGATGTATAATGTCCGAAGATGCGCTGGC	XhoI	
		reverse	AATTGCCAGCGCATCTTCGGACATTATACATCTGTGGCTTCACTATAATGTCCGAAGATGCGCTGGT	EcoRI	
	shRNA_2	forward	TCGACGCCAAGAATAGCACACTCAAATAGTGAAGCCACAGATGTATTTGAGTGTGCTATTCTTGGCA	XhoI	
		reverse	AATTTGCCAAGAATAGCACACTCAAATACATCTGTGGCTTCACTATTTGAGTGTGCTATTCTTGGCG	EcoRI	
	shRNA_3	forward	TCGAGCCAAGAATAGCACACTCAAACTCGAGTTTGAGTGTGCTATTCTTGGCTTTTTG	XhoI	TRCN0000070558
		reverse	AATTCAAAAAGCCAAGAATAGCACACTCAAACTCGAGTTTGAGTGTGCTATTCTTGGC	EcoRI	
	shRNA_4	forward	TCGAGCAGCTCATCAAGCACAACATCTCGAGATGTTGTGCTTGATGAGCTGCTTTTTG	XhoI	TRCN0000070559
		reverse	AATTCAAAAAGCAGCTCATCAAGCACAACATCTCGAGATGTTGTGCTTGATGAGCTGC	EcoRI	
	shRNA_5	forward	TCGACCACTGCTAAAGAGCTTCCAACTCGAGTTGGAAGCTCTTTAGCAGTGGTTTTTG	XhoI	TRCN0000070560
		reverse	AATTCAAAAACCACTGCTAAAGAGCTTCCAACTCGAGTTGGAAGCTCTTTAGCAGTGG	EcoRI	
	shRNA_6	forward	TCGAGCCCTCAGCATCCAAGAATTACTCGAGTAATTCTTGGATGCTGAGGGCTTTTTG	XhoI	TRCN0000070561
		reverse	AATTCAAAAAGCCCTCAGCATCCAAGAATTACTCGAGTAATTCTTGGATGCTGAGGGC	EcoRI	
	Scramble	forward	TCGAGACTCCACGAATCATATTAGACTCGAGTCTAATATGATTCGTGGAGTCTTTTTTG	XhoI	
		reverse	AATTCAAAAAAGACTCCACGAATCATATTAGACTCGAGTCTAATATGATTCGTGGAGTC	EcoRI	
SH2B2	shRNA_1	forward	TCGACCACTCCCATTAGCAGCTATTCTCGAGAATAGCTGCTAATGGGAGTGGTTTTTG	XhoI	TRCN0000100115
		reverse	AATTCAAAAACCACTCCCATTAGCAGCTATTCTCGAGAATAGCTGCTAATGGGAGTGG	EcoRI	
	shRNA_2	forward	TCGACGGCTGATATCACCCTAAGAACTCGAGTTCTTAGGGTGATATCAGCCGTTTTTG	XhoI	TRCN0000100116
		reverse	AATTCAAAAACGGCTGATATCACCCTAAGAACTCGAGTTCTTAGGGTGATATCAGCCG	EcoRI	
	shRNA_3	forward	TCGAGAGCAGAATACATCCTGGAAACTCGAGTTTCCAGGATGTATTCTGCTCTTTTTG	XhoI	TRCN0000100117
		reverse	AATTCAAAAAGAGCAGAATACATCCTGGAAACTCGAGTTTCCAGGATGTATTCTGCTC	EcoRI	
	shRNA_4	forward	TCGAGATCGGCTGATATCACCCTAACTCGAGTTAGGGTGATATCAGCCGATCTTTTTG	XhoI	TRCN0000100118
		reverse	AATTCAAAAAGATCGGCTGATATCACCCTAACTCGAGTTAGGGTGATATCAGCCGATC	EcoRI	
	shRNA_5	forward	TCGAGTGGAGAATCAGTACTCCTTTCTCGAGAAAGGAGTACTGATTCTCCACTTTTTG	XhoI	TRCN0000100119
		reverse	AATTCAAAAAGTGGAGAATCAGTACTCCTTTCTCGAGAAAGGAGTACTGATTCTCCAC	EcoRI	
	Scramble	forward	TCGAGTCCTATCAATCCCCCTACATCTCGAGATGTAGGGGGATTGATAGGACTTTTTTG	XhoI	
		reverse	AATTCAAAAAAGTCCTATCAATCCCCCTACATCTCGAGATGTAGGGGGATTGATAGGAC	EcoRI	

Table 5 shRNAs used for *CUX1* and *SH2B2* knock down

At the beginning, maxipreps were performed with the PureYield Plasmid Maxiprep System for obtaining SFLV-BFP and SFLV-RFP vectors. For this, a colony was inoculated in 2mL of LB+Amp medium and left overnight at 37°C for shaking at 220rpm. After 8 hours, 500µL of the pre-inoculation was put into 180mL of LB+Amp medium and left again overnight at 37°C at 220rpm. Afterwards, the tubes were centrifuged for 10 minutes at 6.000g, and the pellet was resuspended in 12mL of the resuspension solution. The obtained solution was put into a 50mL Falcon tube and 12mL cell lysis solution was added. The tubes were inverted gently and then, 12mL of the neutralization solution was added. The tubes were centrifuged at 14.000g for 20 minutes, and the DNA was purified. For this, the solution was poured into a column and placed on a vacuum. The vacuum was opened, and after drying, 5mL of the endotoxin removal wash was added. Then, the columns were placed into 50mL Falcon tubes, 1,5mL ddH_2O were added to each, and they were spun down at 2.000g for 5 minutes for DNA elution. After that, the concentration of the vector DNA was measured using Nanodrop.

Buffer 2	2,0
XhoI	0,5
EcoRI	0,5
10xBSA	2,0
8µg DNA	1,0
ddH_2O	14,0
	20,0

Table 6 Master mix for vector restriction (for 1 reaction)

The vectors were digested using XhoI and EcoRI restriction enzymes. The digestion mix was left at 37°C for 2-3h. Afterwards, an 0,8% agarose gel was prepared with 1µL ethidium bromide per 100mL agarose and left running for around 1h at 80-90V. The undigested vectors were used as control. Later, the bands with the digested vectors were cut out under UV light and gel extraction with the peqGOLD Gel Extraction Kit was performed. For this, equal volume of binding buffer was added to each gel slice and incubated for 7 minutes at 55°C-65°C. The mixture was vortexed every 2-3 minutes until the agarose was completely dissolved. The samples

were loaded into a column and centrifuged for 1 minute at 10.000g. After that, they were washed once with 300µL binding buffer and twice with 600µL CG wash buffer, incubated 2-3 minutes and centrifuged each time as before. As the next step, a centrifugation for 1 minute at 10.000g was performed for drying the column. Elution was done by addition of 50µL elution buffer and centrifugation for 1 minute at 5.000g.

Subsequently, in order to anneal the forward and reverse shRNA sequence, 1µL of each 10µM oligonucleotide was mixed with 5µL of NEB2 buffer and 43µL ddH_2O (end concentration was 200nM). Then the mixture was cooled down 1°C every 4 minutes from 96°C to 4°C.

The ligation of the annealed shRNAs with the appropriate vector was done at room temperature for 2-3h using the NEB ligase. Different dilutions of the inserts were used.

10xbuffer	1,0
100ng vector	1,1
insert	1,0
NEB ligase	0,5
ddH_2O	6,4
	10,0

Table 7 Master mix for ligation (for 1 reaction)

For transformation, XL10-Gold Ultracompetent Cells were used. Two 14-mL round-bottom tubes were pre-chilled on ice, and the S.O.C. medium was pre-heated to 42°C. The cells were thawed on ice, gently mixed and aliquots of 100µL were made in the pre-chilled tubes. 4µL of a β-mercaptoethanol mix were added to each tube. The tubes were swirled gently and the cells were incubated on ice for 10 minutes, swirling every 2 minutes. Afterwards, 2µL of the ligation mixture were added to the tubes and then incubated on ice for 30 minutes. The tubes were heat-pulsed in a 42°C water bath for 30 seconds and then incubated on ice for 2 minutes. 900µL of the pre-heated S.O.C. medium was added to the tubes, which were then incubated at 37°C for 1 hour with shaking at 200rpm. After that, the cells were plated on LB+Amp plates, and incubated at 37°C overnight.

Afterwards, one colony was inoculated in 2mL of LB+Amp medium each and left at 37°C overnight shaking. Then, miniprep was performed with the QIAprep Spin Miniprep Kit to obtain the vectors. For this, the overnight culture was centrifuged at 6.800g for 3 minutes, and then the pellet was resuspended in 250µL buffer P1, 250µL of buffer P2 was added and mixed by inverting the tube. After that, 350µL of buffer N3 was added and mixed by invertion, and then the tubes were centrifuged for 10 minutes at 17.900g. Subsequently, the supernatant was transferred to a column which was centrifuged for 30 seconds. 500µL of buffer PB was added and again the column was centrifuged. The second washing was done by addition of 750µL buffer PE. Another centrifugation was made afterwards for removal of the residual wash

buffer. Afterwards, the columns were placed in Eppendorf tubes, and 50µL of buffer EB was added for elution of DNA. The concentration was measured by Nanodrop.

Sequence verification was made by performing BigDye reaction with 150ng of vector DNA and two different primers (forward TGTTTGAATGAGCTTCAGTACTTTACAG, reverse AGTGATTTAATTTATACCATTTTAATTCAGCTTTG).

BigDye® master mix	volume [µL] for 1 reaction
5xBigDye® sequencing buffer	1,0
BigDye® Terminator v3.1	1,0
10µM primer	1,0
DNA	150ng
ddH_2O	7-150ng of DNA
	10,0

96°C	1'	
96°C	10''	25 cycles
50°C	5''	
60°C	4'	
4°C	∞	

Table 8 BigDye master mix for shRNA verification, and thermal cycling conditions

For obtaining the required amount of DNA needed for transfection, transformation of DH5α competent cells (made competent using $MgCl_2$ and $CaCl_2$) was made with the final constructs. 3-4 minipreps were performed per pin to obtain enough DNA for the subsequent transfection of 293T cells.

3.2.6 Transfection of 293T cells for virus production

293T cells were splitted a day before transfection, and 1-3h before transfection, the medium (DMEM+10%FBS+1%P/S) was changed. 2xHBS buffer with pH 7.04, filter sterilized before use, was used for transfection. First, a 15mL Falcon tube was prepared with 500µL HBS and an Eppendorf tube with the vector mix, which consisted of 15µg vector DNA, 10µg DR8.91, 3µg VSV.9, 60µL 2M $CaCl_2$, and filtered H_2O up to 500µL. The HBS was bubbled and the vector mix was added drop-wise to the buffer. After 20 minutes at room temperature, the mix was dropped to the cells. After 12h the medium was changed to 3mL and after 12h more, the supernatant with the virus was taken up with a syringe and was replaced with fresh medium. After another 24h, the supernatant was collected again and used for infections. Virus was stored at -80°C.

3.2.7 Viral transduction of Baf3/EpoR cell line

Baf3/EpoR cells, which are an erythropoietin dependent, immortalized murine bone marrow-derived pro-B cell line, were transduced in 24-well, or 96-well plates, using two different schemes. One was a 12h incubation, and the other spin infection consisting of a centrifugation for 2h at 1000g. Two different dilutions of the viral supernatant were tested, 1:4, and 1:2. The medium (IMDM+10%FBS+1%P/S+1:10.000Epo) was changed after 12h.

3.2.8 FACS

In order to check the transfection efficiency, FACS measurement was performed using the LSR Fortessa FACS machine, and the data was analyzed using the FACS Diva software.

3.2.9 Knock-down efficiency measurement by qPCR

The efficiency of knock-down was controlled by comparing the target mRNA level of knocked-down cells to the one of the control cells by real time PCR performed with a 7900HT Real Time PCR System. Each mRNA level was normalized to the mRNA level of β-actin (mouse).

For RNA extraction, 500µL of cells were put in an Eppendorf tube and centrifuged at 675g for 5 minutes. Afterwards, the cell pellet was resuspended in 250µL Trizol and incubated 5 minutes at room temperature. Then, 50µL chloroform were added to each tube, which were vortexed for 15 seconds, and centrifuged at 12.000g for 15 minutes at 4°C. Afterwards, the upper layer with the RNA was separated and transferred to a new tube with 125µL of isopropanol. After that, the samples were incubated for 10 minutes at room temperature and centrifuged again as before. Subsequently, the supernatant was removed, the pellet washed with 1mL 75% ethanol, and then centrifuged at 7.500g for 5 minutes at 4°C. Later, traces of ethanol were removed, the RNA pellet was left for 5 minutes to dry, and then it was resuspended in 30µL ddH_2O (10 minutes at 60°C). RNA was quantified using Nanodrop.

For cDNA preparation, the master mix, consisting of a reverse transcription buffer, dNTP mix, random primers, ddH_2O, and a reverse transcriptase was prepared on ice, and then 10µL of it were added to each well with 10µL of RNA (500ng). After centrifugation, the plate was put into the thermal cycler.

	volume [µL] for 1 reaction
10xRT buffer	2,0
25xdNTP mix (100mM)	0,8
10xRT random primers	2,0
MultiScribe™ reverse transcriptase	1,0
ddH_2O	4,2
500ng RNA	10,0
	20,0

25°C	10'
37°C	120'
85°C	5'
4°C	∞

Table 9 Master mix for reverse transcription, and thermal cycling conditions

For real time PCR, either the SYBR green buffer, primers, cDNA and ddH_2O were mixed for quantification of *CUX1* knock down, or the TaqMan master mix for mouse actin, TaqMan buffer, the cDNA, and ddH_2O for actin expression measurement.

m*Cux1*	volume [µL]
2xSYBR® Green buffer	5,0
10µM forward primer	0,4
10µM reverse primer	0,4
cDNA	1,0
ddH_2O	3,1
	10,0

mActin	volume [µL]
TaqMan buffer	5,0
mActin TaqMan master mix	0,4
cDNA	1,0
ddH_2O	3,6
	10,0

Table 10 Real time PCR master mix for *Cux1* and actin

Cux1	5' to 3' sequence	product size
Forward	CAAGGGGAGATTGATGCACT	149bp
Reverse	TCGTGTAGACGCTGCACTTT	

Table 11 Primer sequences for *Cux1* for real time PCR

In order to obtain cells with about 100% transduction efficiency, they were grown for about 2 weeks in medium with addition of hygromycin, which should lead to the enrichment of cells having the SFLV vector integration.

4 RESULTS

4.1 Sequence analysis

CUX1 and *SH2B2* deletions were found in MPN patients using Genome-wide Human SNP 6.0 arrays (Affymetrix) and gene copy number analysis. UPDs, resulting from mitotic recombination, were also found with this method, using SNP analysis. Regions, in which only one version of a SNP was found, indicated an UPD. Resulting from an analysis of about 444 patients' DNA,[21] 30 cytogenetic aberrations were found, containing either the *CUX1* and *SH2B2* gene, or, as was the case for one patient which carried the smallest deletion, only *CUX1* (figure 14-15). From the cohort of patients which developed post-MPN AML, 11 deletions of chromosome 7q were identified, and in this case, the CDR comprised both genes, *CUX1*, and *SH2B2* (figure 16-17).

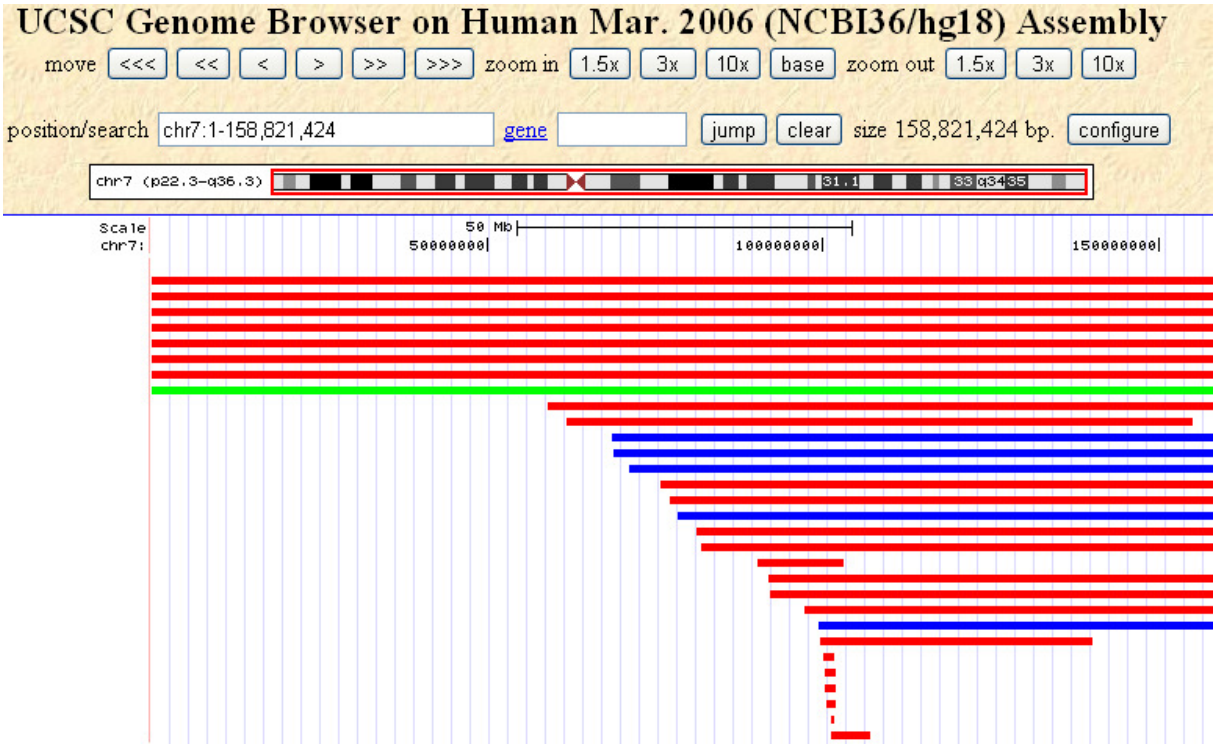


Figure 14 Deletions (red), gains (green), and UPDs (blue), found in all patients using 6.0 arrays

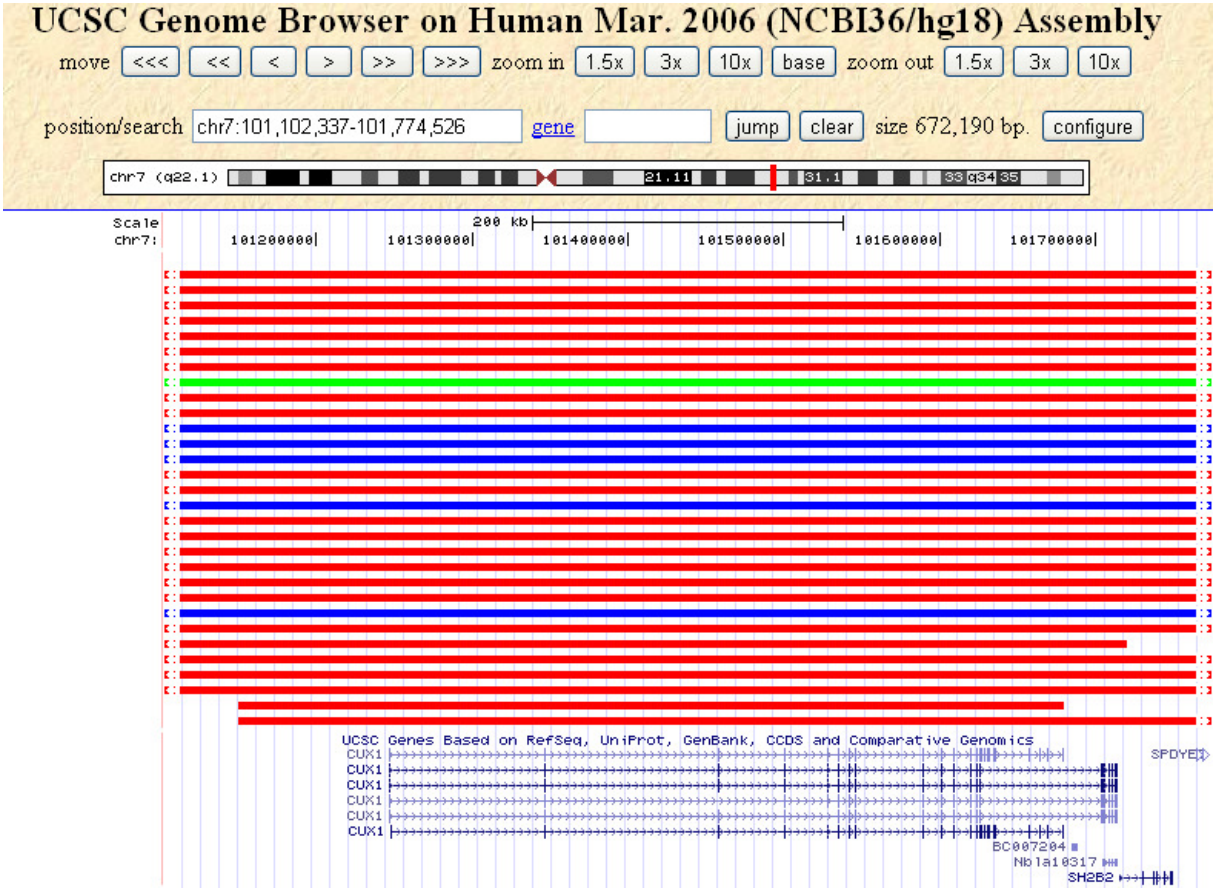


Figure 15 Enlargement of the CDR for depiction of genes located in this region

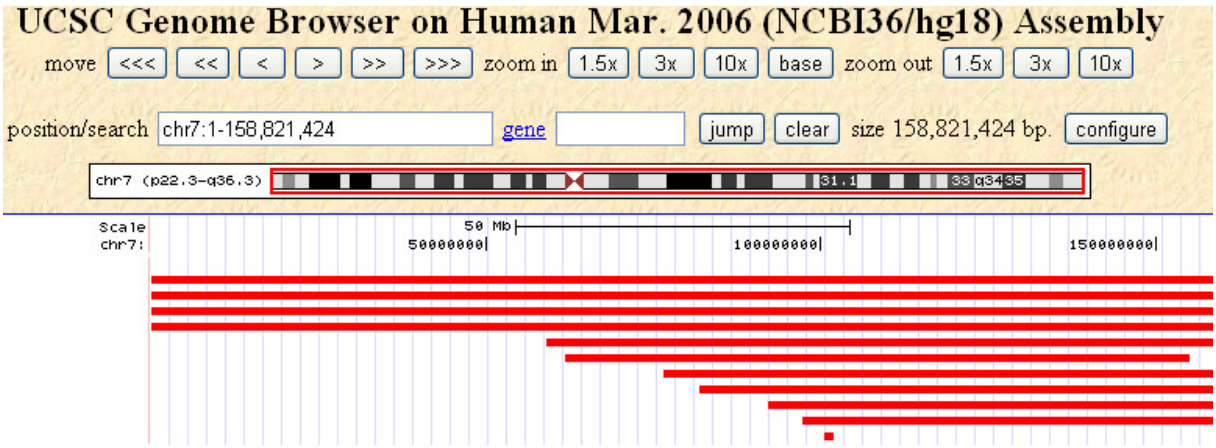


Figure 16 Deletions (red), gains (green), and UPDs (blue), found in patients with post-MPN AML using 6.0 arrays

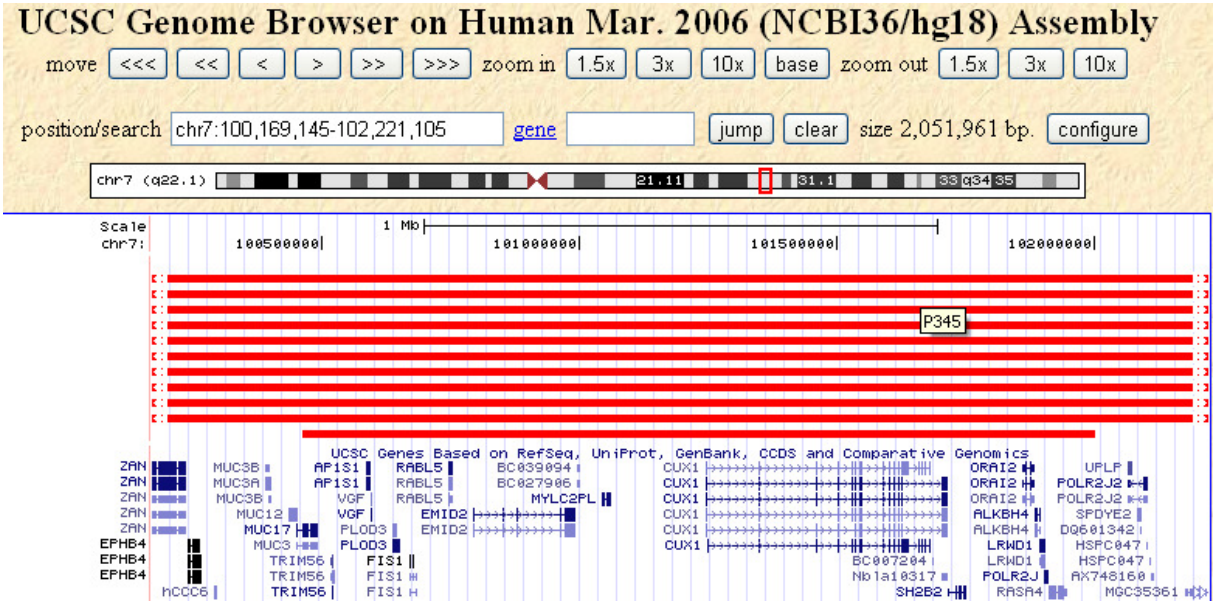


Figure 17 Enlargement of the CDR for depiction of genes located in this region

Based on the finding from the analysis of the samples with 6.0 arrays, deletions of chromosome 7q are associated with transformation to post-MPN AML (figure 4).[21] Granulocytic DNA (GD) from 32 transformed patients, of which 5 carried a deletion on 7q, was sequenced. For validation of mutations, sequences of granulocytic DNA were compared to sequences of buccal DNA (BU), which is the best suited control DNA, or with DNA from T cells (TD) or monocytic cells (MD). This comparison was performed after verification of the mutation in SNP databases (genome.ucsc.edu, www.ensembl.org) to have confirmation that it is not a known polymorphism. For *CUX1*, 23 exons of one splicing variant, and 11 more of another variant, were sequenced. Exon 14 could not be sequenced, also after using the 360 GC enhancer for sequencing of GC-rich sequences, but sequencing of the other exons in all mentioned above patients didn't reveal any somatic mutation. Only two germline mutations (figure 18-19) were found, which however, in accordance to the hypothesis of cancer development, don't play a role in the pathogenesis of MPN.[166] Due to the fact that *SH2B2* is located only ~1kb away from *CUX1*, and belongs to one gene family with *SH2B3*, the 7 exons of *SH2B2* were also sequenced. One somatic mutation in exon 2 of the *SH2B2*

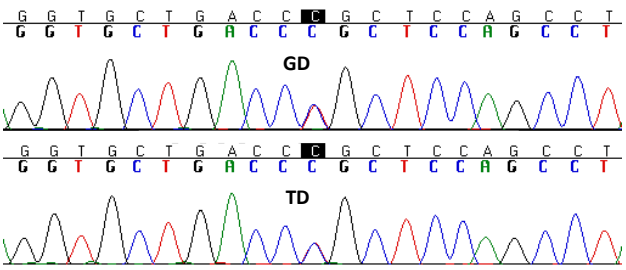


Figure 19 Germline mutation of *CUX1*, found in a patient with post-MPN AML

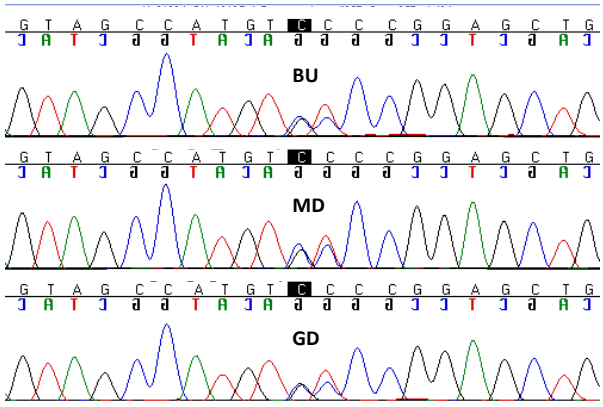


Figure 18 Germline mutation of *CUX1*, found in a patient with a deletion of chromosome 7q

gene was validated by sequencing T-cell DNA (figure 20). The mutation is a cytosine to adenosine substitution which leads to an amino acid change of glycine to cysteine at position 113. The Polyphen prediction is possibly damaging with a PSIC score of 1,944. Apart from sequencing these two genes, located in the CDR of MPN patients analyzed by 6.0 arrays, exon 2 of *SH2B3*, reported to be mutated in MPN patients, was also sequenced.[129-130] In our cohort no mutation in *SH2B3* was found.

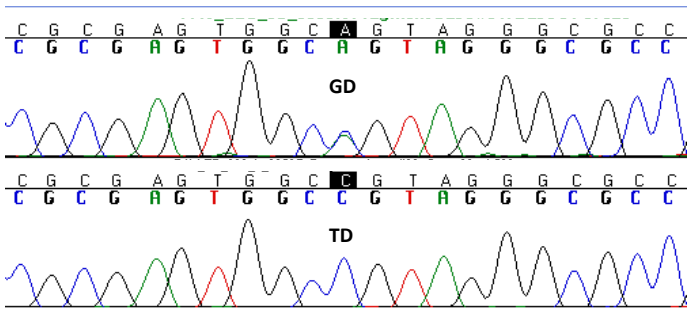


Figure 20 Somatic mutation in *SH2B2* found in a patient with post-MPN AML

4.2 Functional analysis

Based on the sequencing data of *CUX1* and *SH2B2*, an experiment was designed to test the functional impact of these two genes on the pathogenesis of MPN. Because of the somatic mutation in *SH2B2*, which was found in one patient, and no mutation found in *CUX1*, but occurrence of only this gene in the CDR of chromosome 7q, shRNAs were designed against both genes. shRNAs were cloned into a lentiviral vector for their delivery to Baf3/EpoR cells. To test the vectors, 293T cells were transfected only with the SFLV vectors with different fluorescent markers, but without any shRNA and without producing virus. We used different combinations of the markers to see if double and triple transfected cells, can be individually detected using the FACS machine, and if cells containing one, two, or three different markers, can be distinguished from another. On the figures one can see that double and triple transfected cells can be detected, as well as cells transfected with only one or another marker (figure 21). On one picture one can also distinguish double transfected cells from single transfected ones and compare the fluorescence of each, which would have the purpose of comparing the proliferation of cells which have only one or another gene knocked down, or have a knock down of both genes.

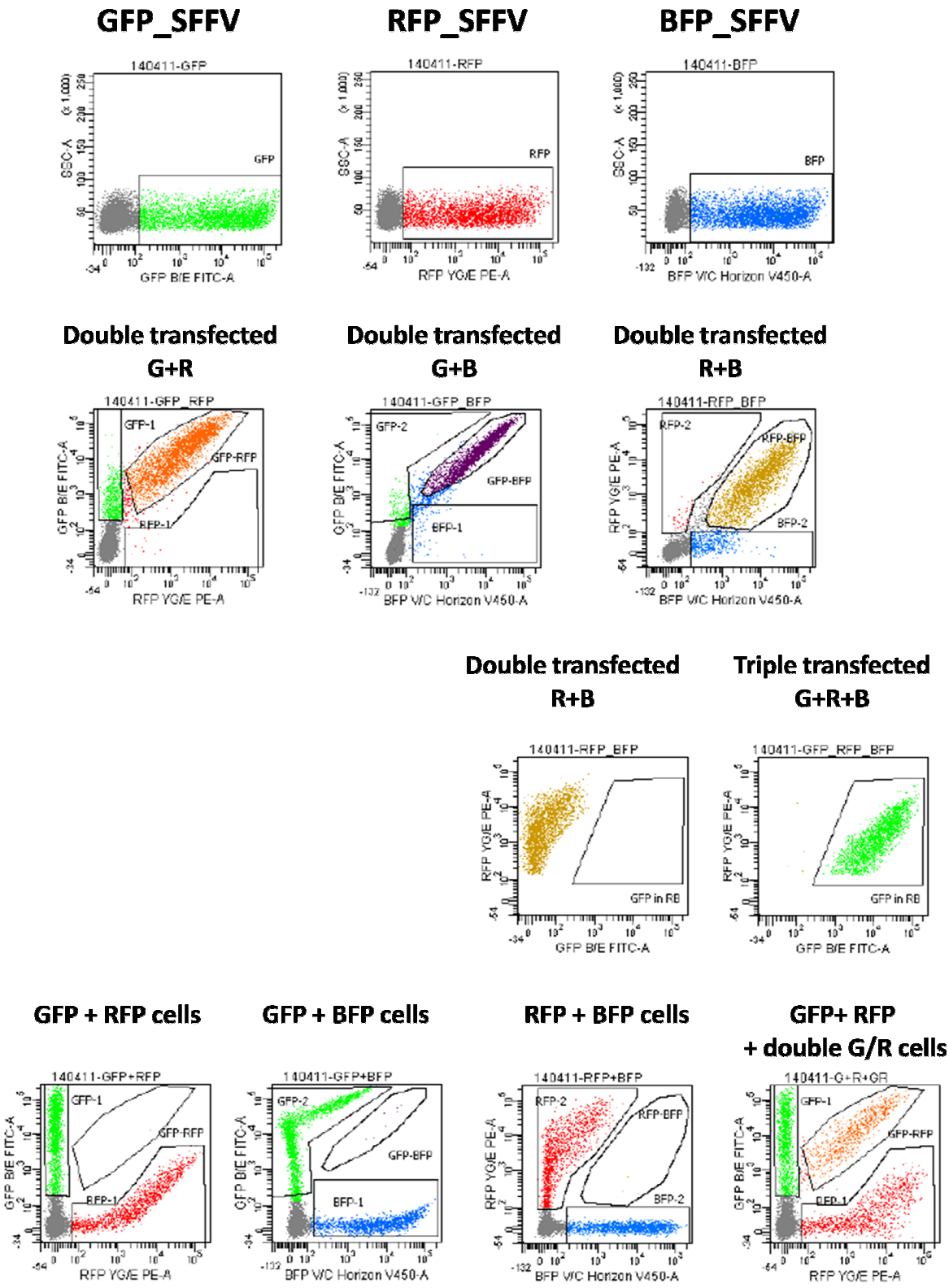


Figure 21 Transfection of 293T cells with different combinations of SFLV-GFP, SFLV-RFP, and SFLV-BFP vectors

The 6 shRNAs against *CUX1*, and 5 shRNAs against *SH2B2* were retrieved from the Sigma-Aldrich library (www.sigmaaldrich.com) and Open Biosystems library (www.openbiosystems.com). Furthermore one scrambled sequence for each gene was cloned in the same vector as the appropriate gene. *CUX1* shRNA pins were cloned into SFLV-BFP, and *SH2B2* into the SFLV-RFP vector. The shRNAs were cloned into the vectors and after entering the cell, they were expressed through the SFFV promoter. According to the hypothesis that the cells with reduced expression of a target gene, mimicking haploinsufficiency, would have proliferative advantage, they would therefore outgrowth the cells that don't have the knock down. The quantity of the cells expressing the pins was measured by the FACS machine. The fluorescence is measured over a period of time to assess if the knock down of one of the genes gives proliferative advantage to the cells. Proliferative advantages or disadvantages can be measured by comparing the initial and final fluorescence of the cells over a period of time.

Over 6 weeks no significant differences were observed in growth dynamics of cells expressing pins targeting *CUX1*, neither in the 24-well plate (figure 22a), nor in the 96-well plate after spin infection (figure 22b). The visible small changes were comparable to those of the cells which either had only the empty vector, or the vector with a scrambled sequence (figure 22). In the figures showing the fluorescence of cells having a vector with a pin against *SH2B2*, an interesting pattern can be seen in the figure showing proliferation of cells in the 96-well plate (figure 23b). In the 24-well plate no significant changes, apart from the drop of fluorescence after 33 days that rose again after 7 more days, were observed (figure 23a). The cells transduced in a 96-well format, however, had very instable percentages of fluorescence. In a period of about 5 to 22 days, the fluorescence of cells with all shRNAs, as well as the empty vector, and the control, raised and fell significantly (figure 23b). However, there was no remarkable change of the fluorescence intensity of any shRNA.

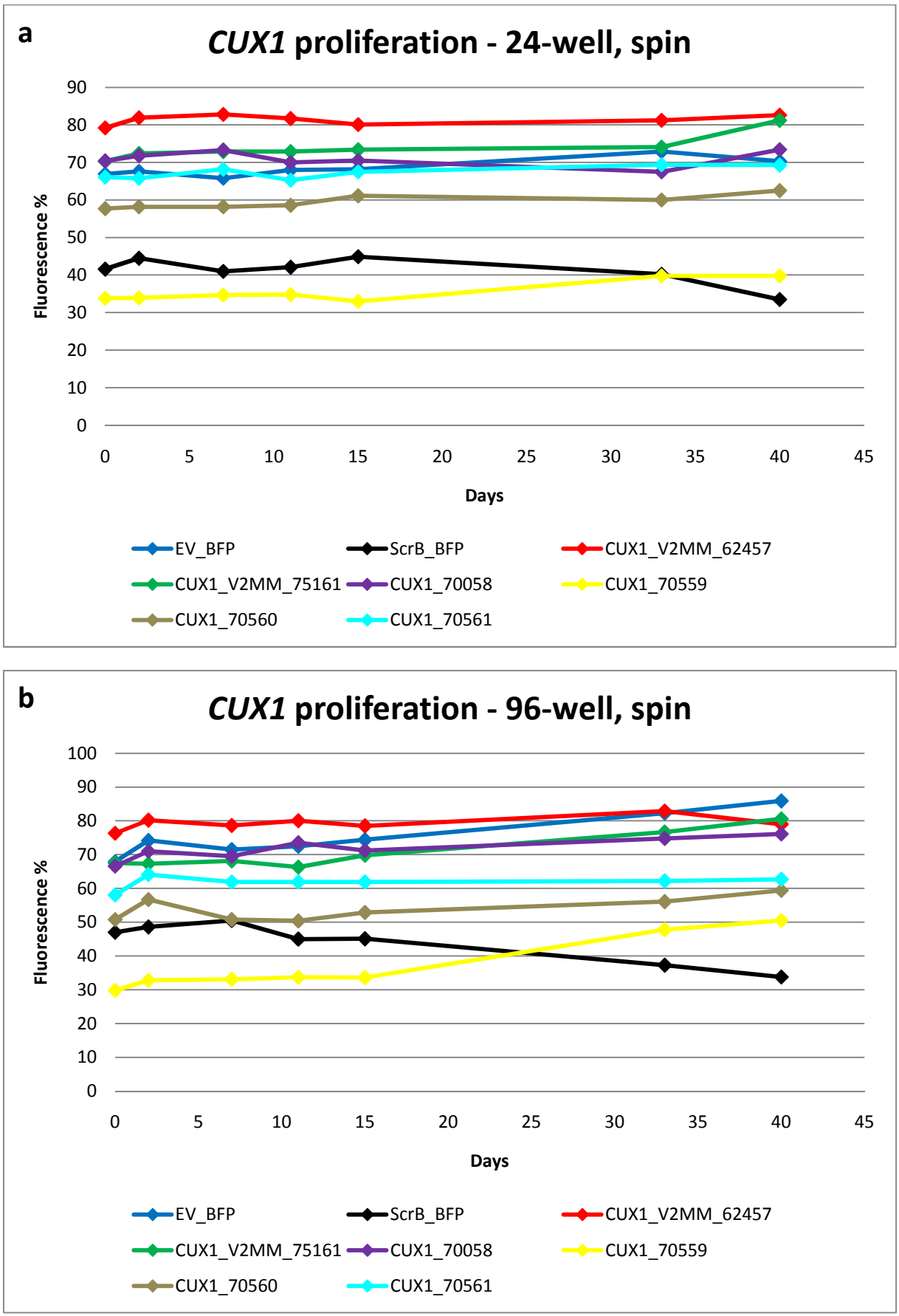


Figure 22 Proliferation of Baf3/EpoR cells transfected with shRNAs against *CUX1*

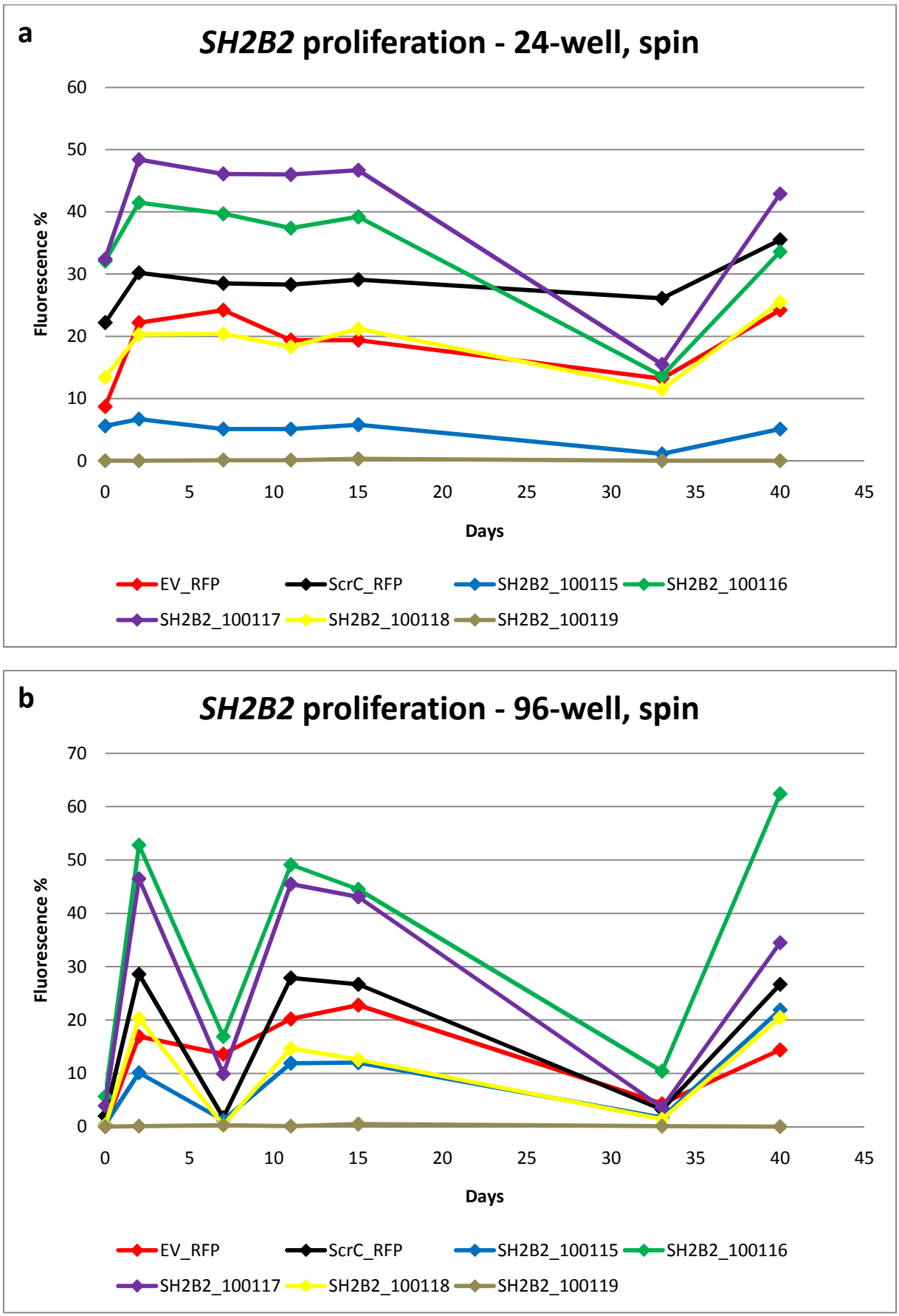


Figure 23 Proliferation of Baf3/EpoR cells transfected with shRNAs against *SH2B2*

Another possibility to screen the different shRNAs and test if one leads to a proliferative advantage, was to stress the transduced cells in a variety of ways. One option was to add 100µM hydroxyurea (HU), which is used for treatment of MPN patients, to the medium, and after 6 days, when the viability of cells strongly decreases, change the medium again to one without HU, and look for proliferative advantage. All cells were killed by HU, but afterwards, cells which have a shRNA which gives an advantage, would outgrowth the others. However, in figures 24 and 25, one can see that none of the pins conferred proliferative advantage to the cells that survived the treatment and reached the initial viability. A second method of stressing cells was to let them grow in medium with reduced concentration of Epo. Instead using a concentration of 1U/mL, 0,01U/mL was used, but again in this experiment, no shRNA conferred proliferative advantage of the cells (figure 26-27).

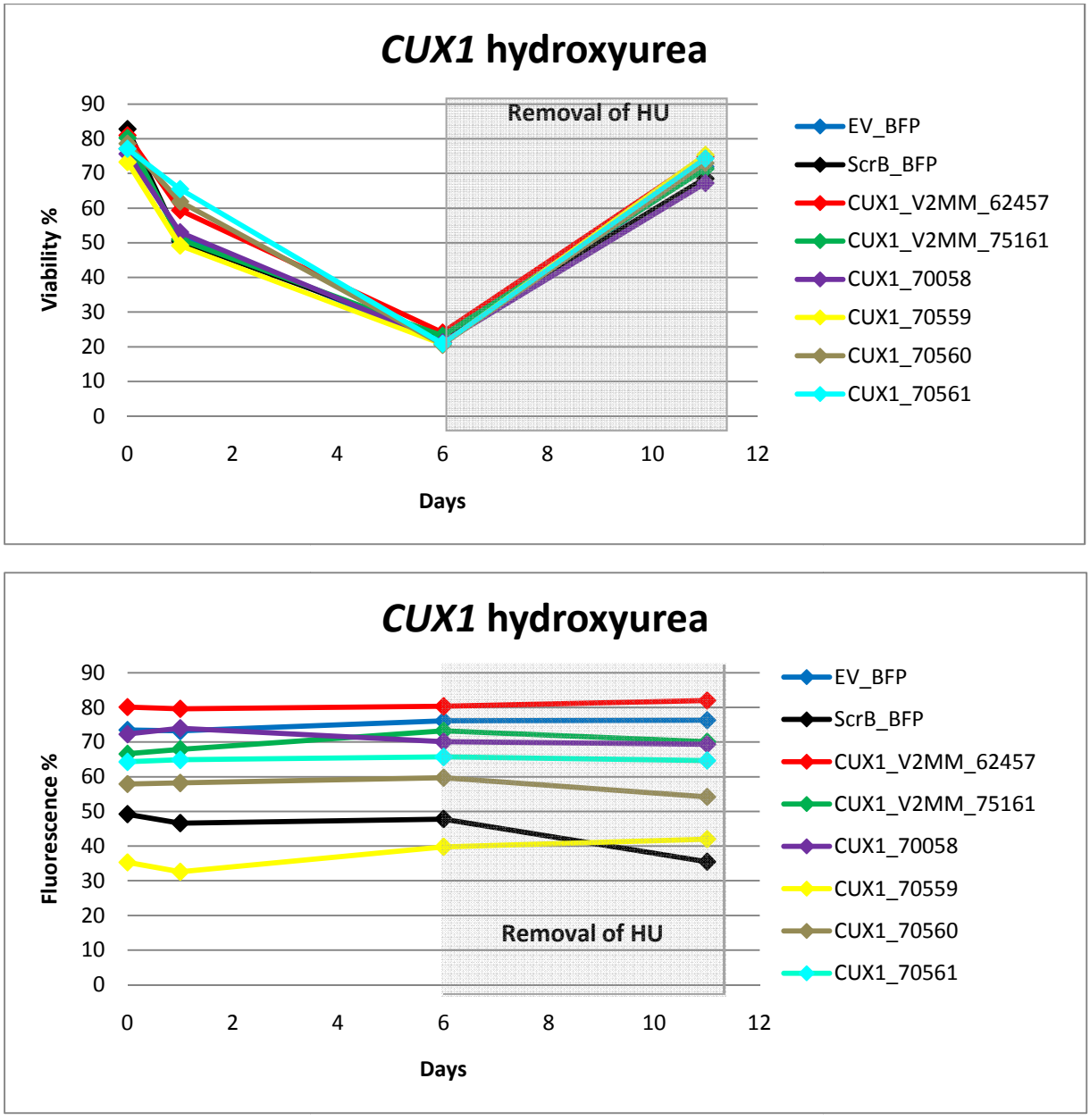


Figure 24 Treatment of Baf3/EpoR cells transfected with shRNAs against *CUX1* with hydroxyurea

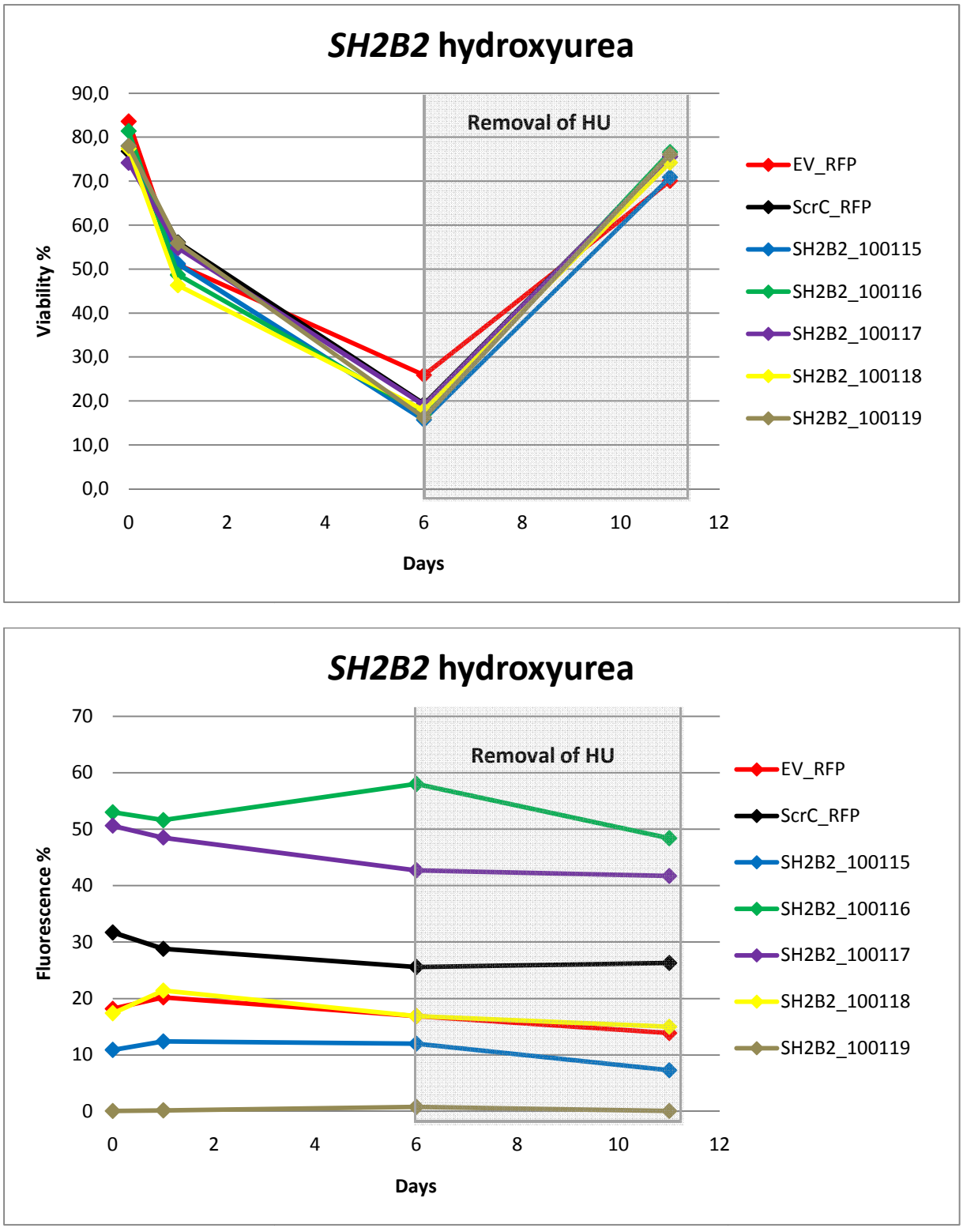
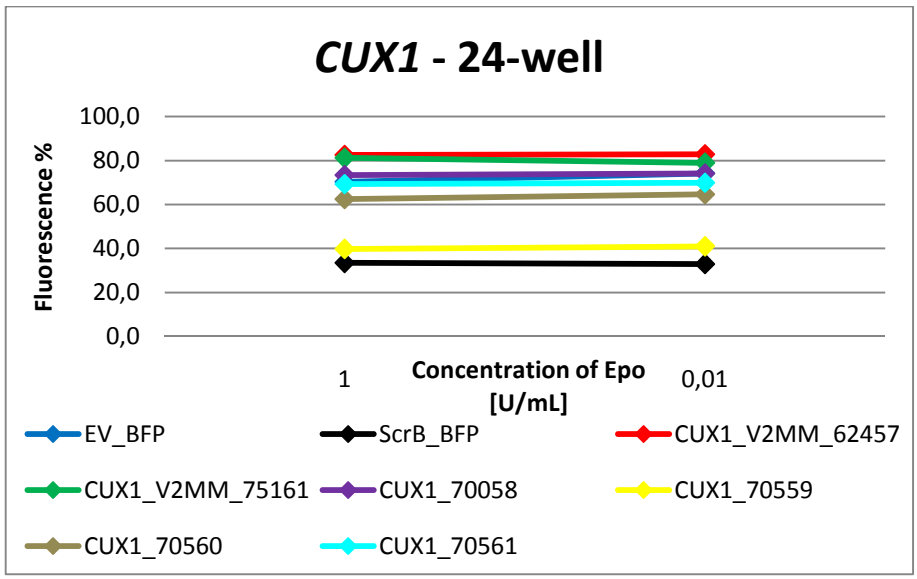


Figure 25 Treatment of Baf3/EpoR cells transfected with shRNAs against *SH2B2* with hydroxyurea



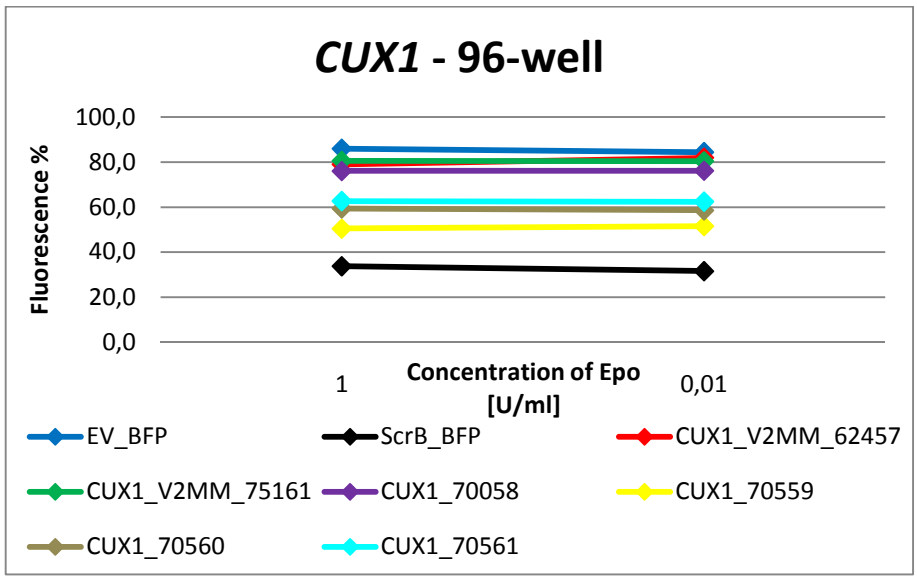
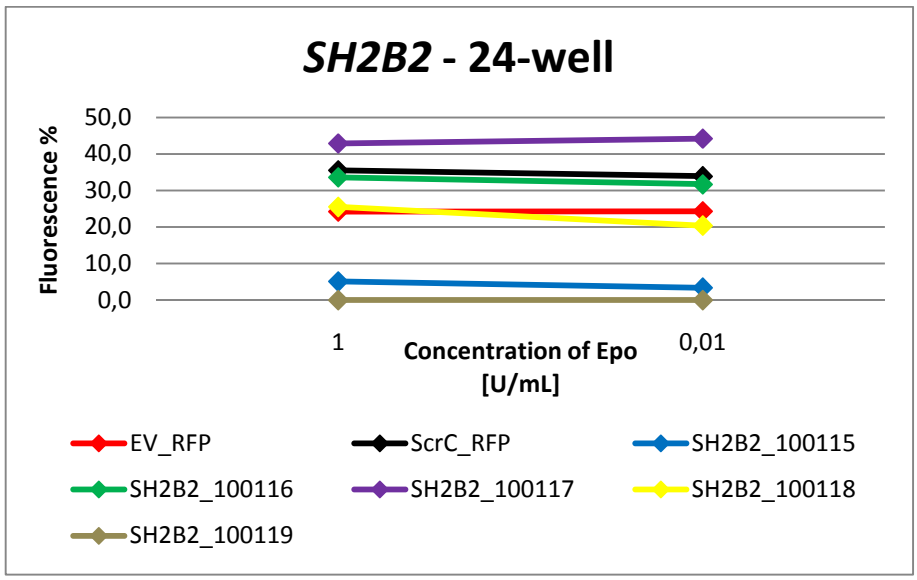


Figure 26 Growth of Baf3/EpoR cells transfected with shRNAs against *CUX1* in media with different concentrations of Epo



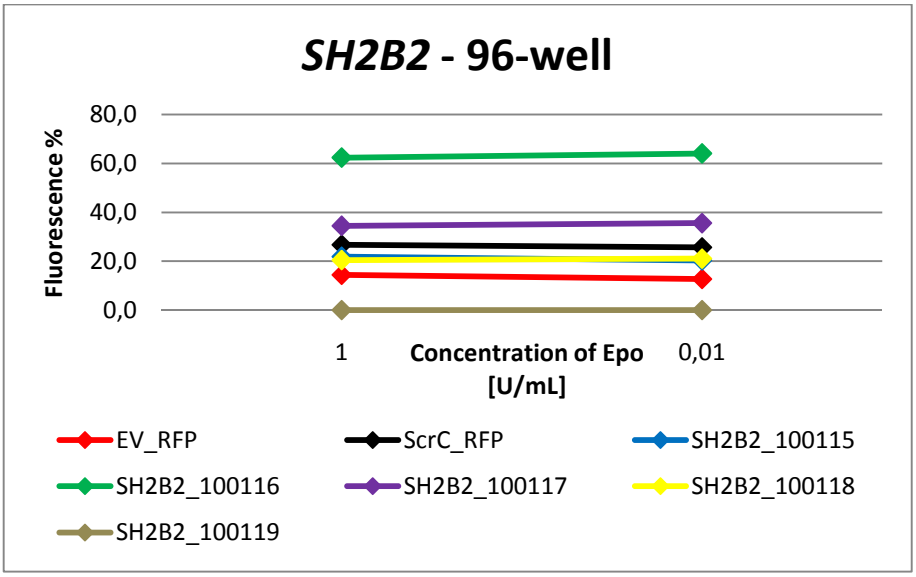


Figure 27 Growth of Baf3/EpoR cells transfected with shRNAs against *SH2B2* in media with different concentrations of erythropoietin

In order to confirm the knock down of *CUX1* and *SH2B2*, RNA from cells was prepared, and by performing reverse transcription, cDNA was synthesized. The expression levels of the two genes, normalized to actin, and measured by real time PCR, are shown in figure 28. No significant change could be seen in the expression of *CUX1*, especially when comparing it to the expression of *CUX1* in cells which had only the empty vector or the scrambled oligonucleotide as control. Only the knock down of *CUX1*was analyzed, because the transduction rates of *SH2B2* were much lower than those of *CUX1*, and because of this, expression analysis could not be performed. For enrichment of transduced cells, the cells were grown in 700µg/mL hygromycin for 9 days. After day 5 no enrichment was observed (data not shown). The SFLV vector contains a resistance gene against hygromycin, and because of this, cells having the vector should be the only ones growing in this medium. After this time, only cells carrying some pins survived and were able to be enriched at >90%. Only the cells with the scrambled sequence, and with three different shRNAs against *CUX1*, survived (figure 29-31). However, also after enrichment of these cells, no decrease of *CUX1* expression could be observed compared to the control (figure 29-31).

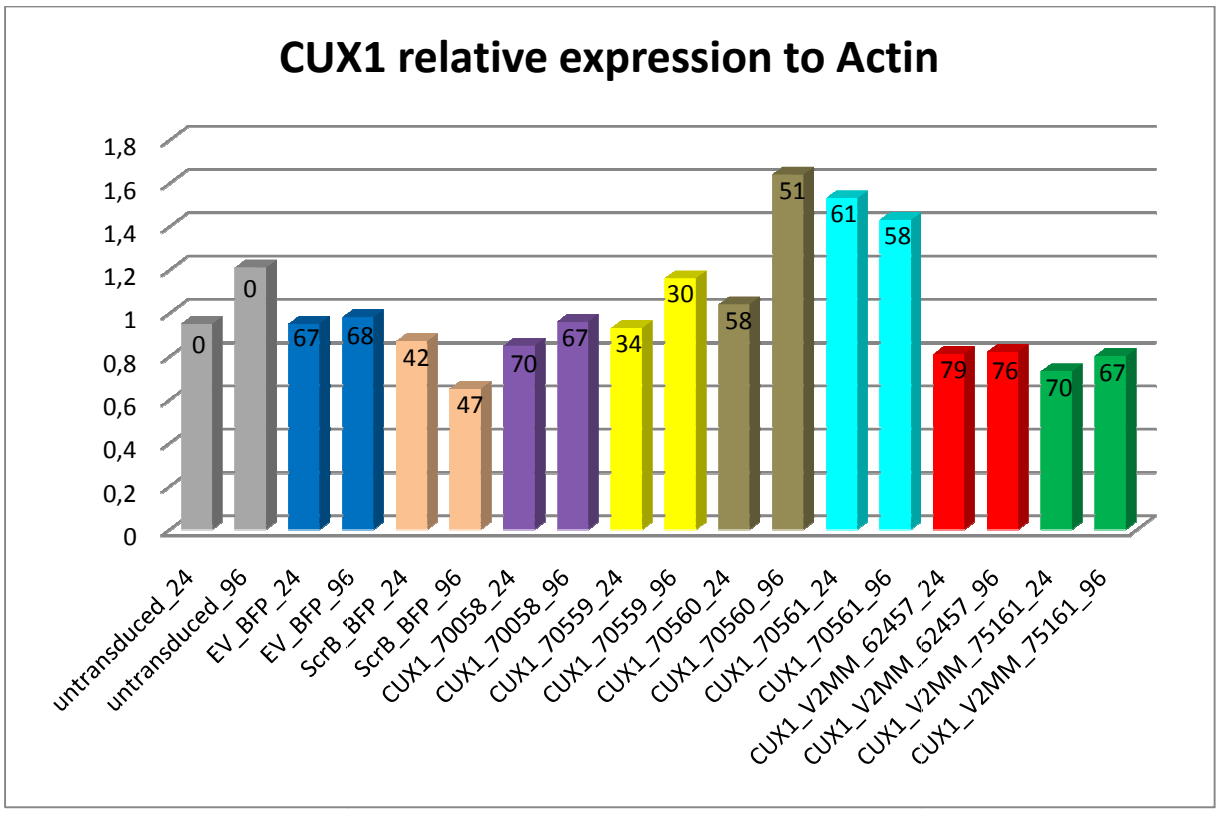


Figure 28 *CUX1* expression in Baf3/EpoR cells transduced with shRNAs against *CUX1* (transduction efficiencies [%] are written on the columns)

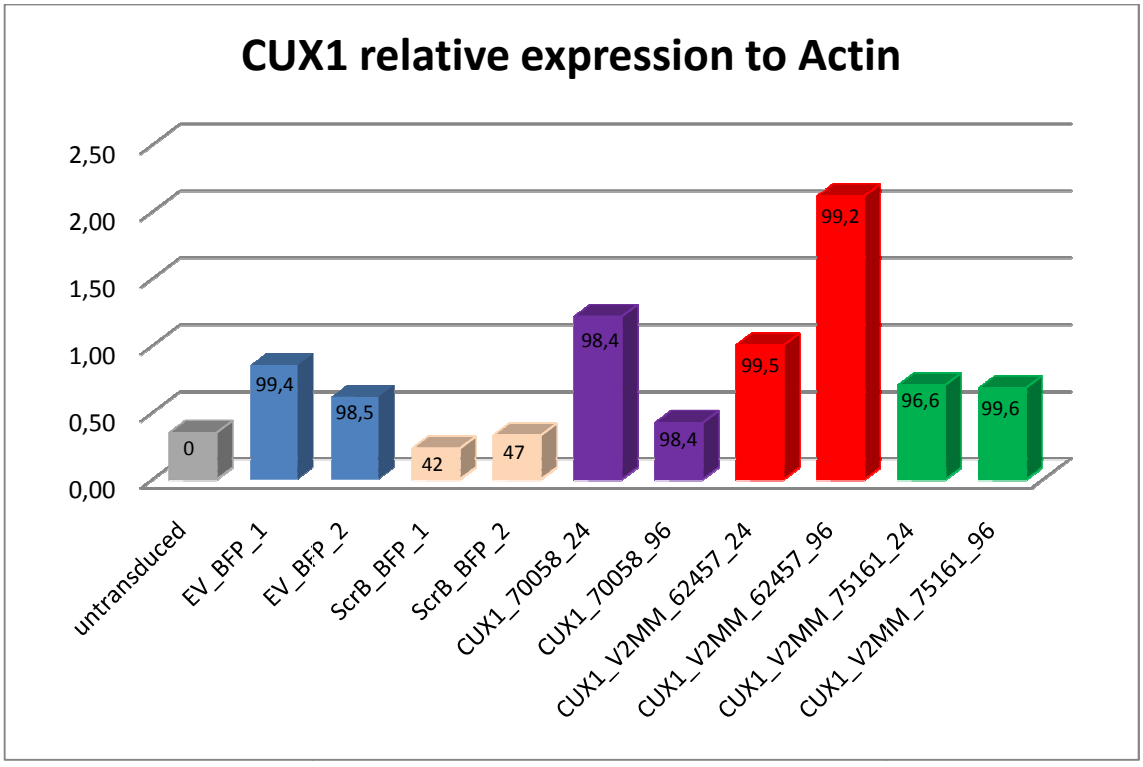


Figure 29 *CUX1* expression in Baf3/EpoR cells transduced with shRNAs against *CUX1* (transduction efficiencies [%] are written on the columns)

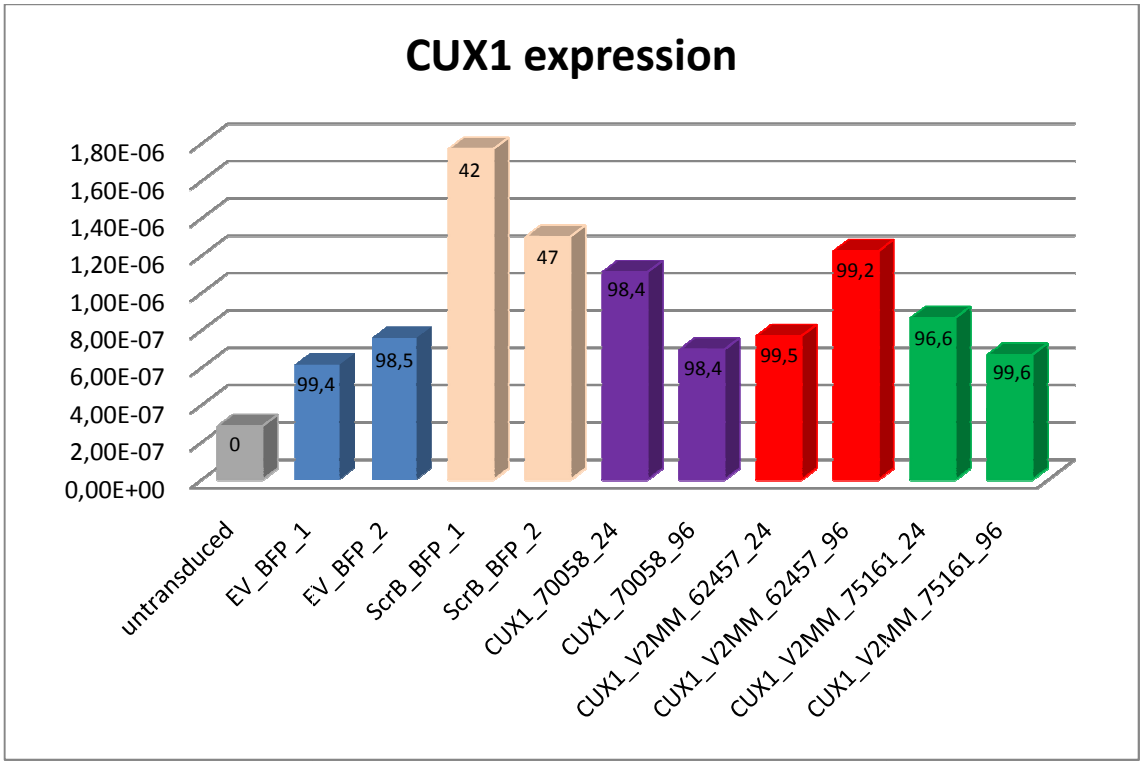


Figure 30 *CUX1* expression in Baf3/EpoR cells transduced with shRNAs against *CUX1*, not normalized to actin (transduction efficiencies [%] are written on the columns)

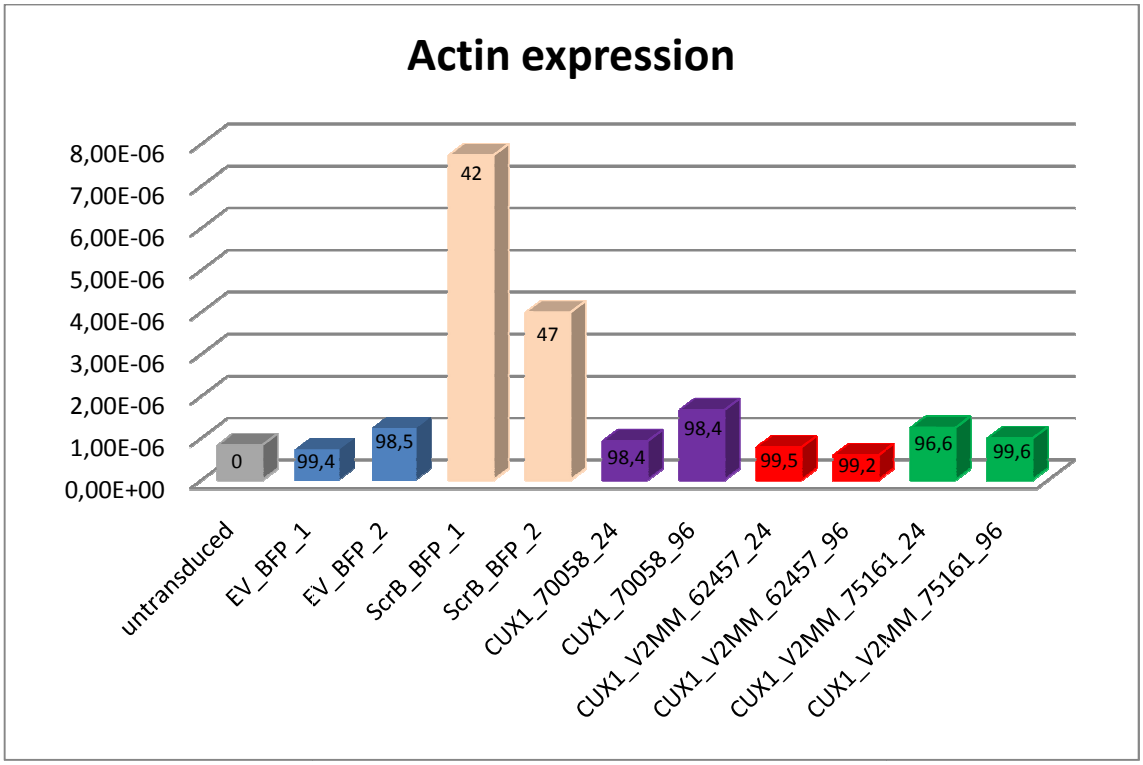


Figure 31 *Actin* expression in Baf3/EpoR cells transduced with shRNAs against *CUX1* (transduction efficiencies [%] are written on the columns)

4.3 TP53

In order to analyze another gene, which could play an important role in MPN pathogenesis, and is associated with transformation to post-MPN AML, the 11 exons of *TP53* were sequenced from 24 patients that experienced disease progression to sMF or post-MPN AML. For all patients there were at least two paired DNA samples available, one collected in the chronic phase of the disease and the second after disease evolution. Five of the patients (21%) had a *TP53* mutation. [24] One patient had a homozygous mutation, two had independent mutations on both *TP53* alleles, and two had a monoallelic mutation (one point mutation and one 19bp deletion) (table 12). For these patients the samples from the chronic phase were also analyzed. Two of them displayed the mutations also in chronic phase, but one carried the mutations in a smaller clone and the other had only one of the two mutations before disease progression.[24]

UPN	Disease phase	*TP53*
UPN 1	MF post-PV	C135S/M246K
	AML	C135S/M246K
UPN 2	MF post-ET	Wild type
	AML	K132E (17pUPD)
UPN 3	PMF	S261T
	AML	S261T/N239D
UPN 4	PMF	Wild type
	AML	R306X
UPN 5	PV	Wild type
	AML	c.450-468-del19

Table 12 Sequencing results of *TP53* in patients with disease progression (UPN unique patient number)[24]

5 DISCUSSION

The purpose of this study was to identify a new tumor suppressor or oncogene which would play a role in the pathogenesis of MPN. MPN are a very heterogeneous disease group,[18] because of the many different genes playing a role in the pathogenesis. Up to now it remains unknown whether a founder mutation driving the clonality exists. In an attempt to find this common mutation, which could lead to better diagnosis and treatment of MPN, three genes with possible involvement in the pathogenesis were studied.

CUX1 and *SH2B2* were found in the CDR of chromosome 7q using 6.0 arrays. *CUX1* was often suggested before to play a role in myeloid malignancies,[99] but only very recently, a mutation was found.[105] *SH2B2* belongs to one gene family with *SH2B3*, which was also recently reported to be a tumor suppressor in MPN.[129-130] Our CDR found in chronic and transformed MPN patients, contained only one gene, *CUX1*. However, because of the relatedness of *SH2B2* to *SH2B3*, it was also interesting to search for mutations in *SH2B2*. It might be, that a deletion of one of the two genes would lead to a weaker phenotype, and a deletion of both would result in a stronger phenotype. Only one mutation in *SH2B2*, but no somatic mutation in *CUX1*, was found in 32 patients with post-MPN AML.

On chromosome 12q a duplication of the chromosomal region of 7q can be found. *CUX2* and *SH2B3* are located in this duplicated region, and both are suggested to play a role in the pathogenesis of MPN.[129-130, 146] A duplication of a gene is a mechanism by which evolution multiplies genes that are important during evolution.[167] This is also an argument in favor of the hypothesis that *CUX1* and *SH2B2* could be important for MPN pathogenesis. It also supports the theory, that MPN are complex diseases, and that the patients constitute a very heterogeneous group.[18] We could see from the 6.0 array analyses that five out of six target genes of the CDRs, *FOXP1*, *IKZF1*, *CUX1*, *ETV6*, and *RUNX1*, are transcription factors. This could mean that transcription factor networks could have a big impact on MPN pathogenesis if disturbed.[21] One possibility to study this, would be to compare the expression levels of different genes in cells, which have a knock down of one important gene for MPN, and search for the common ones with altered expression. It is possible that these genes, which are found down-regulated, constitute a network.

For further analysis of the function of *CUX1* and *SH2B2* in MPN, it was attempted to analyze the impact of shRNA pins against these genes on Baf3/EpoR cell kinetics. For that, a knock down of *CUX1* and *SH2B2* using shRNAs was conducted. A screen of the different pins

showed no significant change in the proliferative advantage of cells containing the different pins. This could be due to an insufficient knock down of the gene of interest, or could mean that these genes do not play a major role in the pathogenesis of MPN. To re-evaluate the hypothesis, more experiments can be envisioned. The irregular pattern of fluorescence of cells containing the vector with the RFP marker could be due to toxicity of the marker, which manifests after reaching a certain concentration, or because of an inefficient translation and folding of the marker protein. These results call for caution when using RFP as a cell tracer. One shRNA against *SH2B2* had a transduction rate of 0% in all experiments, which could be either due to a toxicity of this pin, or due to too low concentrations of DNA used for production of the virus. The results of the real time PCR, showing that there is no knock down of *CUX1*, could be in figure 28 due to the different transduction efficiencies of the cells. Single copy integrations might not be enough to reach good knock down efficiencies, that is why an increase in MOI (multiplicity of infection) might be useful. To study the expression level of *CUX1* and *SH2B2*, it would be necessary to get 100% enriched cells for all the shRNAs, and then check again the knock down efficiency. One of the possibilities to sort the cells according to their fluorescence would be to perform FACS sorting or improve the antibiotic selection. Nevertheless, for 3 shRNAs against *CUX1* a ~100% enrichment of fluorescent cells with hygromycin was achieved. These were the only cells which survived after the hygromycin treatment. Measurement of CUX1 expression in these cells also resulted in no effect, but here the normalization to actin could play a role, because its expression was too high (figure 29-31). Whether the efficiency of the used pins against *CUX1* was enough needs still to be verified. *CUX1* has a very large transcript and multiple variants, which could hinder its knock down.

Another interesting study would be to make double transduction of cells with shRNAs against *CUX1* and *SH2B2*, and look if a cell, which has both genes knocked down, gets proliferative advantage. One could also include a third gene, for example *SH2B3*, or *CUX2*, and look, if there is some cumulative effect that confers stronger proliferative advantage. The shRNAs, which would be proven to confer a knock down of *CUX1* and *SH2B2*, could be also later used for *in vivo* studies in bone marrow transplantation experiments in mice to assess the role of *CUX1* and *SH2B2* in the pathogenesis of MPN. As an alternative to knock down technologies, the careful analysis of mice with *Cux1* and *Sh2b2* deletions might be useful in determining any cooperative effect between the genes.

6 REFERENCES

1. Tefferi A, Vainchenker W (2011) Myeloproliferative neoplasms: molecular pathophysiology, essential clinical understanding, and treatment strategies. J Clin Oncol. 29(5):574-82
2. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, Harris NL, Le Beau MM, Hellström-Lindberg E, Tefferi A, Bloomfield CD (2009) The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 114(5):937-51
3. Dameshek W (1951) Some speculations on the myeloproliferative syndromes. Blood. 6(4):372-5
4. Chen GL, Prchal JT (2007) X-linked clonality testing: interpretation and limitations. Blood. 110(5):1411-9
5. Adamson JW, Fialkow PJ, Murphy S, Prchal JF, Steinmann L (1976) Polycythemia vera: stem-cell and probable clonal origin of the disease. N Engl J Med. 295(17):913-6
6. Fialkow PJ, Faquet GB, Jacobson RJ, Vaidya K, Murphy S (1981) Evidence that essential thrombocythemia is a clonal disorder with origin in a multipotent stem cell. Blood. 58(5):916-9
7. Jacobson RJ, Salo A, Fialkow PJ (1978) Agnogenic myeloid metaplasia: a clonal proliferation of hematopoietic stem cells with secondary myelofibrosis. Blood. 51(2):189-94
8. Goldman JM, Melo JV (2003) Chronic myeloid leukemia – advances in biology and new approaches to treatment. N Engl J Med. 349(15):1451-64
9. Nowell PC, Hungerford DA (1960) A minute chromosome in human chronic granulocytic leukemia. Science. 132:1497
10. Rowley JD (1973) A new consistent chromosomal abnormality in chronic myelogenous leukemia identified by quinacrine fluorescence and Giemsa staining. Nature. 243(5405):290-3
11. Shtivelman E, Lifshitz B, Gale RP, Canaani E (1985) Fused transcript of abl and bcr genes in chronic myelogenous leukemia. Nature. 315(6020):550-4
12. Campbell PJ, Green AR (2006) The myeloproliferative disorders. N Engl J Med. 355(23):2452-66
13. Levine RL, Pardanani A, Tefferi A, Gilliland DG (2007) Role of JAK2 in the pathogenesis and therapy of myeloproliferative disorders. Nat Rev Cancer. 7(9):673-83
14. Ania BJ, Suman VJ, Sobell JL, Codd MB, Silverstein MN, Melton LJ 3rd (1994) Trends in the incidence of polycythemia vera among Olmsted County, Minnesota residents, 1935-1989. Am J Hematol. 47(2):89-93
15. Mesa RA, Silverstein MN, Jacobsen SJ, Wollan PC, Tefferi A (1999) Population-based incidence and survival figures in essential thrombocythemia and agnogenic

myeloid metaplasia: and Olmsted County Study, 1976-1995. Am J Hematol. 61(1):10-5
16. Kralovics R, Skoda RC (2005) Molecular pathogenesis of Philadelphia chromosome negative myeloproliferative disorder. Blood Rev. 19(1):1-13
17. Kralovics R, Stockton DW, Prchal JT (2003) Clonal hematopoiesis in familial polycythemia vera suggests the involvement of multiple mutational events in the early pathogenesis of the disease. Blood. 102(10):3793-6
18. Kralovics R (2008) Genetic complexity of MPN. Leukemia. 22(10):1841-8
19. Passamonti F, Malabarba L, Orlandi E, Barate C, Canevari A, Brusamolino E, Bonfichi M, Arcaini L, Caberlon S, Pascutto C, Lazzarino M (2003) Polycythemia vera in young patients: a study on the long-term risk of thrombosis, myelofibrosis and leukemia. Haematologica. 88(1):13-8
20. Passamonti F, Rumi E, Arcaini L, Boveri E, Elena C, Pietra D, Boggi S, Astori C, Bernasconi P, Varettoni M, Brusamolino E, Pascutto C, Lazzarino M (2008) Prognostic factors for thrombosis, myelofibrosis, and leukemia in essential thrombocythemia: a study of 605 patients. Haematologica. 93(11):1645-51
21. Klampfl T, Harutyunyan A, Berg T, Gisslinger B, Schalling M, Bagienski K, Olcaydu D, Passamonti F, Rumi E, Pietra D, Jäger R, Pieri L, Guglielmelli P, Iacobucci I, Martinelli G, Cazzola M,Vannucchi AM, Gisslinger H, Kralovics R (2011) Genome integrity of myeloproliferative neoplasms in chronic phase and during disease progression. Blood. 118(1):167-76
22. Abdulkarim K, Girodon F, Johansson P, Maynadié M, Kutti J, Carli PM, Bovet E, Andréasson B (2009) AML transformation in 56 patients with Ph-MPD in two well defined populations. Eur J Haematol. 82(2):106-11
23. Harutyunyan A, Klampfl T, Cazzola M, Kralovics R (2011) P53 lesions in leukemic transformation. N Engl J Med. 364(5):488-90
24. Rumi E, Harutyunyan A, Elena C, Pietra D, Klampfl T, Bagienski K, Berg T, Casetti I, Pascutto C, Passamonti F, Kralovics R, Cazzola M. Sequential evaluation of chromosomal aberrations using high-resolution SNP microarrays in patients with myeloproliferative neoplasm experiencing disease progression. In Press
25. Prchal JF, Axelrad AA (1974) Bone-marrow responses in polycythemia vera. N Engl J Med. 290(24):1382
26. Neubauer H, Cumano A, Muller M, Wu H, Huffstadt U, Pfeffer K (1998) JAK2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. Cell. 93(3):397-409
27. Parganas E, Wang D, Stravopodis D, Topham DJ, Marine JC, Teglund S, Vanin EF, Bodner S, Colamonici OR, van Deursen JM, Grosveld G, Ihle JN (1998) Jak2 is essential for signaling through a variety of cytokine receptors. Cell. 93(3):385-395
28. Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, Tichelli A, Cazzola M, Skoda RC (2005) A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med. 352(17):1779-90
29. 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 Project (2005) Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet. 365(9464):1054-61

30. James C, Ugo V, Le Couédic JP, Staerk J, Delhommeau F, Lacout C, Garçon 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 signaling causes polycythaemia vera. Nature. 434(7037):1144-8
31. 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, Fröhling S, Döhner K, Marynen P, Vandenberghe P, Mesa RA, Tefferi A, Griffin JD, Eck MJ, Sellers WR, Meyerson M, Golub TR, Lee SJ, Gilliland DG (2005) Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell. 7(4):387-97
32. Jones AV, Kreil S, Zoi K, Waghorn K, Curtis C, Zhang L, Score J, Seear R, Chase AJ, Grand FH, White H, Zoi C, Loukopoulos D, Terpos E, Vervessou EC, Schultheis B, Emig M, Ernst T, Lengfelder E, Hehlmann R, Hochhaus A, Oscier D, Silver RT, Reiter A, Cross NC (2005) Widespread occurrence of the JAK2 V617F mutation in chronic myeloproliferative disorders. Blood. 106(6):2162-8
33. Tefferi A, Gilliland DG (2007) Oncogenes in myeloproliferative disorders. Cell Cycle. 6(5):550-566
34. Ihle J, Gilliland D (2007) Jak2: normal function and role in hematopoietic disorders. Curr. Opin. Genet. Develop. 17(1):8-14
35. Huang LJ, Constantinescu SN, Lodish HF (2001) The N-terminal domain of Janus kinase 2 is required for Golgi processing and cell surface expression of erythropoietin receptor. Mol Cell. 8(6):1327-38
36. Remy I, Wilson IA, Michnick SW (1999) Erythropoietin receptor activation by a ligand-induced conformation change. Science. 283(5404):990-3
37. Lu X, Gross AW, Lodish HF (2006) Active conformation of the erythropoietin receptor: random and cysteine-scanning mutagenesis of the extracellular juxtamembrane and transmembrane domains. J Biol Chem. 281(11):7002-11
38. Seubert N, Royer Y, Staerk J, Kubatzky KF, Moucadel V, Krishnakumar S, Smith SO, Constantinescu SN (2003) Active and inactive orientations of the transmembrane and cytosolic domains of the erythropoietin receptor dimer. Mol Cell. 12(5):1239-50
39. Witthuhn BA, Quelle FW, Silvennoinen O, Yi T, Tang B, Miura O, Ihle JN (1993) JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell. 74(2):227-36
40. Sandberg EM, Wallace TA, Godeny MD, Vonderlinden D, Sayeski PP (2004) Jak2 tyrosine kinase: a true jak of all trades? Cell Biochem Biophys. 41(2):207-32
41. Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW (2002) Signaling through the JAK/STAT pathway, recent advances and future challenges. Gene. 285(1-2):1-24
42. Valentino L, Pierre J (2006) JAK/STAT signal transduction: regulators and implication in hematological malignancies. Biochem. Pharmacol. 71(6):713-721

43. Saharinen P, Takaluoma K, Silvennoinen O (2000) Regulation of the Jak2 tyrosine kinase by its pseudokinase domain. Mol Cell Biol. 20(10):3387-95
44. Zhao R, Xing S, Li Z, Fu X, Li Q, Krantz SB, Zhao ZJ (2005) Identification of an acquired JAK2 mutation in polycythemia vera. J Biol Chem. 280(24):22788-92
45. Ugo V, Marzac C, Teyssandier I, Larbret F, Lécluse Y, Debili N, Vainchenker W, Casadevall N (2004) Multiple signaling pathways are involved in erythropoietin-independent differentiation of erythroid progenitors in polycythemia vera. Exp Hematol. 32(2):179-87
46. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF (1999) Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. Cell. 98(2):181-91
47. Myklebust JH, Blomhoff HK, Rusten LS, Stokke T, Smeland EB (2002) Activation of phosphatidylinositol 3-kinase is important for erythropoietin-induced erythropoiesis from CD34(+) hematopoietic progenitor cells. Exp Hematol. 30(9):990-1000
48. Bumm T, Elsea C, Corbin A, Loriaux M, Sherbenou D, Wood L, Deininger J, Silver RT, Druker BJ, Deininger MW (2006) Characterization of murine JAK2V617F-positive myeloproliferative disease. Cancer Res. 66(23):11156-11165
49. Lacout C, Pisani DF, Tulliez M, Gachelin FM, Vainchenker W, Villeval JL (2006) JAK2V617F expression in murine hematopoietic cells leads to MPD mimicking human PV with secondary myelofibrosis. Blood. 108(5):1652-1660
50. Shide K, Shimoda H, Kumano T, Karube K, Kameda T, Takenaka K, Oku S, Abe H, Katayose KS, Kubuki Y, Kusumoto K, Hasuike S, Tahara Y, Nagata K, Matsuda T, Ohshima K, Harada M, Shimoda K (2008) Development of ET, primary myelofibrosis and PV in mice expressing JAK2 V617F. Leukemia. 22(1):87-95
51. Xing S, Wanting T, Zhao W, Ma J, Wang S, Xu X, Li Q, Fu X, Xu M, Zhao ZJ (2008) Transgenic expression of JAK2V617F causes myeloproliferative disorders in mice. Blood. 111(10):5109-5117
52. Tiedt R, Hao-Shen H, Sobas MA, Looser R, Dirnhofer S, Schwaller J, Skoda RC (2008) Ratio of mutant *JAK2*-V617F to wild-type Jak2 determines the MPD phenotypes in transgenic mice. Blood. 111(8):3931-3940
53. Antonioli E, Guglielmelli P, Poli G, Bogani C, Pancrazzi A, Longo G, Ponziani V, Tozzi L, Pieri L, Santini V, Bosi A, Vannucchi AM, Myeloproliferative Disorders Research Consortium (MPD-RC) (2008) Influence of JAK2V617F allele burden on phenotype in essential thrombocythemia. Haematologica. 93(1):41-48
54. Vannucchi A, Antonioli E, Guglielmelli P, Longo G, Pancrazzi A, Ponziani V, Bogani C, Ferrini PR, Rambaldi A, Guerini V, Bosi A, Barbui T, MPD Research Consortium (2007) Prospective identification of high-risk polycythemia vera patients based on JAK2V617F allele burden. Leukemia. 21(9):1952-1959
55. Tefferi A, Strand J, Lasho T, Knudson RA, Finke CM, Gangat N, Pardanani A, Hanson CA, Ketterling RP (2007) Bone marrow JAK2V617F allele burden and clinical correlates in polycythemia vera. Leukemia. 21(9):2074-2075

56. Wernig G, Mercher T, Okabe R, Levine R, Lee BH, Gilliland DG (2006) Expression of *JAK2*V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model. Blood. 107(11):4274-4281
57. Zaleskas V, Krause DS, Lazarides K (2006) Molecular pathogenesis and therapy of polycythemia induced in mice by JAK2 V617F. PLoS ONE. 1(1):e18
58. Oh ST, Gotlib J (2010) JAK2 V617F and beyond: role of genetics and aberrant signaling in the pathogenesis of myeloproliferative neoplasms. Expert Rev Hematol. 3(3): 323-37
59. Scott LM, Tong W, Levine RL, Scott MA, Beer PA, Stratton MR, Futreal PA, Erber WN, McMullin MF, Harrison CN, Warren AJ, Gilliland DG, Lodish HF, Green AR (2007) JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. N. Engl. J. Med. 356(5):459-468
60. Pardanani A, Lasho T, Finke C, Hanson C, Tefferi A (2007) Prevalence and clinicopathologic correlates of JAK2 exon 12 mutations in JAK2V617F-negative polycythemia vera. Leukemia 21(9):1960-1963
61. 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(3):1686-1689
62. Wang Y, Vandris K, Jones A, Cross NC, Christos P, Adriano F, Silver RT (2008) *JAK2* mutations are present in all cases of polycythemia vera. Leukemia. 22(6):1289
63. Hagstrom SA, Dryja TP (1999) Mitotic recombination map of 13cen-13q14 derived from an investigation of loss of heterozygosity in retinoblastomas. Proc Natl Acad Sci USA. 96(6):2952-7
64. Zhu X, Dunn JM, Goddard AD, Squire JA, Becker A, Phillips RA, Gallie BL (1992) Mechanisms of loss of heterozygosity in retinoblastoma. Cytogenet Cell Genet. 59(4):248-52
65. Wadey RB, Pal N, Buckle B, Yeomans E, Pritchard J, Cowell JK (1990) Loss of heterozygosity in Wilms' tumour involves two distinct regions of chromosome 11. Oncogene. 5(6):901-7
66. Fitzgibbon J, Iqbal S, Davies A, O'Shea D, Carlotti E, Chaplin T, Matthews J, Raghavan M, Norton A, Lister TA, Young BD (2007) Genome-wide detection of recurring sites of uniparental disomy in follicular and transformed follicular lymphoma. Leukemia. 21(7):1514-20
67. Olcaydu D, Harutyunyan A, Jäger R, Berg T, Gisslinger B, Pabinger I, Gisslinger H, Kralovics R (2009) A common *JAK2* haplotype confers susceptibility to myeloproliferative neoplasms. Nat Genet. 41(4):450-4
68. Olcaydu D, Skoda RC, Looser R, Li S, Cazzola M, Pietra D, Passamonti F, Lippert E, Carillo S, Girodon F, Vannucchi A, Reading NS, Prchal JT, Ay C, Pabinger I, Gisslinger H, Kralovics R (2009) The 'GGCC' haplotype of *JAK2* confers susceptibility to *JAK2* exon 12 mutation-positive polycythemia vera. Leukemia. 23(10):1924-26
69. Pardanani AD, Levine RL, Lasho T, Pikman Y, Mesa RA, Wadleigh M. Steensma DP, Elliott MA, Wolanskyj AP, Hogan WJ, McClure RF, Litzow MR, Gilliland

DG, Tefferi A (2006) MPL515 mutations in myeloproliferative and other myeloid disorder: a study of 1182 patients. Blood. 108(10):3472-6

70. Beer PA, Campbell PJ, Scott LM, Bench AJ, Erber WN, Bareford D, Wilkins BS, Reilly JT, Hasselbalch HC, Bowman R, Wheatley K, Buck G, Harrison CN, Green AR (2008) MPL mutations in myeloproliferative disorders: analysis of the PT-1 cohort. Blood. 112(1):141-9
71. Vannucchi AM, Antonioli E, Guglielmelli P, Pancrazzi A, Guerini V, Barosi G, Ruggeri M, Specchia G, Lo-Coco F, Delaini F, Villani L, Finotto S, Ammatuna E, Alterini R, Carrai V, Capaccioli G, Di Lollo S, Liso V, Rambaldi A, Bosi A, Barbui T (2008) Characteristics and clinical correlates of MPL515W>L/K mutation in essential thrombocythemia. Blood. 112(3):844-7
72. Chaligne R, Tonetti C, Besancenot R, Roy L, Marty C, Mossuz P, Kiladjian JJ, Socié G, Bordessoule D, Le Bousse-Kerdilès MC, Vainchenker W, Giraudier S (2008) New mutations of MPL in primitive myelofibrosis: only the MPL W515 mutations promote a G(1)/S-phase transition. Leukemia. 22(8):1557-66
73. Guglielmelli P, Pancrazzi A, Bergamaschi G, Rosti V, Villani L, Antonioli E, Bosi A, Barosi G, Vannucchi AM, GIMEMA--Italian Registry of Myelofibrosis, MPD Research Consortium (2007) Anaemia characterizes patients with myelofibrosis harbouring Mpl mutation. Br J Haematol. 137(3):244-7
74. Pikman Y, Lee BH, Mercher T, McDowell E, Ebert BL, Gozo M, Cuker A, Wernig G, Moore S, Galinsky I, DeAngelo DJ, Clark JJ, Lee SJ, Golub TR, Wadleigh M, Gilliland DG, Levine RL (2006) MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. PLoS Med. 3(7):e270
75. Loh ML, Sakai DS, Flotho C, Kang M, Fliegauf M, Archambeault S, Mullighan CG, Chen L, Bergstraesser E, Bueso-Ramos CE, Emanuel PD, Hasle H, Issa JP, van den Heuvel-Eibrink MM, Locatelli F, Stary J, Trebo M, Wlodarski M, Zecca M, Shannon KM, Niemeyer CM (2009) Mutations in CBL occur frequently in juvenile myelomonocytic leukemia. Blood. 114(9):1859-63
76. Grand FH, Hidalgo-Curtis CE, Ernst T, Zoi K, Zoi C, McGuire C, Kreil S, Jones A, Score J, Metzgeroth G, Oscier D, Hall A, Brandts C, Serve H, Reiter A, Chase AJ, Cross NC (2009) Frequent CBL mutations associated with 11q acquired uniparental disomy in myeloproliferative neoplasms. Blood. 113(24):6182-92
77. Schmidt MH, Dikic I (2005) The Cbl interactome and its functions. Nat. Rev. Mol. Cell Biol. 6(12):907-18
78. Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Massé A, Kosmider O, Le Couedic JP, Robert F, Alberdi A, Lécluse Y, Plo I, Dreyfus FJ, Marzac C, Casadevall N, Lacombe C,Romana SP, Dessen P, Soulier J, Viguié F, Fontenay M, Vainchenker W, Bernard OA (2009) Mutation in TET2 in myeloid cancers. N Engl J Med. 360(22):2289-2301
79. Tefferi A, Pardanani A, Lim KH, Abdel-Wahab O, Lasho TL, Patel J, Gangat N, Finke CM, Schwager S, Mullally A, Li CY, Hanson CA, Mesa R, Bernard O, Delhommeau F, Vainchenker W, Gilliland DG, Levine RL (2009) TET2 mutations and their clinical correlates in polycythemia vera, essential thrombocythemia and myelofibrosis. Leukemia. 23(5):905-11

80. Abdel-Wahab O, Manshouri T, Patel J, Harris K, Yao J, Hedvat C, Heguy A, Bueso-Ramos C, Kantarjian H, Levine RL, Verstovsek S (2010) Genetic analysis of transforming events that convert chronic myeloproliferative neoplasms to leukemias. Cancer Res. 70(2):447-52
81. Carbuccia N, Murati A, Trouplin V, Brecqueville M, Adélaïde J, Rey J, Vainchenker W, Bernard OA, Chaffanet M, Vey N, Birnbaum D, Mozziconacci MJ (2009) Mutations of ASXL1 gene in myeloproliferative neoplasms. Leukemia. 23(11):2183-6
82. Tefferi A, Lasho TL, Abdel-Wahab O, Guglielmelli P, Patel J, Caramazza D, Pieri L, Finke CM, Kilpivaara O, Wadleigh M, Mai M, McClure RF, Gilliland DG, Levine RL, Pardanani A, Vannucchi AM (2010) IDH1 and IDH2 mutation studies in 1473 patients with chronic-, fibrotic- or blast-phase essential thrombocythemia, polycythemia vera or myelofibrosis. Leukemia. 24(7)1302-9
83. Jäger R, Gisslinger H, Passamonti F, Rumi E, Berg T, Gisslinger B, Pietra D, Harutyunyan A, Klampfl T, Olcaydu D, Cazzola M, Kralovics R (2010) Deletions of the transcription factor Ikaros in myeloproliferative neoplasms. Leukemia. 24(7):1290-8
84. Georgopoulos K, Moore D, Derfler B (1992) Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. Science. 258(5083):808-12
85. Winandy S, Wu P, Georgopoulos K (1995) A dominant mutation in the Ikaros gene leads to rapid development of leukemia and lymphoma. Cell. 83(2):289-99
86. Wang J, Nichogiannopoulou A, Wu L, Sun L, Sharpe AH, Bigby M, Georgopoulos K (1996) Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. Immunity. 5(6):537-49
87. Kirstetter P, Thomas M, Dierich A, Kastner P, Chan S (2002) Ikaros is critical for B cell differentiation and function. Eur J Immunol. 32(3):720-30
88. Lopez RA, Schoetz S, DeAngelis K, O'Neill D, Bank A (2002) Multiple hematopoietic defects and delayed globin switching in Ikaros null mice. Proc Natl Acad Sci USA. 99(2):602-7
89. 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(8):722-726
90. Nikoloski G, Langemeijer SM, Kuiper RP, Knops R, Massop M, Tönnissen 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(8):665-667
91. Jäger R, Kralovics R (2010) Molecular basis and clonal evolution of myeloproliferative neoplasms. Haematologica. 95(4):526-9
92. Skoda R, Prchal JT (2005) Lessons from familial myeloproliferative disorders. Semin Hematol. 42(4):266-73

93. Bellanne-Chantelot C, Chaumarel I, Labopin M, Bellanger F, Barbu V, De Toma C, Delhommeau F, Casadevall N, Vainchenker W, Thomas G, Najman A (2006) Genetic and clinical implications of the Val617Phe JAK2 mutation in 72 families with myeloproliferative disorders. Blood. 108(1):346-52
94. Cario H, Goerttler PS, Steimle C, Levine RL, Pahl HL (2005) The JAK2V617F mutation is acquired secondary to the predisposing alteration in familial polycythaemia vera. Br J Haematol. 130(5):800-1
95. Sansregret L, Nepveu A (2008) The multiple roles of CUX1: Insights from mouse models and cell-based assays. Gene. 412(1-2):84-94
96. Michl P, Ramjaun AR, Pardo OE, Warne PH, Wagner M, Poulsom R, D'Arrigo C, Ryder K, Menke A, Gress T, Downward J (2005) CUTL1 is a target of TGF(beta) signaling that enhances cancer cell motility and invasiveness. Cancer Cell. 7(6):521-32
97. Rong Zeng W, Soucie E, Sung Moon N, Martin-Soudant N, Berube G, Leduy L, Nepveu A (2000) Exon/intron structure and alternative transcripts of the CUTL1 gene. Gene. 241(1):75-85
98. Truscott M, Harada R, Vadnais C, Robert F, Nepveu A (2008) P110 CUX1 cooperates with E2F transcription factors in the transcriptional activation of cell cycle-regulated genes. Mol Cell Biol. 28(10):3127-38
99. Cadieux C, Fournier S, Peterson AC, Bédard C, Bedell BJ, Nepveu A (2006) Transgenic mice expressing the p75 CCAAT-displacement protein/Cut homeobox isoform develop a myeloproliferative disease-like myeloid leukemia. Cancer Res. 66(19):9492-501
100. Coqueret O, Berube G, Nepveu A (1998) The mammalian Cut homeodomain protein functions as a cell-cycle-dependent transcriptional repressor which downmodulates P21 (Waf1/Cip1/Sdi1) in S phase. EMBO J. 17(16):4680-94
101. Moon NS, Premdas P, Truscott M, Leduy L, Berube G, Nepveu A (2001) S-phase specific proteolytic cleavage is required to activate stable dna binding by the CDP/Cut homeodomain protein. Mol Cell Biol. 21(18):6332-45
102. Goulet B, Watson P, Poirier M, Leduy L, Bérubé G, Meterissian S, Jolicoeur P, Nepveu A (2002) Characterization of a tissue-specific CDP/Cux isoform, p75, activated in breast tumor cells. Cancer Res. 62(22):6625-33
103. De Vos J, Thykjaer T, Tarte K, Ensslen M, Raynaud P, Requirand G, Pellet F, Pantesco V, Rème T, Jourdan M, Rossi JF, Ørntoft T, Klein B (2002) Comparison of gene expression profiling between malignant and normal plasma cells with oligonucleotide arrays. Oncogene. 21(44):6848-57
104. Tsutsumi S, Taketani T, Nishimura K, Ge X, Taki T, Sugita K, Ishii E, Hanada R, Ohki M, Aburatani H, Hayashi Y (2003) Two distinct gene expression signatures in pediatric acute lymphoblastic leukemia with MLL rearrangements. Cancer Res. 63(16):4882-87
105. Thoennissen NH, Lasho T, Thoennissen GB, Ogawa S, Tefferi A, Koeffler HP (2011) Novel CUX1 missense mutation in association with 7q- at leukemic transformation of MPN. Am J Hematol. 86(8):703-5

106. Tufarelli C, Fujiwara Y, Zappulla DC, Neufeld EJ (1998) Hair defects and pup loss in mice with targeted deletion of the first cut repeat domain of the Cux/CDP homeoprotein gene. Dev Biol. 200(1):69-81
107. Sinclair AM, Lee JA, Goldstein A, Xing D, Liu S, Ju R, Tucker PW, Neufeld EJ, Scheuermann RH (2001) Lymphoid apoptosis and myeloid hyperplasia in CCAAT displacement protein mutant mice. Blood. 98(13):3658-67
108. Luong MX, van der Meijden CM, Xing D, Hesselton R, Monuki ES, Jones SN, Lian JB, Stein JL, Stein GS, Neufeld EJ, van Wijnen AJ (2002) Genetic ablation of the CDP/Cux protein C terminus results in hair cycle defects and reduced male fertility. Mol Cell Biol. 22(5):1424-37
109. Ellis T, Gambardella L, Horcher M, Tschanz S, Capol J, Bertram P, Jochum W, Barrandon Y, Busslinger M (2001) The transcriptional repressor CDP (Cutl1) is essential for epithelial cell differentiation of the lung and the hair follicle. Genes Dev. 15(17):2307-19
110. Ledford AW, Brantley JG, Kemeny G, Foreman TL, Quaggin SE, Igarashi P, Oberhaus SM, Rodova M, Calvet JP, Vanden Heuvel GB (2002) Deregulated expression of the homeobox gene Cux-1 in transgenic mice results in downregulation of p27(kip1) expression during nephrogenesis, glomerular abnormalities, and multiorgan hyperplasia. Dev Biol. 245(1):157-71
111. Ozisik YY, Meloni AM, Surti U, Sandberg AA (1993) Deletion 7q22 in uterine leiomyomas. A cytogenetic review. Cancer Genet Cytogenet. 71(1):1-6
112. Fenaux P, Preudhomme C, Lai JL, Morel P, Beuscart R, Bauters F (1989) Cytogenetics and their prognostic value in de novo acute myeloid leukaemia: a report on 283 cases. Br J Haematol. 73(1):61-7
113. Heim S (1992) Cytogenetic findings in primary and secondary MDS. Leuk Res. 16(1):43-6
114. 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, Müller-Tidow C, Mesa R, Haferlach T, Gilliland DG, Tefferi A, Ogawa S, Koeffler HP (2010) Prevalence and prognostic impact of allelic imbalances associated with leukemic transformation of Philadelphia chromosome-negative myeloproliferative neoplasms. Blood. 115(14):2882-90
115. Rui L, Mathews LS, Hotta K, Gustafson TA, Carter-Su C (1997) Identification of SH2-Bβ as a substrate of the tyrosine kinase JAK2 involved in growth hormone signaling. Mol Cell Biol. 17(11):6633-44
116. Nishi M, Werner ED, Oh BC, Frantz JD, Dhe-Paganon S, Hansen L, Lee J, Shoelson SE (2005) Kinase activation through dimerization by human SH2-B. Mol Cell Biol. 25(7):2607-21
117. Li M, Li Z, Morris DL, Rui L (2007) Identification of SH2B2β as an inhibitor for SH2B1- and SH2B2α-promoted Janus Kinase-2 activation and insulin signaling. Endocrinology. 148(4):1615-21
118. Dhe-Paganon S, Werner ED, Nishi M, Hansen L, Chi YI, Shoelson SE (2004) A phenylalanine zipper mediates APS dimerization. Nat Struct Mol Biol. 11(10):968-74

119. Ahmed Z, Pillay TS (2003) Adapter protein with a pleckstrin homology (PH) and an Src homology s (SH2) domain (APS) and SH2-B enhance insulin-receptor autophosphorylation, extracellular-signal-regulated kinase and phosphoinositide 3-kinase-dependent signaling. Biochem J. 371(Pt 2):405-12
120. Li M, Ren D, Iseki M, Takaki S, Rui L (2006) Differential role of SH2-B and APS in regulating energy and glucose homeostasis. Endocrinology. 147(5):2163-70
121. Minami A, Iseki M, Kishi K, Wang M, Ogura M, Furukawa N, Hayashi S, Yamada M, Obata T, Takeshita Y, Nakaya Y, Bando Y, Izumi K, Moodie SA, Kajiura F, Matsumoto M, Takatsu K, Takaki S, Ebina Y (2003) Increased insulin sensitivity and hypoinsulinemia in APS knockout mice. Diabetes. 52(11):2657-65
122. Quian X, Ginty DD (2001) SH2-B and APS are multimeric adapters that augment TrkA signaling. Mol Cell Biol. 21(5):1613-20
123. Hu J, Hubbard SR (2005) Structural characterization of a novel Cbl phosphotyrosine recognition motif in the APS family of adapter proteins. J Biol Chem. 280(19):18943-9
124. Wakioka T, Sasaki A, Mitsui K, Yokouchi M, Inoue A, Komiya S, Yoshimura A (1999) APS, an adaptor protein containing Pleckstrin homology (PH) and Src homology-2 (SH2) domains inhibits the JAK-STAT pathway in collaboration with c-Cbl. Leukemia. 13(5):760-767
125. Ahmed Z, Smith BJ, Pillay TS (2000) The APS adapter protein couples the insulin receptor to the phosphorylation of c-Cbl and facilitates ligand-stimulated ubiquitination of the insulin receptor. FEBS Lett. 475(1):31-4
126. Iseki M, Takaki S, Takatsu K (2000) Molecular cloning of the mouse APS as a member of the Lnk family adaptor proteins. Biochem Biophys Res Commun. 272(1):45-54
127. Iseki M, Kubo-Akashi C, Kwon SM, Yamaguchi A, Takatsu K, Takaki S (2005) APS, an adaptor molecule containing PH and SH2 domains, has a negative regulatory role in B cell proliferation. Biochem Biophys Res Commun. 330(3):1005-13
128. Yokouchi M, Suzuki R, Masuhara M, Komiya S, Inoue A, Yoshimura A (1997) Cloning and characterization of APS, an adaptor molecule containing PH and SH2 domains that is tyrosine phosphorylated upon B-cell receptor stimulation. Oncogene. 15(1):7-15
129. Oh ST, Simonds EF, Jones C, Hale MB, Goltsev Y, Gibbs KD Jr, Merker JD, Zehnder JL, Nolan GP, Gotlib J (2010) Novel mutations in the inhibitory adaptor protein LNK drive JAK-STAT signaling in patients with myeloproliferative neoplasms. Blood. 116(6):988-992
130. Pardanani A, Lasho T, Finke C, Oh ST, Gotlib J, Tefferi A (2010) LNK mutation studies in blast-phase myeloproliferative neoplasms, and in chronic-phase disease with TET2, IDH, JAK2 or MPL mutations. Leukemia.24(10):1713-1718
131. Takaki S, Morita H, Tezuka Y, Takatsu K (2002) Enhanced hematopoiesis by hematopoietic progenitor cells lacking intracellular adaptor protein, Lnk. J Exp Med. 195(2):151-160

132. Velazquez L, Cheng MA, Fleming EH, Furlonger C, Vesely S, Bernstein A, Paige CJ, Pawson T (2002) Cytokine signaling and hematopoietic homeostasis are disrupted in Lnk-deficient mice. J Exp Med. 195(12):1599-1611
133. Baran-Marszak F, Magdoud H, Desterke C, Alvarado A, Roger C, Harel S, Mazoyer E, Cassinat B, Chevret S, Tonetti C, Giraudier S, Fenaux P, Cymbalista F, Varin-Blank N, Le Bousse-Kerdilès MC, Kiladjian JJ, Velazquez L (2010) Expression level and differential JAK2-V617F-binding of the adaptor protein Lnk regulates JAK2-mediated signals in myeloproliferative neoplasms. Blood. 116(26):5961-71
134. Bersenev A, Wu C, Balcerek J, Tong W (2008) Lnk controls mouse hematopoietic stem cell self-renewal and quiescence through direct interactions with JAK2. J Clin Invest. 118(8):2832-44
135. Buza-Vidas N, Antonchuk J, Qian H, Månsson R, Luc S, Zandi S, Anderson K, Takaki S, Nygren JM, Jensen CT, Jacobsen SE (2006) Cytokines regulate postnatal hematopoietic stem cell expansion: opposing roles of thrombopoietin and LNK. Genes Dev. 20(15):2018-23
136. Ema H, Sudo K, Seita J, Matsubara A, Morita Y, Osawa M, Takatsu K, Takaki S, Nakauchi H (2005) Quantification of self-renewal capacity in single hematopoietic stem cells from normal and Lnk-deficient mice. Develop Cell. 8(6):907-14
137. Seita J, Ema H, Ooehara J, Yamazaki S, Tadokoro Y, Yamasaki A, Eto K, Takaki S, Takatsu K, Nakauchi H (2007) Lnk negatively regulates self-renewal of hematopoietic stem cells by modifying thrombopoietic-mediated signal transduction. Proc Natl Acad Sci USA. 104(7):2349-54
138. Takizawa H, Eto K, Yoshikawa A, Nakauchi H, Takatsu K, Takaki S (2008) Growth and maturation of megakaryocytes is regulated by Lnk/Sh2b3 adaptor protein through crosstalk between cytokine- and integrin-mediated signals. Exp Hematol. 36(7):897-906
139. Tong W, Lodish FH (2004) Lnk inhibits Tpo-mpl signaling and Tpo-mediated megakaryocytopoiesis. J Exp Med. 200(5):569-80
140. Tong W, Lodish FH (2005) Lnk inhibits erythropoiesis and Epo-dependent JAK2 activation and downstream signaling pathways. Blood. 105(12):4604-12
141. Gery S, Gueller S, Chumakova K, Kawamata N, Liu L, Koeffler HP (2007) Adaptor protein Lnk negatively regulates the mutant MPL, MPLW515L associated with myeloproliferative disorders. Blood. 110(9):3360-64
142. Gery S, Qi C, Gueller S, Hongtao X, Tefferi A, Koeffler HP (2009) Lnk inhibits myeloproliferative disorder-associated JAK2 mutant, JAK2V617F. J Leuk Biol. 85(6):957-65
143. Nepveu A (2001) Role of the multifunctional CDP/Cut/Cux homeodomain transcription factor in regulating differentiation, cell growth and development. Gene. 270(1-2):1-15
144. Cubelos B, Sebastian-Serrano A, Beccari L, Calcagnotto ME, Cisneros E, Kim S, Dopazo A, Alvarez-Dolado M, Redondo JM, Bovolenta P, Walsh CA, Nieto M (2010) Cux1 and Cux2 regulate dendritic branching, spine morphology, and synapses of the upper layer neurons of the cortex. Neuron. 66(4):523-35

145. Iulianella A, Sharma M, Vanden Heuvel GB, Trainor PA (2009) *Cux2* functions downstream of Notch signaling to regulate dorsal interneuron formation in the spinal cord. Development. 136(14):2329-34
146. Stegelmann F, Bullinger L, Griesshammer M, Holzmann K, Habdank M, Kuhn S, Maile C, Schauer S, Döhner H, Döhner K (2010) High-resolution single-nucleotide polymorphism array-profiling in myeloproliferative neoplasms identifies novel genomic aberrations. Haematologica. 95(4):666-9
147. Laurie NA, Donovan SL, Shih CS, Zhang J, Mills N, Fuller C, Teunisse A, Lam S, Ramos Y, Mohan A, Johnson D, Wilson M, Rodriguez-Galindo C, Quarto M, Francoz S, Mendrysa SM, Guy RK,Marine JC, Jochemsen AG, Dyer MA (2006) Inactivation of the p53 pathway in retinoblastoma. Nature. 444(7115):61-6
148. Veerakumarasivam A, Scott HE, Chin SF, Warren A, Wallard MJ, Grimmer D, Ichimura K, Caldas C, Collins VP, Neal DE, Kelly JD (2008) High-resolution array-based comparative genomic hybridization of bladder cancers identifies mouse double minute 4 (*MDM4*) as an amplification target exclusive of *MDM2* and *TP53*. Clin Cancer Res. 14(9):2527-34
149. Kralovics R, Teo SS, Li S, Theocharides A, Buser AS, Tichelli A, Skoda RC (2006) Acquisition of the V617F mutation of JAK2 is a late genetic event in a subset of patients with myeloproliferative disorders. Blood. 108(4):1377-80
150. Tefferi A, Thiele J, Orazi A, Kvasnicka HM, Barbui T, Hanson CA, Barosi G, Verstovsek S, Birgegard G, Mesa R, Reilly JT, Gisslinger H, Vannucchi AM, Cervantes F, Finazzi G, Hoffman R,Gilliland DG, Bloomfield CD, Vardiman JW (2007) Proposals and rationale for revision of the World Health Organization diagnostic criteria for polycythemia vera, essential thrombocythemia, and primary myelofibrosis: recommendations from an ad hoc international expert panel. Blood. 110(4):1092-7
151. Tefferi A, Sirhan S, Lasho TL, Schwager SM, Li CY, Dingli D, Wolanskyj AP, Steensma DP, Mesa R, Gilliland DG (2005) Concomitant neutrophil JAK2 mutation screening and PRV-1 expression analysis in myeloproliferative disorders and secondary polycythaemia. Br J Haematol. 131(2):166-71
152. James C, Delhommeau F, Marzac C, Teyssandier I, Couédic JP, Giraudier S, Roy L, Saulnier P, Lacroix L, Maury S, Tulliez M, Vainchenker W, Ugo V, Casadevall N (2006) Detection of JAK2 V617F as a first intention diagnostic test for erythrocytosis. Leukemia. 20(2):350-3
153. Finazzi G, Barbui T (2008) Evidence and expertise in the management of polycythemia vera and essential thrombocythemia. Leukemia. 22(8):1494-1502
154. Gangat N, Caramazza D, Vaidya R, George G, Begna K, Schwager S, Van Dyke D, Hanson C, Wu W, Pardanani A, Cervantes F, Passamonti F, Tefferi A (2011) DIPSS plus: a refined dynamic international prognostic scoring system for primary myelofibrosis that incorporates prognostic information from karyotype, platelet count, and transfusion status. J Clin Oncol. 29(4):392-7
155. Di Nisio M, Barbui T, Di Gennaro L, Borrelli G, Finazzi G, Landolfi R, Leone G, Marfisi R, Porreca E, Ruggeri M, Rutjes AW, Tognoni G, Vannucchi AM, Marchioli R, European Collaboration on Low-dose Aspirin in Polycythemia

Vera (ECLAP) Investigators (2007) The haematocrit and platelet target in polycythemia vera. Br J Haematol. 136(2):249-59
156. Passamonti F, Rumi E, Pungolino E, Malabarba L, Bertazzoni P, Valentini M, Orlandi E, Arcaini L, Brusamolino E, Pascutto C, Cazzola M, Morra E, Lazzarino M (2004) Life expectancy and prognostic factors for survival in patients with polycythemia vera and essential thrombocythemia. Am J Med. 117(10):755-61
157. Gangat N, Strand J, Li CY, Wu W, Pardanani A, Tefferi A (2007) Leucocytosis in polycythaemia vera predicts both inferior survival and leukaemic transformation. Br J Haematol. 138(3):154-8
158. Gangat N, Wolanskyj AP, McClure RF, Li CY, Schwager S, Wu W, Tefferi A (2007) Risk stratification for survival and leukemic transformation in essential thrombocythemia: a single institutional study of 605 patients. Leukemia. 21(2):270-6
159. Landolfi R, Marchioli R, Kutti J, Gisslinger H, Tognoni G, Patrono C, Barbui T, European Collaboration on Low-Dose Aspirin in Polycythemia Vera Investigators (2004) Efficacy and safety of low-dose aspirin in polycythemia vera. N Engl J Med. 350(2):114-24
160. Cortelazzo S, Finazzi G, Ruggeri M, Vestri O, Galli M, Rodeghiero F, Barbui T (1995) Hydroxyurea for patients with essential thrombocythemia and a high risk of thrombosis. N Engl J Med. 332(17):1132-6
161. Harrison CN, Campbell PJ, Buck G, Wheatley K, East CL, Bareford D, Wilkins BS, van der Walt JD, Reilly JT, Grigg AP, Revell P, Woodcock BE, Green AR, United Kingdom Medical Research Council Primary Thrombocythemia 1 Study (2005) Hydroxyurea compared with anagrelide in high-risk essential thrombocythemia. N Engl J Med. 353(1):33-45
162. Quintas-Cardama A, Kantarjian H, Manshouri T, Luthra R, Estrov Z, Pierce S, Richie MA, Borthakur G, Konopleva M, Cortes J, Verstovsek S (2009) Pegylated interferon alfa-2a yields high rates of hematologic and molecular response in patients with advanced essential thrombocythemia and polycythemia vera. J Clin Oncol. 27(32):5418-24
163. Kiladjian JJ, Cassinat B, Chevret S, Turlure P, Cambier N, Roussel M, Bellucci S, Grandchamp B, Chomienne C, Fenaux P (2008) Pegylated interferon-alfa-2a induces complete hematologic and molecular responses with low toxicity in polycythemia vera. Blood. 112(8):3065-72
164. Shvidel L, Sigler E, Haran M, Klepfish A, Duek A, Berrebi A, Shtalrid M (2007) Busulphan is safe and efficient treatment in elderly patients with essential thrombocythemia. Leukemia. 21(9):2071-2
165. "Leukemia and Hematosarcoma" Cooperative Group, European Organization for Research on Treatment of Cancer (E.O.R.T.C.) (1981) Treatment of polycythaemia vera by radiophosphorus or busulphan: a randomized trial. Br J Cancer. 44(1):75-80
166. Knudson AG Jr. (1971) Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci USA. 68(4):820-3
167. Zhang J (2003) Evolution by gene duplication: an update. TRENDS in ecology and evolution. 18(6):292-8

7 Author contribution

Genome-Wide Human SNP 6.0 arrays (Affymetrix) were performed by Tiina Berg, Ashot Harutyunyan, Thorsten Klampfl, Jelena Milosevic and Ana Puda.

Help during preparation of functional experiments was kindly provided by Luis Guachalla.

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Research Experience

04/2010	Internship at the Laboratory of Membranes and the Cytoskeleton, MFPL, Vienna, Austria Laboratory of Prof. Marcela Hermann
11/2009	Internship at the Center for Brain Research, Vienna, Austria
08/2009 – 09/2009	Internship at the Laboratory of Asymmetric Cell Division in Drosophila and Mouse Stem Cells, IMBA, Vienna, Austria Laboratory of Jürgen Knoblich, Ph.D.
08/2008	Internship at the Laboratory of Chromosome Biology, MFPL Laboratory of Verena Jantsch-Plunger, Ph.D.

Publications

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

Harutyunyan A, Gisslinger B, Klampfl T, Berg T, **Bagienski K**, Gisslinger H, Kralovics R (2011) Rare germline variants in regions of loss of heterozygosity may influence clinical course of hematological malignancies. Leukemia. Epub ahead of print. doi:10.1038/leu2011.150

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. In press.