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# **JAK2V617F-positive Myeloproliferative Neoplasms: KI mouse models, Interferon- $\alpha$ therapy and clonal architecture**

Salma Hasan

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ÉCOLE DOCTORALE : Cancérologie

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Hématopoïèse Normales et pathologiques

*DISCIPLINE Cancérologie*

THÈSE DE DOCTORAT

Soutenue le 27/11/2013

par

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**JAK2<sup>V617F</sup>-positive Myeloproliferative Neoplasms: KI mouse  
models, interferon- $\alpha$  therapy and clonal architecture**

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'Tis a common proof,  
That lowliness is young ambition's ladder,  
Whereto the climber-upward turns  
His face ...

-William Shakespeare, *Julius Caesar*

---

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“Thankfulness brings you to the place where the Beloved lives”  
-Rumi

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*Present is the moment just passed...*



Mummy  
&  
Abbu



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## **RESUME**

## English

This work concerns malignant myeloid hemopathies called classical BCR-ABL-negative Myeloproliferative Neoplasms (MPN) and include Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF). They result from the transformation of a multipotent hematopoietic stem cell (HSC) with hyperproliferation but no blockade of differentiation. The most common molecular defect is the acquired point mutation  $JAK2^{V617F}$  resulting into the activation of the cytokine receptor/JAK2 pathway. We have developed a mouse constitutive and a conditional  $JAK2^{V617F}$  knock-in (KI) mouse models. These animals developed a disease mimicking human PV evolving into secondary MF. They also displayed an age dependent increase in the total numbers of early hematopoietic cells (phenotype LK, LSK and SLAM: LSK/CD48-/CD150+). Using *In vivo* competitive repopulation assays we demonstrated that cells from KI origin outcompeted their WT counterparts and that a low number of  $JAK2^{V617F}$  KI SLAM cells propagates the disease. These results show that the sole  $JAK2^{V617F}$  mutation, without any additional mutations, is sufficient for disease phenotype and emergence. Using this KI mouse model, we tested the effect of interferon- $\alpha$  (IFN $\alpha$ ) treatment on MPN development. We found that IFN $\alpha$  treats the disease phenotype by blocking the propagation of early  $JAK2^{V617F}$  cells and eradicates disease-initiating cells, showing that IFN $\alpha$  could cure the disease in mice, as shown in some PV patients. Finally, we developed a new method combining the measurement of 46/1 SNPs and  $JAK2^{V617F}$  allele burdens in blood predicting the frequency of normal, heterozygous and homozygous  $JAK2^{V617F}$  clones in PV patients. This study suggested that IFN $\alpha$  preferentially targets the homozygous  $JAK2^{V617F}$  clone in PV patients suggesting a link between the levels of JAK2 signaling and the success of the IFN $\alpha$  response.

**KEYWORDS:** Myeloproliferative Neoplasms,  $JAK2^{V617F}$ , HSC, Interferon-  $\alpha$ , haplotype 46/1

## Français

Ce travail concerne des hémopathies myéloïdes malignes appelés Néoplasmes Myéloprolifératifs qui incluent les Polyglobulies de Vaquez (PV), les Thrombocythémies Essentielles (TE) et les Myélofibroses Primaires (MFP). Ces maladies résultent de la transformation d'une cellule souche hématopoïétique (CSH) avec hyperprolifération mais sans blocage de différenciation. Leur défaut moléculaire le plus fréquent est la mutation  $JAK2^{V617F}$  résultant dans l'activation de la signalisation des récepteurs aux cytokines utilisant JAK2. Au cours de ce travail, nous avons développé un modèle murin « Knock-In » (KI) constitutif et conditionnel pour la mutation  $JAK2^{V617F}$ . Ces animaux développent une maladie mimant la PV humaine évoluant vers la MF secondaire. Ces animaux présentent augmentation en fonction de l'âge du nombre de cellules immatures (phénotypes Lin-, LSK et SLAM: LSK/CD48-/CD150+). Dans un système compétitifs *in vivo* nous montrons que les cellules KI ont un avantage prolifératif dès le stade CSH et qu'un faible nombre de CSH peuvent déclencher la maladie. Ces résultats suggèrent que la mutation  $JAK2^{V617F}$  seule est suffisante pour (1) le phénotype et (2) l'émergence de ces maladies. Nous avons aussi testé l'effet de l'interféron- $\alpha$  (IFN $\alpha$ ) sur le développement des NMP en utilisant ces souris  $JAK2^{V617F}$  KI. Nous montrons que l'IFN $\alpha$  traite le phénotype de la maladie en bloquant la propagation des cellules KI dès le stade immature avec éradication des cellules souches néoplasiques, entraînant comme chez certains patients PV une rémission hématologique et aussi moléculaire. Enfin, en combinant l'analyse quantitative de l'haplotype 46/1 et de la mutation  $JAK2^{V617F}$  sur les cellules sanguines nous développons une nouvelle méthode prédictive de la fréquence des clones hétérozygotes et homozygotes  $JAK2^{V617F}$  chez les patients PV. Cette étude suggère que l'IFN $\alpha$  cible préférentiellement le clone homozygote  $JAK2^{V617F}$  et que sa réponse est fonction de l'intensité de la signalisation JAK2.

**MOTS CLÉS :** Néoplasies Myéloprolifératifs,  $JAK2^{V617F}$ , CSH, Interféron-  $\alpha$ , haplotype 46/1

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## **LIST OF ABBREVIATIONS**

2-HG = 2 hydroxyglutarate

$\alpha$ -KG = Alpha ketoglutarate

AML = Acute myeloid leukemia

AMMoL = Acute myelomonocytic leukemia

Ana = Anagrelide

ASXL1 = Additional sex-comb like 1

Allo-SCT = Allogeneic stem cell transplantation

ALL = Acute lymphoblastic leukemia

Bfn = Busulfan

BM = Bone marrow

BFU-E = Blast forming unit-erythroid

BCR-ABL = Break point cluster-Abelson kinase

bFGF = Basic fibroblast growth factor

CD = Cluster of differentiation

CDK = Cyclin dependent kinase

CFU-E = Colony forming unit-erythroid

CFU-GM = Colony forming unit-granulocyte macrophage

CCR = Complete cytogenic remission

CHR = Complete hematologic remission

CMR = Complete molecular remission

CTL = Cytotoxic T cells

CMP = Common myeloid progenitor

CLP = Common lymphoid progenitor

CMML = Chronic myelomonocytic leukemia

CML = Chronic myeloid leukemia

CEL = Chronic eosinophilic leukemia

CNL = Chronic neutrophilic leukemia

CSF3R = Colony stimulating factor 3 receptor

CBL = Casitas B-lymphoma

CDKi = Cyclin dependent kinase inhibitor

CFU-GEMM = Colony forming unit-granulocyte erythrocyte macrophage megakaryocyte

DC = Dendritic cells

DGS = De Guglielmo's syndrome

DNMT3a = DNA (cytosine-5)-methyltransferase 3A

EEC = Endogenous erythroid colonies

EZH2 = Enhancer of Zeste homology 2

ET = Essential thrombocythemia

Epo = Erythropoietin

F = Phenylalanine

FGFR1 = Fibroblast growth factor receptor 1

G = Guanine

GHR = Growth hormone receptor

G-6PD = Glucose-6 phosphate dehydrogenase

GMP = Granulocyte-macrophage progenitor

GM-CSF = Granulocyte macrophage-colony stimulating factor

G-CSF = Granulocyte-colony stimulating factor

GAS = IFN- $\gamma$  activated site

hmC = Hydroxymethyl Cytosine

HP- $\alpha$ 1 = Heterochromatin Protein-alpha 1

HSC = Hematopoietic stem cell

HU = Hydroxy Urea  
HSPC = Hematopoietic stem and progenitor cells  
HR = Homologous recombination

IL = Interleukin  
IFN $\alpha$  = Interferon alpha  
IGF-1 = Insulin-like growth factor receptor 1  
ISG = Interferon stimulated gene  
ISRE = IFN stimulated response element  
IGSF3 = IFN stimulated gene factor 3  
IFN- $\gamma$  = Interferon gamma  
IFN- $\alpha$  = Interferon alpha  
IKZF1 = IKAROS family zinc finger 1  
IDH1/2 = Isocitrate dehydrogenase 1/2

JH = Janus homology  
JAK2 = Just another kinase/Janus kinase 2

KI = Knock-In  
KO = Knock-Out  
KIT = Tyrosine-protein kinase

LSK = Lineage<sup>-</sup>Sca1<sup>+</sup>cKit<sup>+</sup>  
LT-HSC = Long term-hematopoietic stem cell  
LTC-IC = Long term culture-initiating cell  
LOH = Loss heterozygosity  
LD = Linkage disequilibrium  
LK = Myeloid progenitor

MEP50 = Methylosome protein 50  
MEP = Myelo-erythroid progenitor  
MPP = Multipotent progenitor  
MDS = Myelodysplastic syndrome  
MPD = Myeloproliferative disorder  
MPN = Myeloproliferative neoplasm  
MDM2 = Mouse double minute 2 homolog

NGS = Next generation sequencing  
NAC = N-acetylcystein  
NOD/SCID = Nonobese Diabetic/Severe Combined Immunodeficiency

PRMT5 = Protein Arg methyltransferase 5  
PV = Polycythemia vera  
PRC2 = Polycomb repressive complex 2  
PR-DUB = Polycomb repressive – deubiquitinase  
P3 = Proteinase 3  
PMF = Primary myelofibrosis  
Ph = Philadelphia  
PDGFRA = Platelet derived growth factor alpha  
PDGFRB = Platelet derived growth factor beta  
PIAS = Protein inhibitors of activated STAT  
PI3K = Phosphoinositol-3-Kinase

ROS = Reactive oxygen species  
RV = Retroviral

SM = Systemic mastocytosis  
SOCS = Suppressor of cytokine receptor  
SRC = SCID repopulating cells  
SNP = Single nucleotide polymorphism

ST-HSC = Short term-hematopoietic stem cell  
SLAM = Signaling lymphocyte activation molecules  
SCA-1 = Stem cell antigen-1

T = Thymine  
TGF- $\beta$  = Transforming growth factor beta  
TET2 = Ten eleven translocation 2  
T-reg = Regulatory T cells  
TG = Transgenic  
Tpo = Thrombopoietin  
TF = Transcription factor

UPD = Uniparental disomy

V = Valine

WHO = World health organization  
WBC = White blood cell  
WT = Wild type

XCIP = X chromosome inactivation pattern

Y = Tyrosine

## **INTRODUCTION**

## 1. Hematopoiesis – an overview

**B**lood is the most highly regenerative tissue with approximately  $10^{12}$  cells arising daily from adult human bone marrow (BM) and lymphoid tissues. The cells in the hematopoietic system are continually generated from a low number of self-renewing cells called hematopoietic stem cells (HSCs). As multi-potent HSCs, at the apex of hematopoietic system, differentiate they give rise to progenitors, precursors and finally, terminally differentiated cells with increasing lineage-restricted capacity down the hierarchy. This lineage relationship between stem cell, progenitors and mature cells form a complex road map that is guided by strict interplay of chromatin remodeling, transcription factors, signaling molecules and cytokine receptors. In order to perpetuate as well as differentiate, to sustain the homeostasis in hematopoietic system, HSCs maintain a balance between self-renewal, differentiation, proliferation and apoptosis.

### 1.1. Definition

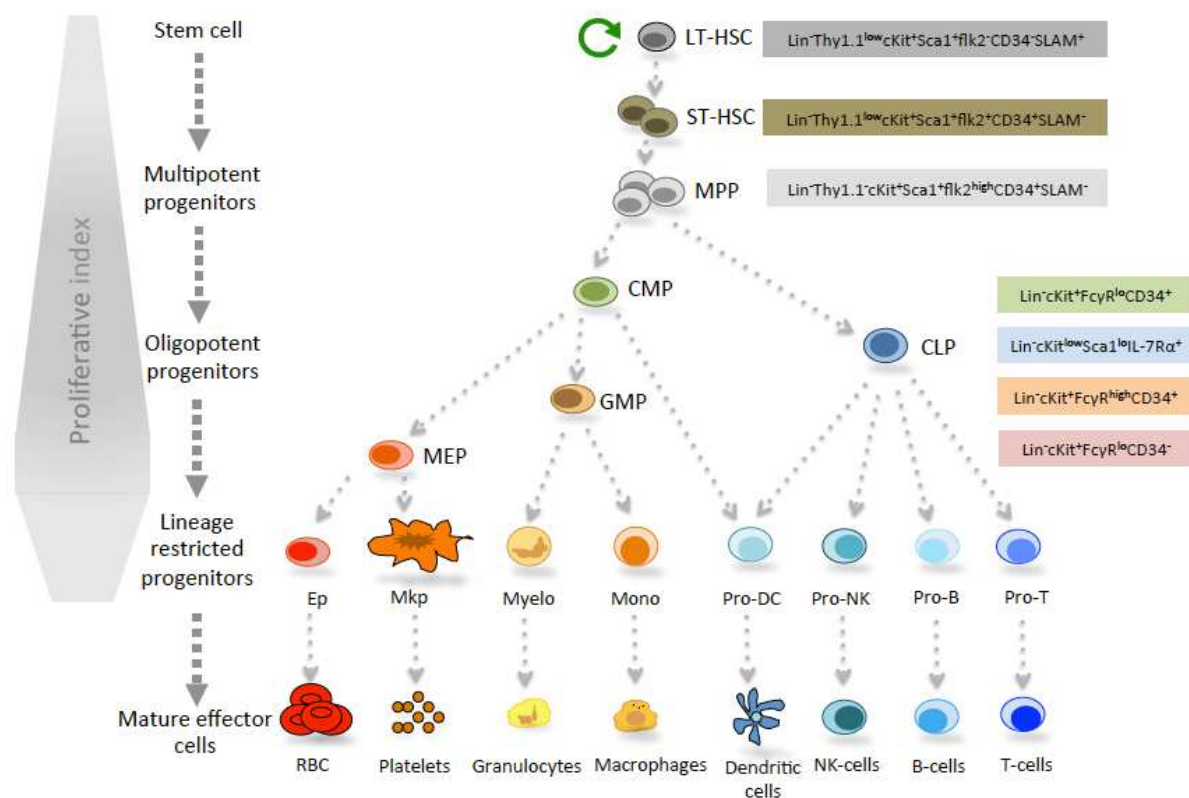
Hematopoiesis is the process of generating all functional effector blood cell types from HSC through successive differentiation events. These effector cells include at least 8 blood lineages i.e., erythrocytes, platelets, granulocyte, macrophages, dendritic cells, T cells, natural killer cells and B cells. Prenatally, hematopoiesis occurs in yolk sac then in liver and finally in BM while in adults it takes place primarily in BM and lymphoid tissues.

### 1.2. Hematopoietic hierarchy

HSC resides at the top of the hematopoietic pyramid. Murine HSC compartment is confined within lineage marker-negative, c-kit-positive and Sca-1-positive (LSK:  $\text{Lin}^- \text{c-kit}^+ \text{Sca-1}^+$ ) population and represents a very small fraction of cells of total BM with the capacity of self-renewal<sup>1,2</sup>. As the dormant HSC with most enriched self-renewing potential enters into an active cycling state they acquire the expression of CD34<sup>3</sup>. HSCs express Thy1.1 (CD90) at very low levels and the expression of Flk2 (also called Flt3 or CD135) tyrosine kinase differentiates the long term HSC (LT-HSC:  $\text{Thy1.1}^{\text{low}} \text{Lin}^- \text{c-kit}^+ \text{Sca-1}^+ \text{CD34}^- \text{flk}^-$ ) with strong self-renewal and differentiating potential, from the short term HSC (ST-HSC:  $\text{Thy1.1}^{\text{low}} \text{Lin}^- \text{c-kit}^+ \text{Sca-1}^+ \text{CD34}^+ \text{flk}^+$ ) with limited self-renewal and full multilineage differentiating potential<sup>4</sup>. Kiel *et al.* identified a more sophisticated set of SLAM family cell surface receptors (CD150 and CD48) to distinguish between most primitive and highly purified LT-HSC (SLAM:  $\text{Lin}^- \text{cKit}^+ \text{Sca1}^+ \text{CD150}^+ \text{CD48}^-$ ) from multipotent progenitors (MPP:  $\text{Lin}^- \text{cKit}^+ \text{Sca1}^+ \text{CD150}^- \text{CD48}^+$ ). In mice these SLAM cells compose ~0.0058% of total BM and single SLAM cell injection into lethally irradiated mice revealed that every 1 out of 2.1 SLAM cells has a long term multilineage reconstitution capacity<sup>5</sup>. ST-HSCs generate MPPs ( $\text{Lin}^- \text{Thy1.1}^+ \text{cKit}^+ \text{Sca1}^+ \text{flk2}^{\text{high}} \text{CD34}^+ \text{SLAM}^-$ )<sup>6</sup> with retained full lineage potential but limited self-renewal capacity. This appears to be the branching point in hematopoietic hierarchy giving rise to progenitors with differential and restricted differentiation capacity. MPPs give rise to two kinds of oligolineage-restricted progenitors with no self-renewal capacity: the common lymphoid progenitor (CLP:  $\text{Lin}^- \text{cKit}^{\text{low}} \text{Sca1}^{\text{low}} \text{IL-7R}\alpha^+$ ) with lymphoid potential<sup>7</sup> and the common myeloid progenitor within the  $\text{Lin}^- \text{cKit}^+$  (LK) population (CMP:  $\text{Lin}^- \text{cKit}^+ \text{Fc}\gamma\text{R}^{\text{lo}} \text{CD34}^+$ ) with myeloid potential that eventually give rise to megakaryocytic-erythroid progenitor (MEP:  $\text{Lin}^- \text{cKit}^+ \text{Fc}\gamma\text{R}^{\text{lo}} \text{CD34}^-$ ) and common granulocyte-macrophage



progenitors (GMP: Lin<sup>c</sup>Kit<sup>+</sup>FcγR<sup>hi</sup>CD34<sup>+</sup>)<sup>8</sup>. The CLPs have the capability to produce further lineage-restricted progenitors i.e., Pro-dendritic cells (DC), Pro-T cells, pro-natural killer cells (NK) and pro-B cells. Likewise, GMPs give rise to precursors that will eventually be able to produce granulocyte and macrophages. MEPs further differentiate to give megakaryocyte progenitors and erythroid precursors. Interestingly, both CLP and CMP carry the potential to generate pro-DC. This multilayered differentiation forms the base of hematopoietic pyramid: mature blood cells. Because these effector cells have very short life span, mature blood cell production is an ongoing process demanding the production of 1.5 X 10<sup>6</sup> blood cells every second in an adult human **Figure 1**.



**Figure 1. Hematopoietic pyramid** At the apex of pyramid, long term-hematopoietic stem cell (LT-HSC) asymmetrically divides to give rise to self-renewable HSC and short term-HSC (ST-HSC), which in turn gives rise to multipotent progenitor (MPP). MPP differentiates into oligopotent common myeloid (CMP) and common lymphoid progenitors (CLP). CMP further engages to produce megakaryocyte-erythroid (MEP) and granulocyte-macrophage progenitors (GMP). These oligopotent progenitors give rise to specific lineage restricted precursors i.e., erythroid (Ep), megakaryocytic (Mkp), myeloid (myelo), monocytic (mono), Dendritic (Pro-DC), Natural killer (Pro-NK), B-cells (Pro-B) and T-cells (Pro-T) precursors that finally differentiate into mature effector cells to form the base of pyramid. As cells assume their lineage fate down the hierarchy their proliferative potential also increases. Colored panels in right show surface markers use to isolate hematopoietic stem and progenitor cells. Inspired by Bryder et al. (2006) and Keil et al. (2005).

### 1.3. Lineage selection – a guide to hematopoietic road map

HSC differentiation is a unidirectional process in which it loses its pluripotency to assume the process of commitment. However, how the HSC makes decision whether to differentiate and acquire a lineage identity or to self-renew is largely unknown. This HSC differentiation to opt a lineage fate can be explained by different models. In ‘Stochastic model’<sup>9,10</sup> it is assumed that the cell’s intrinsic genetic and epigenetic factors as well as

external stimuli determines the cell's fate 'randomly', say cytokines, cytokine receptors and transcription factors (TFs) play a permissive role rather than instructive. In contrast, 'Determinism model'<sup>11</sup> emphasizes on the predetermined role of these factors and stimuli in HSC differentiation and lineage selection. As HSC differentiation is a highly complex stepwise process, for being conducive to differentiation process, it first enters in the state of 'priming' where chromatin remodeling allows the expression of lineage specific genes and repression of undesired genes<sup>12,13</sup>. These primed cells are now ready for the transcriptional response, expressing positive and negative TFs, under the influence of external stimuli i.e., binding of cytokines to lineage specific cytokine receptors<sup>14,15,16</sup>. Cytokines and their cognate receptors finely tune the regulatory pathways that eventually decide the fate of HSC.

#### **1.4. Homeostasis - self renewal, cell cycle and apoptosis**

Functional effector cells of hematopoietic system have very short life span demanding efficient homeostatic control mechanisms. In order to perpetuate as well as produce progeny, HSCs maintain a balance between self-renewal and differentiation through asymmetric cell division. However, the molecular mechanisms that control HSC self-renewal are poorly understood. Interestingly, several signaling pathways that are postulated to be involved in HSC self-renewal, like Hox genes<sup>17</sup>, Notch<sup>18</sup>, Sonic hedgehog and Wnt signaling pathways<sup>19,20</sup>, are also hypothesized to be associated with oncogenesis emphasizing on the fact that normal and cancer stem cell share the ability to self-renew.

Most of the HSC resides in G<sub>0</sub> phase of cell cycle<sup>21</sup>. The minimal proliferative pressure on HSC protects them from mutagenic hazards of DNA replication and damage-inducing metabolic side products. As HSC quits self-renewing potential and enters into differentiation it gradually becomes mitotically active. As the proliferative advantage increases down the hierarchy this add an advantage of fine-tuning the homeostasis of a given effector cell type.

Like other cell types, HSCs are also subjected to apoptosis and programmed cell death. In fact, overexpression of oncogene bcl-2 prevented apoptosis, increased LT-HSC frequency and radio-resistance in transgenic mice<sup>22</sup> showing the role of apoptosis in hematopoietic homeostasis.

Overall, hematopoiesis is a highly orchestrated process of producing and maintaining high turnover of all effector cell types from a single HSC under the control of intrinsic and extrinsic stimuli of hematopoietic system

## 2. Myeloproliferative neoplasms

The concept of disease with excessive proliferation of blood cells was already conceived when Hippocrates, *The Father Of Medicine*, (460-370 BC) and Galen, the most prominent physician after Hippocrates, (129-200 AD) postulated the *humoral theory*. ‘Blood’ is one of the four humors (three being phlegm: white blood cells, yellow bile: serum and black bile: deoxygenated red blood cells) and ‘plethora’ is the imbalance of humors with blood dominating over the others<sup>23</sup>. Since then, many studies to understand the origin, evolution, diversity and complexity of blood malignancies have shown that this *plethora* is a result of alteration in genetic and epigenetic factors that perturb the key process such as self-renewal, proliferation and differentiation of cells.

### 2.1. Myeloid neoplasms

Myeloid neoplasms (MN) are clonal HSC disorders of myeloid cell hyper proliferation. MN can be categorized into three broad clinicopathological groups: acute myeloid leukemia (AML), myelodysplastic syndrome (MDS) and myeloproliferative neoplasm (MPN).

AML are characterized by proliferation of blast cells, principally in marrow and resulting in impaired production of normal blood cells e.g. acute myelomonocytic leukemia (AMMoL) with excess production of myeloblasts and monoblasts.

MDS, on the other hand, are characterized by ineffective hematopoiesis, dysmorphogenesis of myeloid cells and cytopenias e.g. refractory cytopenias with multilineage dysplasia (RCMD) that is characterized by cytopenias and dysplasia in more than one myeloid lineage and <1% blast cells in blood stream.

In contrast to AML and MDS, MPN are disorders with increased numbers of functional and terminally differentiated myeloid elements e.g. polycythemia vera in which red blood cells are over produced.

However, such classification is not precise; as some patients present with symptoms that overlap MPN and MDS and, they are assigned to ‘MPN/MDS overlap’ subgroup e.g. chronic myelomonocytic leukemia (CMML) with increased numbers of monocytes: characteristic of MPN and dysplasia of monocytes and granulocytes: characteristic of MDS.

Moreover, both MDS and MPN have a propensity to evolve into AML.

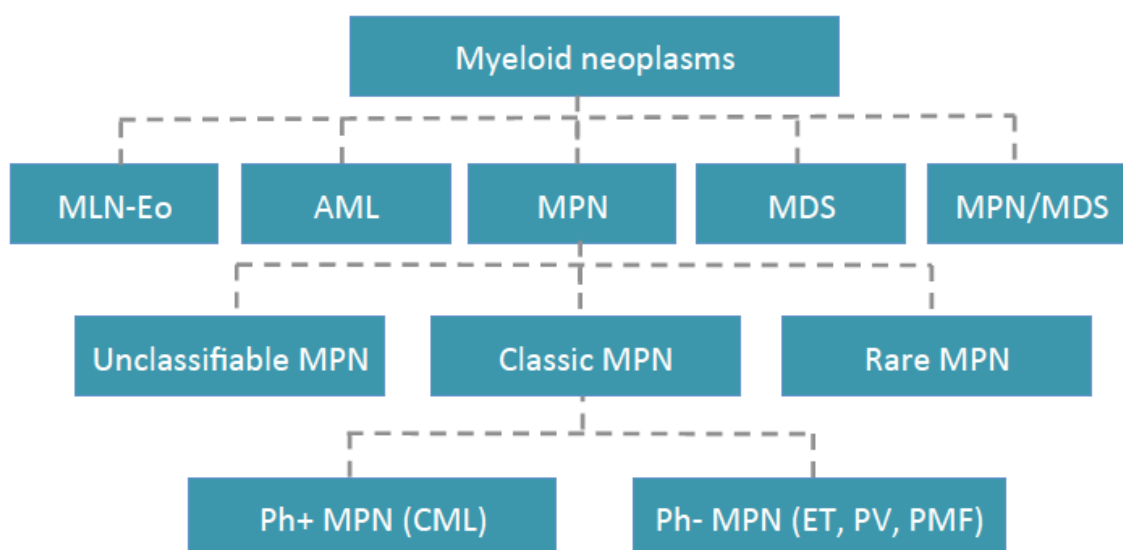
### 2.2. Classification – from the age of Dameshek to the epoch of genome

Chronic myeloid leukemia (CML), primary myelofibrosis (PMF), polycythemia vera (PV), Di Guglielmo’s syndrome (DGS) and essential thrombocythemia (ET) were already recognized in early 19<sup>th</sup> century as distinct disorders of hyper proliferation of three main myeloid lineages but predominate in one of them: the granulocytic lineage in CML, the megakaryocytic/granulocytic lineages in PMF, the erythroid lineage in PV and DGS and the megakaryocytic/platelet lineage in ET.

Proper classification of myeloproliferative disorder (MPD) began when in 1951 William Dameshek has highlighted the overlapping clinical and laboratory features of these entities. He argued that although erythrocytosis is the key characteristic of PV, PV is also presented with ‘pancytosis’, over production of erythroid, megakaryocytic and granulocytic lineages. Moreover, PV patients often display erythroblasts in

peripheral blood, BM fibrosis and splenomegaly mimicking PMF. Considering these disorders distinct yet closely related Dameshek grouped them for the first time under the term ‘myeloproliferative disorders’ (MPD)<sup>24</sup>. Over the time, DGS was recognized as erythroid leukemia and the rest of the members remained under the umbrella of classic MPDs. In 1960, with the discovery of Philadelphia (Ph) chromosome<sup>25</sup>, classic MPDs were further sub-grouped into Ph positive MPD (CML) and classic Ph negative MPD (PV, ET and PMF).

In 2008, the WHO has revised its classification<sup>26</sup> of myeloid malignancies and coined the term ‘myeloproliferative neoplasm’ (MPN). Furthermore, it has sub-categorized the myeloid malignancies into 5 well-defined groups: 1-Myeloproliferative neoplasms (MPNs), 2-Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of PDGFRA, PDGFRB, and FGFR1, 3-MDS, 4-MPN/MDS and 5-AML **Figure 2**.



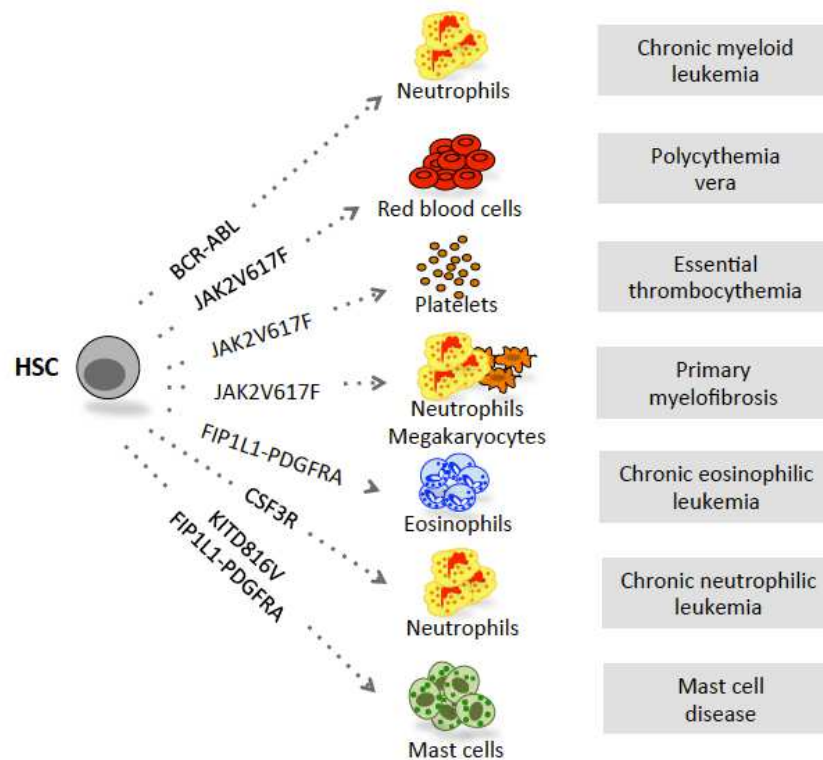
**Figure 2. 2008 WHO classification of myeloid neoplasm** Myeloid neoplasms are classified into five broad categories: 1- Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of PDGFRA, PDGFRB and FGFR1 (MLN-Eo), 2- acute myeloid leukemia (AML), 3- myeloproliferative neoplasm (MPN), 4- myelodysplastic syndrome (MDS) and 5. MPN/MDS overlaps. MPN are further divided into rare MPN, classic MPN and unclassifiable MPN. Classic MPN encompass Philadelphia positive chronic myeloid leukemia (Ph+ CML) and Philadelphia negative (Ph-) MPN i.e., essential thrombocythemia (ET), polycythemia vera (PV) and primary myeloid fibrosis (PMF).

### 2.3. Main etiological factors in MPN

MPNs are acquired and clonal myeloid malignancies of HSC origin with the over production of mature myeloid cells due to cytokine hypersensitivity. The group comprises of Ph positive CML, Ph negative MPN (PV, ET and PMF), less frequent non-classic MPN (CNL, CEL/NOS, and MCD) and unclassifiable MPN.

John Hughes Bennett has reported CML as a new clinical entity with hypertrophy of spleen and liver in 1845<sup>27</sup> and X-linked glucose-6-phosphate dehydrogenase (G-6PD) locus analysis demonstrated its clonal hematopoietic stem cell origin<sup>28</sup>. CML's etiological factor, the fusion tyrosine kinase ‘BCR-ABL’<sup>29</sup>, resulting from a Philadelphia translocation between chromosome 9 and 22 ; t(9;22)(q34;q11)<sup>25,30</sup>, was the first and long time unique, constitutively active mutated kinase identified and characterized in MPNs. Since its discovery, BCR-

ABL was thoroughly studied in in-vitro and in-vivo systems and shown to transform mouse BM derived IL-3 dependent cells and to induce CML-like disease in retrovirally transduced BM transplantation mouse models<sup>31-33</sup>. Following the discovery of oncogenic translocation in CML many other fusion tyrosine kinases including fusions of platelet derived growth factor beta (PDGFβ) in CMML<sup>34</sup> and PDGFRα in SM and CEL<sup>35</sup> were later unveiled. The fact that KIT tyrosine kinase is highly expressed in mast cells led to the discovery of KITD816V mutant allele in SM<sup>36</sup>. All these observations showed that MPNs are tyrosine kinase disorders.



**Figure 3. Main etiological factors in MPN** A predominate terminally differentiated myeloid lineage is involved in each clinically distinct MPN. Activated alleles resulting in constitutive tyrosine kinase signaling are identified in each disorder. HSC: hematopoietic stem cell, BCR-ABL: Break point cluster region-Abelson tyrosine kinase, JAK2: Janus kinase, FIP1L1-PDGFR: FIP1-like-1-Platelet derived growth factor receptor, CSF3R: Colony stimulating factor 3 receptor, KIT: Tyrosine-protein kinase.

Classic Ph- MPNs are closely linked diseases that were initially grouped on the basis of their shared clinical and laboratory features. Ph- MPNs also display deregulated clonal myeloid proliferation as observed in other MPNs harboring constitutively active tyrosine kinases. Inhibition of endogenous erythroid colonies (EEC) with imatinib mesylate<sup>37</sup> (a tyrosine kinase inhibitor that also inhibits the kinase activity of ABL, KIT and PDGFR) and JAK2 inhibitor AG490<sup>38</sup> suggested a tyrosine kinase as an etiological factor involved in signaling of myeloid cells. Moreover, because ET can transform into PV and PV into MF, their pattern of clinical evolution strongly suggested a common etiological factor involved in signaling through different receptors of myeloid lineage in all three Ph- MPNs. In 2005 several teams have published the discovery of a somatic guanine-to-thymine (G>T) mutation encoding a valine-to-phenylalanine substitution at position 617 (V617F) in the auto inhibitory JH2 (JAK homology 2) domain of non-receptor tyrosine kinase JAK2. The mutation JAK2<sup>V617F</sup> results

in the abrogation of auto inhibition leading to constitutive activation of kinase JAK2<sup>39-43</sup> (Structure and functional consequences of JAK2 are discussed in detail in chapter 3).

Recently point mutations in colony stimulating factor 3-receptor (CSF3R) gene have been identified in patients suffering either CNL or aCML. These mutations lead to preferential downstream kinase signaling through SRC family-TNK2 or JAK kinases and possess differential sensitivity to kinase inhibitors<sup>44</sup> **Figure 3**.

## **2.4. Classic Ph- MPNs**

### **2.4.1. Main molecular characteristics of Ph- MPN**

In past two decades, causative lesion in classic Ph+ CML and rare MPNs, like CMML, SM and CEL, were identified to be mutated tyrosine kinases. These constitutively active tyrosine kinases like BCR-ABL in CML and FIP1L1-PDGFR $\alpha$  in SM are found to activate several signaling pathways like STATs, MAPK and PI3k which lead to constitutively active cell survival, proliferation and differentiation pathways. Classic Ph- MPNs also share these features and the discovery of JAK2<sup>V617F</sup> in a tyrosine kinase JAK2 as a genetic cause in more than half of Ph- MPN patients has provided a great deal of knowledge about molecular pathogenesis of this disease.

#### **2.4.1.1. Ph- MPNs are acquired clonal HSC disorders**

In 1970s, X-chromosome linked polymorphism studies in women have already demonstrated that Ph- MPN are clonal disorders with the involvement of multiple myeloid lineages and, therefore, have probable multipotent stem cells origin<sup>45-47,48</sup>. The presence of mutant JAK2<sup>V617F</sup> allele in all the myeloid lineages supported the notion that this clonal mutation has occurred at the apex of hematopoietic hierarchy and also, the absence of mutation in non-hematopoietic cells showed that mutation is somatically acquired<sup>40</sup>. Indeed, just after the discovery of JAK2<sup>V617F</sup>, Jamieson *et al.* investigated for the hematopoietic compartment that harbor the mutation using flow cytometry technique. They found that JAK2<sup>V617F</sup> is not only present in myeloid progenitors (CMP, GMP and MEP) but also in HSC compartment (Lin-CD34+CD38-CD90+) of PV patients<sup>49</sup>.

However, this mutation is also reported in mature lymphoid lineage (B cells, NK cells and very rarely in T cells) in a subset of PV and PMF patients<sup>66</sup>. This suggests, first, the cell that has acquired JAK2<sup>V617F</sup> mutation is capable of differentiating in all hematopoietic lineages and second, this mutation renders a downstream selective advantage unique to myeloid lineage, likely due to the use of JAK2 by cytokine receptors of these lineages. Interestingly, the subsets of JAK2<sup>V617F</sup> negative ET and PMF patients also exhibited clonal myelopoiesis pointing towards the yet-unknown genetic alterations that phenocopy JAK2<sup>V617F</sup> and contribute to MPN pathogenesis<sup>52</sup>.

#### **2.4.1.2. Ph- MPNs are characterized by mature myeloid cell hyper proliferation**

JAK2 has shown to play a crucial role in pre- and post-natal hematopoiesis in mouse models<sup>53,54</sup>. Mice knockout for JAK2 (JAK2<sup>-/-</sup>) are embryonically lethal at day 12.5 of gestation due to failure of definitive erythropoiesis. The phenotype of JAK2<sup>-/-</sup> embryos was comparable to that of EpoR null or Epo null mice, showing a non-redundant function of JAK2 in Epo-R signaling<sup>55</sup>. Moreover, fetal liver cells from JAK2<sup>-/-</sup> embryos failed to respond IL-3, GM-CSF, IL-5 and Tpo, but not to G-CSF, in colony formation assays demonstrating the essential role of JAK2 in signalization via receptors of these cytokines. Furthermore, signaling of type II interferon

receptor (IFN-gamma) but not type I interferon receptor (Interferon-alpha) was abolished showing their dependence on JAK2.

Cytokine independent or hypersensitive growth of hematopoietic progenitors is a cardinal feature of ET, PV and PMF. Endogenous erythroid colonies (EECs)<sup>56,57</sup> and endogenous megakaryocyte colonies (EMCs)<sup>58</sup> are evidenced in these patients along with the hypersensitivity to IGF-1, IL-3, GM-CSF, Epo and Tpo<sup>59-62</sup>. The mutation JAK2<sup>V617F</sup> renders the tyrosine kinase JAK2 an authority to constitutively auto phosphorylate<sup>41</sup> with increased kinase activity<sup>43</sup>. To check whether this constitutive auto phosphorylation was responsible for hematopoietic transformation in PV patients, JAK2<sup>V617F</sup> was expressed in BaF3 and FDC-P1 primary cell lines, both dependent on IL-3 for proliferation, but they were not resulted into cytokine independent growth. However, co-expression of EpoR, a homodimeric type I receptor, transformed these cell lines to cytokine independency<sup>39,41</sup>. Because the megakaryocytic and granulocytic lineages are also involved in Ph- MPNs and their proliferation is also mediated by homodimeric type I receptor it was worth investigating the role of JAK2<sup>V617F</sup> in signalization via Tpo receptor (Mpl) and granulocyte-macrophage colony stimulating factor receptor (GCSFR). Indeed like EpoR, co-expression of JAK2<sup>V617F</sup> with Mpl or GCSFR transformed the BaF3 cell line<sup>63</sup>. Overall, mutant tyrosine kinase JAK2<sup>V617F</sup> renders a proliferative advantage to myeloid cells via signalization through homodimeric type I receptors: EpoR, Mpl and GCSFR, which are present on erythroid, megakaryocyte and granulocyte lineages, respectively.

Constitutive activation of JAK2 results in activation of downstream signaling pathways i.e., JAK-STAT, MAPK/ERK and PI3/AKT, which are crucial to cell survival and proliferation<sup>39,41-43,63</sup>.

## 2.4.2. Main entities in Ph- MPN

### 2.4.2.1. Essential thrombocythemia

Long before its recognition as Dameshek defined classic MPD; Emil Epstein and Alfred Goedel already formally described ET as ‘hemorrhagic thrombocythemia’ in 1934. XCIPs studies by polymerase chain reaction (PCR) demonstrated that, in a large proportion of patients, ET can occur as clonally heterogeneous disease<sup>64,65</sup> and sometimes clonality is only restricted to megakaryocytic lineage<sup>65</sup>.

Clinically, ET is featured with vasomotor symptoms: thrombosis, headache, dizziness, syncope, acrocyanosis, tingling and visual changes, however, 25-35% cases are asymptomatic and are diagnosed accidentally. In laboratory, ET is characterized by thrombocytosis and BM megakaryocyte hyperplasia with hyperlobated nuclei without associated erythrocytosis and leukoerythroblastosis. Megakaryocyte precursors show hypersensitivity to thrombopoietin (TPO)<sup>58</sup>. Mild splenomegaly may occur in about 40% of patients. JAK2<sup>V617F</sup> is the major molecular lesion of ET with a frequency of 55%<sup>39-43</sup> **Figure 4 and 5**. Because this mutation is also present in other MPNs, the 2008 WHO JAK2<sup>V617F</sup> positive ET diagnostic criteria requires the presentation of thrombocytosis with the exclusion of other JAK2<sup>V617F</sup> positive MPNs diagnostic proofs.

Deregulation of megakaryocyte lineage in ET provided the rationale to screen thrombopoietin receptor (MPL), which led to the discovery of the mutations of codon 515 (MPLW515K, MPLW515L and MPLS505N) in minor sub-set of ET patients<sup>66-68</sup>. However, the etiology of large proportion of ET patients without JAK2 or MPL mutations still remains unknown.

The median age at diagnosis is 65-67 years but it can occur at any age with the disease incidence of 1.5/100,000 cases in the general population<sup>69</sup>. 5-6% ET evolve into PV after 2.5 years<sup>70</sup> and 3% ET after 5 years, 8% ET after 10 years and 15% ET after 15 years evolve into MF<sup>71</sup>. Transformation to AML is a life-threatening complication in around 0.6-6% of cases<sup>72</sup> **Figure 6**. Median survival is found to be 20 years in ET patients. Risk of thrombosis and major hemorrhage is shown to be 6.6%<sup>73</sup> and 0.33% patients /year<sup>74</sup>, respectively.

### **Polycythemia Vera**

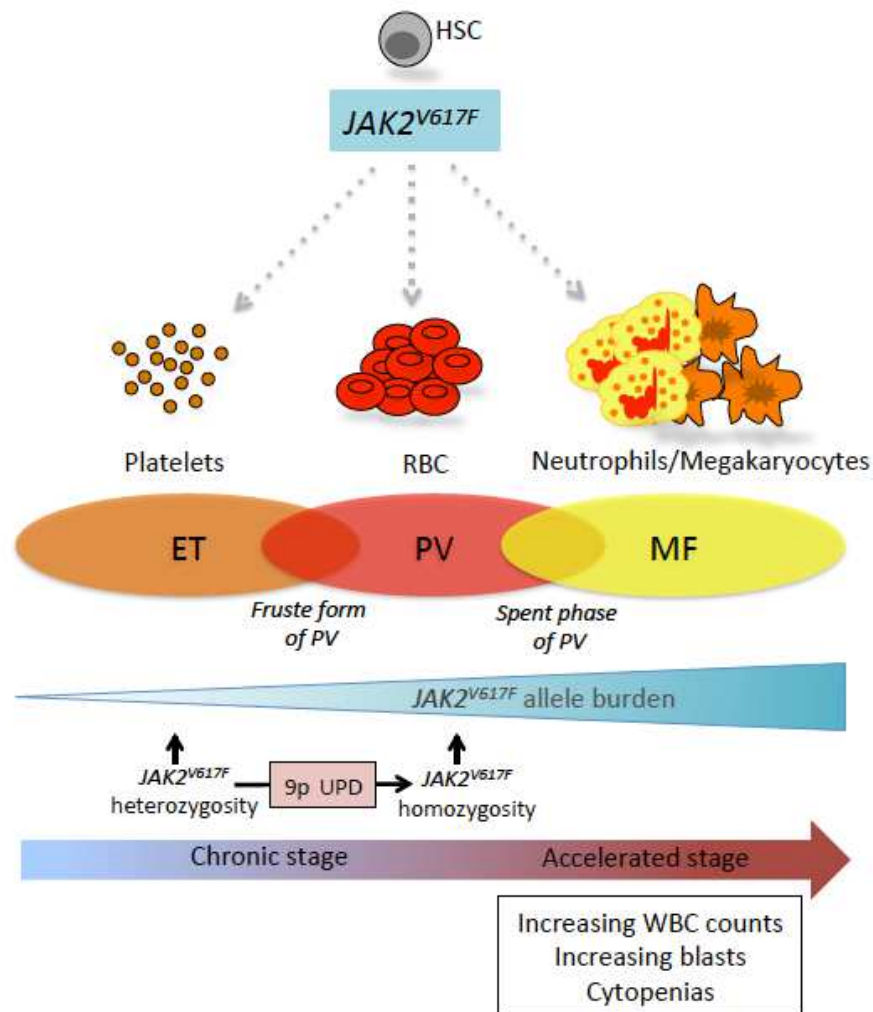
Initially, Louis Henri Vaquez, a French physician, described polycythemia as ‘maladie de Vaquez’ in 1892. Later on, in 1903, William Osler, a renowned physician, presented a descriptive landmark of this disease as chronic cyanosis, splenomegaly and erythrocytosis: a new clinical entity.

PV patients are often presented with headache, pruritus, vaso-occlusive symptoms like tinnitus, paresthesia and erythromelalgia, and in some patients, splenomegaly is present with its degree depending upon the stage of disease. High hematocrit (Hct), erythrocytosis and increased hemoglobin (Hb) are the Sine-qua-non of PV. BM is hyper cellular due to tri-lineage hyperplasia (RBC, granulocytes and megakaryocytes) with morphologically normal looking erythroid and myeloid precursors but hyper lobulated loosely clustered megakaryocytes. A variable increase in reticulin fibers is also observed specially over disease progression.

An important finding that erythroid progenitors from BM of PV patients are capable of growth without added exogenous erythropoietin (Epo)<sup>56</sup>, laid the basis of the idea that suspected etiological factor is likely to be present in cytokine regulation. Mutation in JAK2, a tyrosine kinase down stream to erythropoietin receptor (EpoR) signaling, finally explained the molecular etiology in almost 95% of PV patients<sup>39-42</sup> **Figure 4 and 5**. Moreover, chromosome 9 uniparental disomy (UPD 9), resulting in the duplication of mutant allele, is a common phenomenon in PV (~70%), in contrast to ET, patients<sup>75</sup>. Presence of JAK2<sup>V617F</sup> in majority of PV patients has influenced its diagnosis and it is reflected in the 2008 WHO diagnostic criteria guidelines. Other mutations leading to constitutive activation of signaling pathways are also discovered in exon 12 of JAK2 in JAK2<sup>V617F</sup> negative PV patients with an overall frequency of around 3% in PV.

The annual incidence of PV is ~2/100,000 cases in general population<sup>69</sup> and it can occur in all ages but risk increases over 60 years. Natural evolution of PV into MF is noted in 30% of patients after 10-12 years of diagnosis<sup>76</sup>. 2-5% of PV patients develop AML in the course of disease progression<sup>77</sup>. Although genotoxic therapies increase the risk of AML transformation, it also occurs in treatment naïve patients emphasizing on its natural evolution **Figure 5**. PV patients bear the risk of thrombotic events specially over 60 year and with previous history of such complication<sup>78</sup> **Figure 6**. Leukocytosis may impact thrombotic events maybe considered as an average risk factor<sup>79</sup>. Median survival approaches to 20 years in PV patients.





**Figure 4. Schematic representation of  $JAK2^{V617F}$ -positive MPN** The mutation  $JAK2^{V617F}$  appears in hematopoietic stem cell (HSC) and results in clonal proliferation of terminally mature myeloid cells. This mutation is found in three clinically distinct MPN i.e., essential thrombocythemia (ET), polycythemia vera (PV) and myelofibrosis (MF) that are characterized by the hyper proliferation of platelets, red blood cells (RBC) / neutrophils and neutrophils/megakaryocytes, respectively. However, a small proportion of ET may overlap PV phenotype depicting the *fruste form* of PV and PV can evolve into myelofibrosis by passing through a *spent phase* featuring some symptoms MF. Of note, PV is also characterized by hyperproliferation of granulocytes and platelets in contrast to erythrocytosis that is characterized by the sole hyperproliferation of erythroid cells.  $JAK2^{V617F}$  heterozygosity is associated with ET and  $JAK2^{V617F}$  homozygosity due to chromosome 9 uniparental disomy (9p UPD) is associated with PV.  $JAK2^{V617F}$  allele burden increases across the spectrum of Ph- MPN i.e., lowest in ET and highest in MF. These disorders form a continuum where ET and PV depicts the chronic phase of disease and accelerated phase may encompass the transformation of ET and PV into myelofibrosis along with other manifestations such as increasing white blood cells (WBC), blasts and neutropenia and thrombocytopenia.

#### 2.4.2.2. Primary myelofibrosis

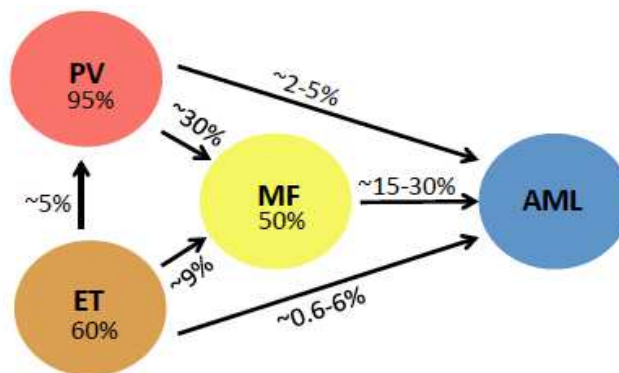
In 1879, Gustav Heuck presented the pathologic description of primary myelofibrosis (PMF) in his two patients as massive splenomegaly, circulating nucleated red blood cells and increased numbers of morphologically abnormal leukocytes, marrow fibrosis, osteosclerosis and extensive extra medullary hematopoiesis<sup>27</sup>.

PMF patients may present with dyspnea, fatigue, night sweats, cachexia, fever and bleeding. More than 90% of patients present splenomegaly. However, 30% of patients are asymptomatic at the time of diagnosis. PMF patients may exhibit anemia, leukocytosis and/or thrombocytosis. Initial stage PMF, 'pre-fibrotic stage', is difficult to diagnose because of thrombocytosis that resembles ET. The *sine-qua-non* of myelofibrosis, whether

PMF or post ET/PV MF, is accumulation of reticulin fibers in marrow accompanied by hyper cellular BM with expanded left-shifted granulopoiesis and decreased erythropoiesis. Megakaryocyte hyperplasia is one of the major criteria in PMF diagnosis. BM harbors densely packed clustered of cytologically bizarre megakaryocytes with hypolobated nuclei. Peripheral blood smears show leukoerythroblastosis and anisopoikilocytosis with teardrop shaped red cells **Figure 4**.

PMF shares etiological factor of ET and PV i.e.  $JAK2^{V617F}$  in around 50% of PMF patients **Figure 5**. Although the discovery of  $JAK2^{V617F}$  promised a unifying mechanism to be targeted effectively, recent molecular studies have identified several other molecular defects, for example in MPL, SOCS1, TET2, EZH2 and IDH1/2, which have provided further opportunity to comprehend the molecular underpinnings of PMF pathophysiology. However, still a large subset of PMF patients harbors a yet-unknown causative factor of disease. Clonal myeloproliferation in PMF is followed by secondary stage with stromal changes and abnormal cytokine profiles that are believed to provoke an inflammatory response and may contribute to the clinical phenotype, including bone marrow fibrosis, angiogenesis, extra medullary hematopoiesis, constitutional symptoms and cachexia<sup>80</sup>.

The annual incidence of PMF is 1.5 cases per 100,000 people and it commonly occurs over the age of 60 years with same frequency in both genders<sup>76</sup>. Around 15-30% of PMF patients may transform into AML<sup>77</sup> **Figure 5 and 6**. Median survival is approximately 6 years. It was not established until recently that aged PMF patients (over 60 years) and those with leukocytosis and  $JAK2^{V617F}$  are at an elevated risk of cardiovascular thrombotic events<sup>81</sup>. In a recent cytokine profiling study of PMF patients certain specific plasma cytokine signatures are shown to be related with overall and leukemia free survival<sup>82</sup>.

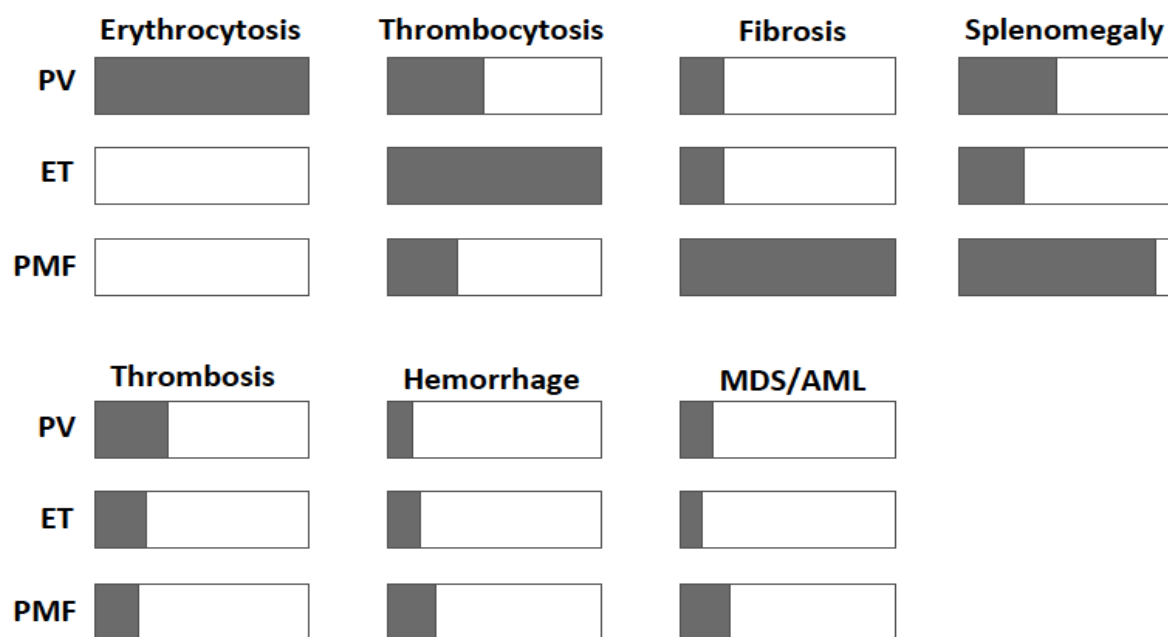


**Figure 5. Natural evolution of Ph- MPN** Around 95% polycythemia vera (PV) patients and 50-60% essential thrombocythemia (ET) and myelofibrosis (MF) patients harbor  $JAK2^{V617F}$  mutation (VF). In the natural course of disease progression ET may evolve into PV and PV into secondary myelofibrosis and all three of them can directly transform into acute myeloid leukemia (AML). Value on each flash represents the approximate percentage of each type of progression.

As Dameshek speculated 64 years ago, these classical Ph- MPNs share core cardinal features and form a spectrum of closely related disease entities. Although erythrocytosis is the hallmark of PV, ET and PMF share thrombocytosis. Likewise, splenomegaly is a common finding in PMF but is also present in approximately 30% ET and PV patients. Marrow fibrosis is sine-qua-non of PMF but ET and PV patients may accumulate reticulin fiber over the natural course of disease evolution. Both ET and PV have the propensity to develop myelofibrosis and all three of them can transform into AML **Figure 5**.

Discovery of the mutation  $JAK2^{V617F}$  in the epoch of genome has proved to be an important twist in the story of

Ph- MPN. The finding of JAK2<sup>V617F</sup>, on one hand, simplified the concept of relatedness of three diseases under the umbrella of classic Ph- MPN and, on the other hand, raised the question of how one mutation can cause three distinct disease entities. JAK2<sup>V617F</sup> positive ET as compared to JAK2<sup>V617F</sup> negative ET present many features resembling PV such as high hemoglobin, leukocytosis, BM erythropoiesis and granulopoiesis, low serum Epo levels and higher rates of venous thrombosis<sup>83</sup> thus, JAK2<sup>V617F</sup> positive ET depicts the ‘fruste form’ of PV. JAK2<sup>V617F</sup> homozygosity is an event mostly associated with PV as compared to ET suggesting the contribution of JAK2<sup>V617F</sup> allele burden in disease phenotype and evolution<sup>75,84</sup>. Evolution of PV is also stepwise with splenomegaly in absence of myelofibrosis, cytopenias of one or several cell lines, unabated erythrocytosis and myeloid metaplasia being the ‘spent phase’ of PV and a propensity to transform into secondary myelofibrosis or AML<sup>85</sup>.



**Figure 6. Overlaps in clinical presentation and complications in classic Ph- MPN** Clinical features and complications are shared over the spectrum of Ph- MPN. Grey bars represent the proportion of specific features and complications found overlapping in essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF). Leukocytosis often present in PV and PMF patients and cytopenia of some PMF patients is not represented. (Credit: Jean-Jacques Kiladjian, Hematology, 2012)

In clinical studies, the JAK2<sup>V617F</sup> allele burden is found to increase across the spectrum of these three disease entities, with the lowest level in ET and highest level during fibrotic transformation<sup>86,87</sup>. All these observations suggest that JAK2<sup>V617F</sup> positive ET, PV and PMF form a continuum model being three stages of Ph- MPN: chronic stage ET and PV precede PMF in the accelerated stage **Figure 4**. JAK2<sup>V617F</sup> provided a simple molecular profile of Ph- MPNs, nevertheless, around 50% JAK2<sup>V617F</sup> negative ET and PMF demand a more complex molecular signature predicting additional events in the pathogenesis and evolution of Ph- MPNs.

### 3. The JAK<sup>V617F</sup> mutation

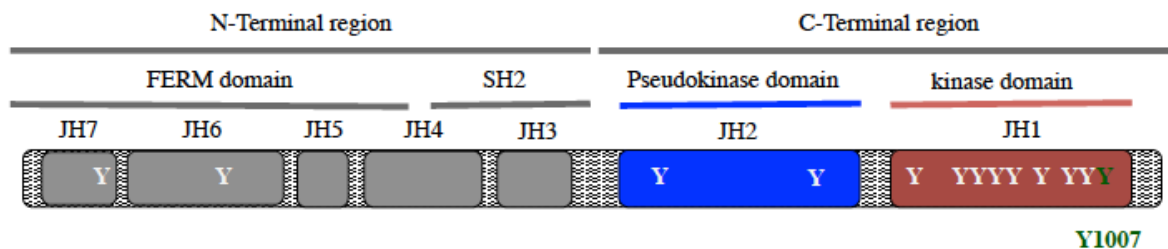
#### 3.1. JAK2 as a signaling molecule

Just another kinase (JAK) or *Janus* kinase, as later named after a roman God, is a family of non-receptor tyrosine kinases crucial for cytokine receptor, devoid of intrinsic kinase activity, mediating signal transduction of variety of hematopoietic and immune responses. Ligand binding to their cognate receptor induces auto/trans phosphorylation of JAKs, which in turn triggers a cascade of downstream signalizations that lead to the cell survival, differentiation and proliferative pathways. The phosphorylation process is tightly regulated and abnormalities that lead to constitutive phosphorylation result in deregulated myeloid cell proliferation, a hallmark of MPNs.

##### 3.1.1. JAK2 structure and cytokine receptor interaction

JAK is one of ten families identified as non-receptor tyrosine kinases, comprising of four family members JAK1, JAK2, JAK3 and TYK2. In human, JAK1 gene is located on ch. 1p31.3, JAK2 on ch. 9p24, JAK3 on ch. 19p13.1 and TYK2 on ch. 19p13.2. They are large proteins of approximately 120-140 KDa. JAK1, JAK2 and TYK2 are ubiquitously expressed while JAK3 is restricted to hematopoietic tissues and they are localized to endosomes and beneath the plasma membrane in close proximity of their cognate receptors.

Structurally, JAKs have seven well-defined homologous domains (JH1-JH7) **Figure 7**.



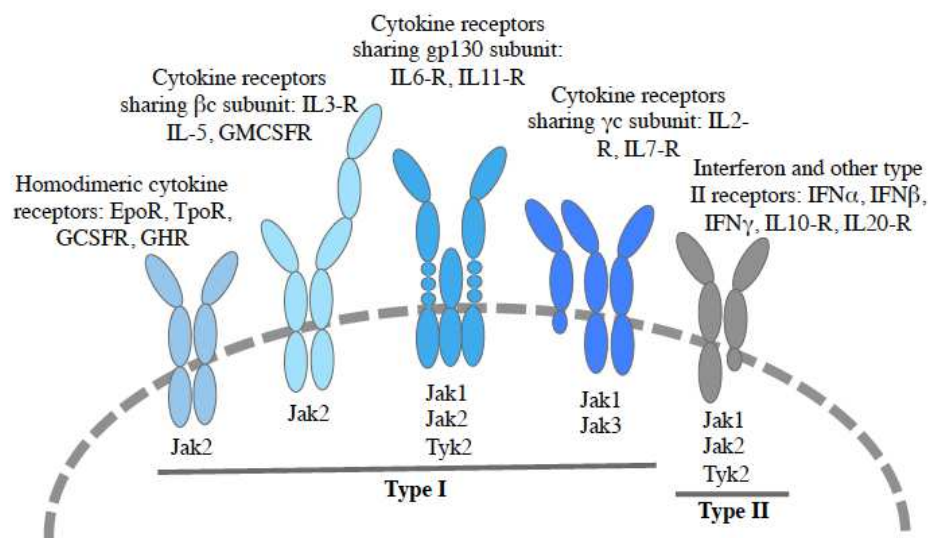
**Figure 7. Structure of JAK2 protein** The protein JAK2 encodes seven well-conserved Janus homology (JH) domains namely JH1-JH7. JH1 is a kinase domain required for phosphorylation of target proteins, JH2 is a pseudokinase domain that is implicated in tyrosine kinase auto-inhibition. Src-homology domain (SH2) interacts with diverse signaling intermediates and FERM domain which mediates receptor interaction. Y represents different tyrosine residues. Kinase domain is activated upon phosphorylation of tyrosine 1007 (Y1007).

JH1 domain at C-terminal possess all the characteristic features of catalytic tyrosine kinase including tyrosine residues in the activation loop region, the canonical GXGXXG motif in the nucleotide-binding loop and a conserved aspartic acid residue involved in the phosphotransfer reaction in the catalytic loop. The JH1 domain is activated upon phosphorylation of tyrosine 1007 (Y1007) situated in the activation loop of JAK2 protein<sup>88</sup>. Next to JH1 domain is its highly homologous JH2 domain and is presumed to be catalytically inactive due to lack of residues required for tyrosine kinase activity, therefore called pseudokinase or kinase-like domain. JH1 and JH2 domain interaction studies predicted an auto regulation mechanism where JH2 inhibits JH1 tyrosine kinase activity<sup>89</sup>. However, recently it has been shown that it actually possess a kinase activity to phosphorylate two negative regulatory site in JAK2: Ser523 and Tyr 570. Mutations that block JH2 activity, like JAK2V617F,

indeed reduce phosphorylation of Tyr570 in MPN patient cells suggesting that loss of JH2 activity may contribute to the pathogenesis of MPN<sup>90</sup>. Moreover, crystal structure of JH2 domain revealed that V617F induces a structural change so that JH2 adopts the fold of prototypical pretein kinase and binds to Mg-ATP. This configuration of JH2 helps trans-phosphorylation of JH1. These observations point towards JH2 as a novel therapeutic target in MPN treatment<sup>91</sup>.

JH3 and JH4 domain form SH2 domain homologous to Src-homology-2 domain. At N-terminal JH5-JH7 domains make FERM (Band-4.1, ezrin, radixin and meosin) domain that is implicated in mediating interactions with cytokines receptors. Moreover, FERM domain of JAK2 is also implicated in cell surface localization of Mpl and EpoR<sup>92,93</sup>.

The cytokine receptor superfamily is comprised of two types of single span membrane receptors: type I and type II, both lacking enzymatic activity in their cytosolic domains. Type I receptors share a conserved cysteine residue and WSXWS motif in their extracellular domain and can be further divided into four sub groups based on the architecture of their activated complex: homodimers (EpoR, Mpl, GCSFR, GHR, etc.), heterodimers sharing common beta chain (GM-CSF, IL-3, IL-5), multimeric receptors that contain the gp-130 signaling chain (IL-6R, IL-11R, etc.) and heterodimers containing the common gamma chain (IL-2R, IL-4R, IL7R, etc.). Type II receptors are activated as heterodimers (IFNR, IL-22R, IL-24R, IL-29R, etc.) **Figure 8**. One or more JAKs may interact with each receptor subunit by interacting through Box1 (proline-rich) and Box2 (hydrophobic and charged) sequences in the cytosolic domain of the receptor.



**Figure 8. JAKs and cytokine receptors** The cytokine superfamily is divided into type I and type II cytokine receptors. Type I receptors are further sub-divided into homodimeric receptors and receptor groups sharing  $\beta$ c, gp130 or  $\gamma$ c subunits. JAK2 is almost exclusively activated by homodimeric and  $\beta$ c subunit sharing type I cytokine receptors. Type I cytokine receptors sharing gp130 or  $\gamma$ c subunits may utilize various members of JAK family. Type II cytokine receptors activate JAK1, JAK2 and TYK2. (Credit: Vainchenker et al., Seminars in cell and developmental biology, 2008)

JAKs are constitutively present in the close proximity of the cytosolic domain of receptors. Ligand binding to their cognate receptor induces a change in receptor conformation that facilitates JAK's approach to the receptor. This positioning of receptor and JAKs results in auto and/or trans phosphorylation of two juxtapositioned JAKs

and this in turn phosphorylates the receptor. Once activated, cytokine receptors promote the phosphorylation of several signal transducers and initiate the downstream signaling cascades **Figure 9**.

### 3.1.2. Negative regulation of JAK2 signaling

The magnitude and the duration of JAK2 signaling are tightly regulated by several mechanisms in order to prevent unnecessary signalization resulting in deregulated cell proliferation and malignant transformation. Multiple proteins including phosphotyrosine phosphatase (PTP), suppressor of cytokine signaling (SOCS), LNK, CBL and protein inhibitors of activated STAT (PIAS) are involved in the negative regulation of JAK2 mediated signaling.

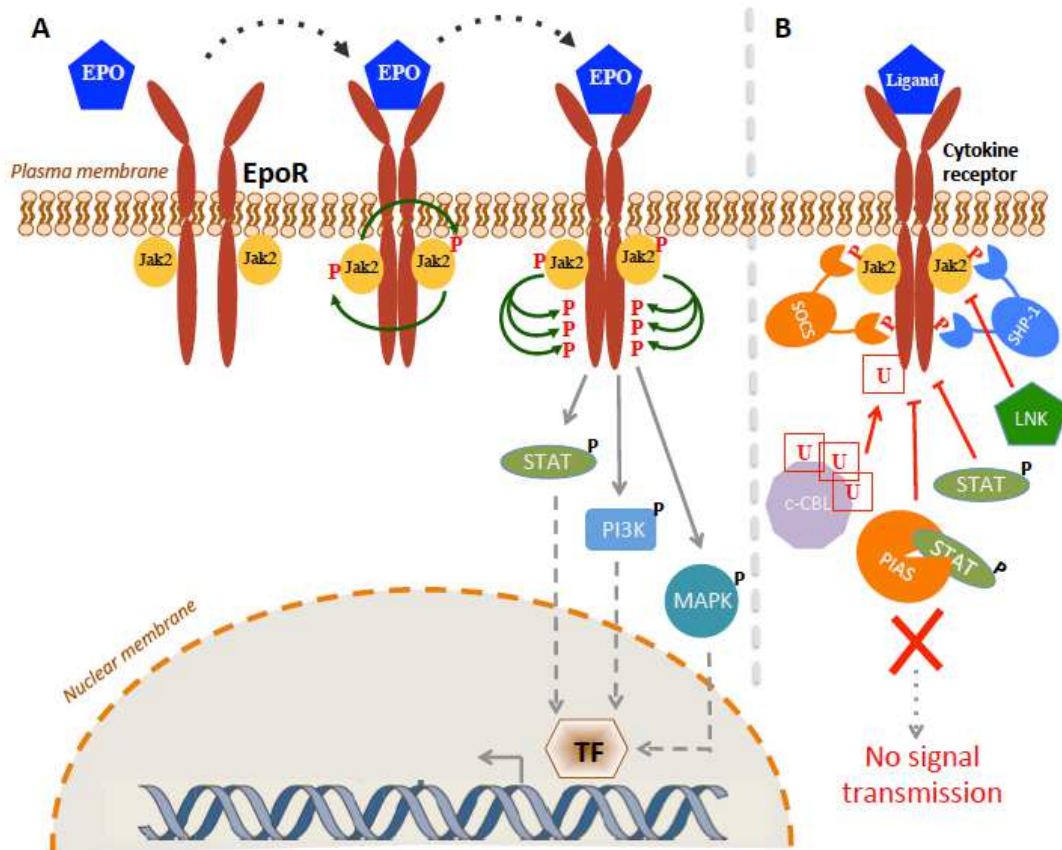
SHP-1, a PTP, uses its SH2 domain to dock onto a phosphorylated tyrosine (pY429) of EpoR that in turn promotes the dephosphorylation of associated JAK2 and eventually results in signal termination<sup>94</sup>. SHP-1 can also directly bind to JAK2, independent of SH-2 domain, and mediate JAK2 dephosphorylation<sup>95</sup>.

Another mechanism of cytokine signaling control uses SOCS (suppressor of cytokine signaling) family members, like CIS, SOCS3 and SOCS1. CIS, a cytokine inducible SH2 containing protein, is a target of JAK2-STAT pathway. Moreover, it contains an SH2 domain and binds to tyrosine-phosphorylated EPO and IL-3 receptors. Thus, CIS is a feedback modulator of STAT5; its expression is induced by STAT5 and it negatively modulates STAT5 activation resulting in attenuation of JAK-STAT pathway<sup>96</sup> (Akira). SOCS3 binds directly to Tyr (401) in the cytoplasmic domain of the EPOR and inhibits EPO-dependent proliferation and STAT5 activation in BaF3-EpoR cells and erythroid progenitors. Tyr (401) is also a binding site of STAT5<sup>97</sup>. SOCS3 interacts with JAK2 and inhibits its kinase activity by binding to phosphorylated Tyr<sup>1007</sup> in the activation loop<sup>98</sup>. Thus, binding of SOCS3 to the EPOR and JAK2 augments the inhibitory effect of SOCS3 in cytokine signaling. SOCS1, in response to IFN $\alpha$ , is shown to impair megakaryopoiesis by inhibiting TPO induced signaling<sup>99</sup>.

LNK, also known as SH2B3, is a member of SH2B family of adapter proteins that is involved in negative regulation of cytokine receptor signaling. The LNK Src homology 2 (SH2) domain is essential for its inhibitory function. LNK is shown to inhibit TpoR and EpoR mediated signaling inhibited by attenuating JAK2 activation resulting in blunting of AKT, STAT and MAPK pathways<sup>100,101</sup> **Figure 9**.

Ubiquitination is another way to control JAK2 signalization. The CBL are E3 ubiquitin ligases that attach a ubiquitin molecule, *kiss of death*, to growth receptors, tyrosine kinases and other molecules and may targets them for recycling or degradation<sup>102</sup>. TpoR is rapidly ubiquitinated on both of its intracellular lysine (K) residues (K<sub>553</sub> and K<sub>573</sub>) and degraded by both the lysosomal and proteasomal pathways<sup>103</sup>. This degradation provides a way to negatively regulate Tpo mediated signaling.

Inactivating downstream signal transmitters, like STAT proteins, can also hamper JAK2 signaling. PIAS, protein inhibitors of activated STAT, are a family of genes involved in inhibiting the JAK-STAT signaling pathway. PIAS1 and PIAS3 interact with STAT1 and STAT3, respectively. This PIAS-STAT interaction inhibits STAT binding and STAT-mediated gene activation<sup>104</sup>.



**Figure 9. JAK2 signaling** **A.** In the absence of ligand, JAK2 molecules bind to cytokine receptor (EpoR: erythropoietin receptor) as an inactive dimer. Ligand (Epo: erythropoietin) binding to cytokine receptor induces the conformational change in the receptor resulting in auto and trans phosphorylation on JAK2 and cytokine receptor. Once activated, cytokine receptor then recruits and phosphorylates several signal transmitter molecules that lead to signaling through signal transducers and activators (STAT), phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. **B.** JAK2 signaling is regulated by dephosphorylation (SHP-1), by competing for STAT binding (PIAS or SOCS), cytokine receptor ubiquitination (c-CBL) and JAK2 inactivation (LNK).

### 3.1.3. Constitutive activation of JAK2

Two main types of genetic alterations in JAK2 resulting in aberrant tyrosine kinase activity are involved in several hematopoietic malignancies: chromosomal rearrangements and point mutations.

In a subset of leukemia and lymphoma patients aberrant JAK2 fusion proteins are found leading to constitutive signal transduction **Figure 10**. Translocation Ets leukemia (TEL)-JAK2 fusion protein is reported in T-cell childhood ALL, pre-B ALL<sup>105</sup> and atypical CML (aCML)<sup>105</sup>. TEL is a transcription factor and its Helix-loop-Helix (HLH) oligomerization domain is found fused with JH2 and/or JH1 domain of JAK2. This fusion protein is shown to transform primary BaF3 cells line IL-3 independent<sup>106,107</sup>. Moreover, TEL-JAK2 fusion protein induces the constitutive activation of JAK-STAT<sup>107</sup>, PI3K<sup>108</sup> and NF- $\kappa$ B<sup>109</sup> signaling pathways. In a murine model STAT5 activation is demonstrated necessary for the myelo- and lympho-proliferative disease induced by TEL/JAK2<sup>110</sup>.

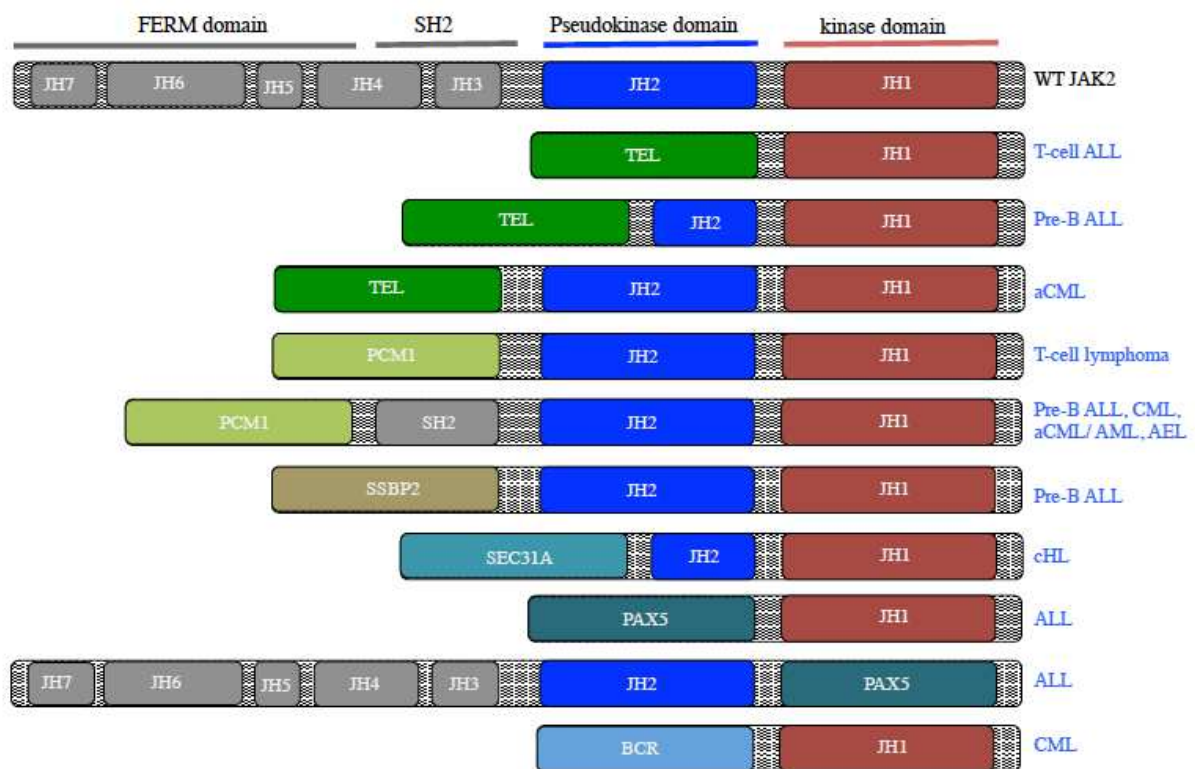
Human autoantigen pericentriolar material (PCM1)-JAK2 fusion is evidenced in atypical CML (aCML)<sup>111</sup>, acute erythroid leukemia (AEL)<sup>112</sup>, secondary AML<sup>111</sup>, pre-B ALL<sup>111</sup> and T-cell lymphoma<sup>113</sup>. The coiled-coil domains of PCMI are fused with the JH1 domain of JAK2 to form an aberrant tyrosine kinase.

Poitras *et al.* discovered single-stranded DNA binding protein 2 (SSBP2) as a new fusion partner of JAK2 in Pre-B ALL, where its LISH domain is found to be combined with JH1 and JH2 domains of JAK2<sup>114</sup>.

A protein transport protein SEC31A is recently identified as a new fusion partner of JAK2 in classical Hodgkin lymphoma (cHL). This constitutive tyrosine kinase was able to transform IL-3 dependent BaF3 cells cytokine independent. Interestingly, BaF3 cell line transformed by SEC31A-JAK2 showed growth inhibition when treated with anti-JAK inhibitors via normalization of JAK-STAT, AKT and MAPK signaling pathways<sup>115</sup>.

B-lineage specific transcription factor PAX5 is involved in several chromosomal rearrangements antagonizing its transcriptional activity, one involving JAK2 gene in childhood ALL<sup>116</sup>.

In one interesting patient of CML, BCR-JAK2 fusion protein is reported instead of BCR-ABL. The coiled-coil dimerization domain of BCR was found fused with JH1 domain of JAK2 and consistent with this patient did not respond to imatinib as well suggesting that constitutive activation of this fusion protein was due to JH1 domain of JAK2<sup>117</sup>.



**Figure 10. Schematic representation of wildtype (WT) JAK2 and its activated fusion proteins found in various hematologic malignancies** WT JAK2 comprises of seven well defined Janus homology domains (JH1-JH7). TEL-JAK2 fusion proteins involve entire JH1 domain and different combinations of JH2 domain region. PCMI-JAK2 fusion proteins are comprised of intact JH1 and JH2 domains and in some cases a part of SH2 domain. SSBP2-JAK2 fusion protein contains entire JH1 and JH2 domains. SEC31A-JAK2 involves entire whole JH1 and a part of JH2 domain. PAX5 may fuse with JH1 domain or may form a reciprocal fusion with rest of JAK2 protein. BCR-JAK2 fusion involves the JH1 domain of JAK2. Right to every fusion protein, in blue fonts, are hematological malignancies in which each respective fusion protein is found. ALL: acute lymphoblastic leukemia, aCML: atypical chronic myeloid leukemia, AML: acute myeloid leukemia, cHL: classical Hodgkin lymphoma, AEL: acute erythroid leukemia and CML: chronic myeloid leukemia.

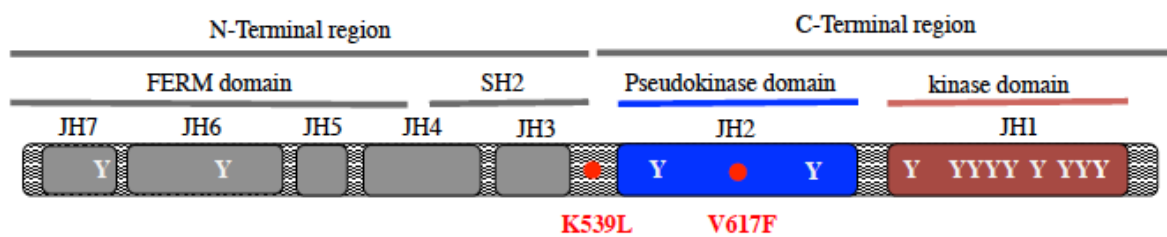


Likewise, JAK2 mutations are also reported in rare ALL and in several myeloid malignancies. Mutations at or around an arginine residue (R683) of JH1 domain of JAK2 are found to be present in B-cell leukemic blast of 19% Down syndrome associated acute lymphoblastic leukemia (DS-ALL)<sup>118,119</sup> and in 7% of sporadic B-cell precursor acute lymphoblastic leukemia (BCP-ALL)<sup>120</sup>.

The mutation JAK2<sup>V617F</sup> is present in 50-60 ET and PMF patients and around 95% of PV patients<sup>39-43</sup>. JAK2<sup>V617F</sup> is also reported in <10% of rare myeloid malignancies<sup>121-124</sup>. V617F homologous mutations in JAK1 (V658F) and TYK2 (V678F) lead to their constitutive activation supporting the notion that V617F relieves the JH1 domain from auto inhibition<sup>125</sup>. JAK2 binds to cytokine receptor through FERM domain and mutation (Y114A) in this domain disrupts this interaction<sup>92</sup>. Functional studies with double-mutant JAK2<sup>V617F</sup> Y114A in BaF3-EpoR cell line did not induce constitutive signaling, emphasizing on fact that physical interaction of JAK2<sup>V617F</sup> to receptor is obligatory for constitutive signaling<sup>126</sup>. This observation is further supported when JH1-JH2 construct with V617F did not lead to constitutive signaling in BaF3 cell line<sup>127</sup>.

Moreover, PV patients negative for JAK2<sup>V617F</sup> mutations harbor other JAK2 mutant variants with mutations in exon 12 (such as K539L)<sup>128</sup>. In vitro, these mutations are capable of transforming factor dependent cell lines to factor independent growth while constitutively activating multiple signaling pathways, like JAK/STAT and their effects can be counteracted by treatment with JAK inhibitors **Figure 11**.

Germline JAK2 mutations are reported in families with hereditary thrombocytosis. Mead *et al.* reported a family with JAK2<sup>V617I</sup> mutation. This mutation did not result in cytokine independence (no cytokine independent colonies) but in cytokine hypersensitivity in CD33+ and CD34+ cells with enhanced phosphorylation of STAT3 over low GCSF exposures. Peripheral blood of these patients displayed higher numbers of HSC<sup>129,130</sup>. Recently, our team also found three JAK2 germline mutations in two different families with hereditary thrombocytosis. These mutations are present in pseudokinase (JAK2 R938Q) and kinase domain (JAK2 R867Q and S755R) of JAK2 and result in constitutive activation of TPO-JAK2-MPL axis leading to spontaneous growth of progenitors. Moreover, these mutations are found to be resistant to JAK2 and HSP90 inhibitors (Marty *et al.*, paper submitted).



**Figure 11. Schematic representation of JAK2 activating mutations** The mutation JAK2<sup>V617F</sup> is present in JH2-pseudokinase domain. Exon 12 mutations, like K539L, are present in the region linking JH2 and JH3 domains. These mutations result in constitutive activation of JAK2.

### 3.1.4. JAK2<sup>V617F</sup> mutation and its downstream signaling in Ph- MPNs

Activated tyrosine kinases are a common feature found in MPN, like BCR-ABL tyrosine kinase in CML, which result in constitutive downstream signaling. Hematopoietic progenitors from Ph- MPN patients also show cytokine hypersensitivity or independence. Indeed, JAK2<sup>V617F</sup> activates constitutive signal transduction downstream to JAK2, like JAK-STAT, PI3K, and MAPK pathways, resulting into uncontrolled cell proliferation.

#### 3.1.4.1. JAK-STAT pathway

STAT (signal transducer and activator of transcription) protein family consists of seven family members STAT1-4, 5A-B and 6, implicated in various cell survival, differentiation and proliferative pathways. Extracellular stimulation to cytokine receptor activates JAK2 that in turn binds to STAT through their SH2 domain leading to their phosphorylation. Once phosphorylated STAT homo- or hetero-dimerize and displaces to nucleus where they bind to DNA in order to activate transcription of various target genes.

Erythropoietin independent erythroid growth is the hallmark of PV. Silva M *et al.* found that during erythroid differentiation in PV, erythroid progenitors increase the expression of Bcl-xL, an anti-apoptotic protein, in order to survive even in erythropoietin free condition, in contrast to erythroid progenitor from normal subjects that under went apoptosis<sup>131</sup>. In another detailed study of STAT5a<sup>-/-</sup>STAT5b<sup>-/-</sup> mouse model demonstrated that these mice are severely anemic at embryonic stages with less numbers of erythroid progenitors due to increased apoptosis. Early spleen erythroblast from these mice showed decreased Bcl-xL expression and increased apoptosis. Furthermore, this study demonstrated that STAT5 directly binds to Bcl-xL promoter region and enhances Epo dependent transcription suggesting that STAT5 regulates Bcl-xL levels during erythropoiesis<sup>132,133</sup>. As JAK2 is essential for EpoR signaling, probably constitutively activated JAK2<sup>V617F</sup> promotes the erythropoietin independent growth by enhancing the STAT5 regulated Bcl-xL expression.

Cyclin D2, a major regulator of G1/S phase transition in cell cycle, is found to be upregulated in BaF3 cell line over-expressing JAK2<sup>V617F1</sup><sup>34</sup>. In another study on HEL cell line (human erythroid leukemia cell line constitutively expressing JAK2<sup>V617F</sup>), it has been demonstrated that JAK2 inhibitor treatment disrupts cell growth while decreasing levels of cyclin D2, pSTAT5 and ROS (reactive oxygen species) and increasing cell cycle inhibitor p27<sup>kip</sup>. Previously, ROS was suggested to contribute in BCR-ABL oncogenic transformation<sup>135</sup>. In this study the authors showed the N-acetylcystein (NAC) treatment, blocking ROS production, arrested the HEL cells in G1 phase in result of decreased cyclin D2 and increased p27<sup>kip</sup> inhibitor. Moreover NAC treatment strongly inhibited STAT5 phosphorylation. To check if STAT5 is directly involved in ROS regulated cell cycle arrest, authors exploited doxycyclin-inducible constitutively active STAT5 system in IL-3 dependent BaF3 cell line. Activation of STAT5 was sufficient to induce ROS levels in absence of IL-3 while clearly increasing cyclin D2 and decreasing p27<sup>kip</sup>. Overall, these observations clearly suggest that JAK2<sup>V617F</sup> exploits constitutively activated JAK/STAT pathway to promote cell cycle by regulating cyclin D2 and p27<sup>kip</sup> in redox dependent manner<sup>136</sup>.

Hypersensitivity to insulin like growth factor 1 (IGF1) is described in PV<sup>59,137,138</sup> but remains controversial<sup>139</sup>. In IL-3 dependent BaF3 cell line it has been shown that JAK2<sup>V617F</sup> positive cells immediately respond to IGF1 stimulation and acquire IGF1 hypersensitive growth. Moreover, a subset of IL-3 dependent BaF3 cells acquired

autonomous growth by constitutively inducing JAK2, STAT3 and STAT5 phosphorylation. This study suggests that IGF-1R may transmit proliferative signals through constitutively active JAK/STAT pathway in the pathogenesis of PV<sup>125</sup>. It is also possible that IGF-1 induced by STAT5 leads to an autocrine stimulation<sup>140</sup>.

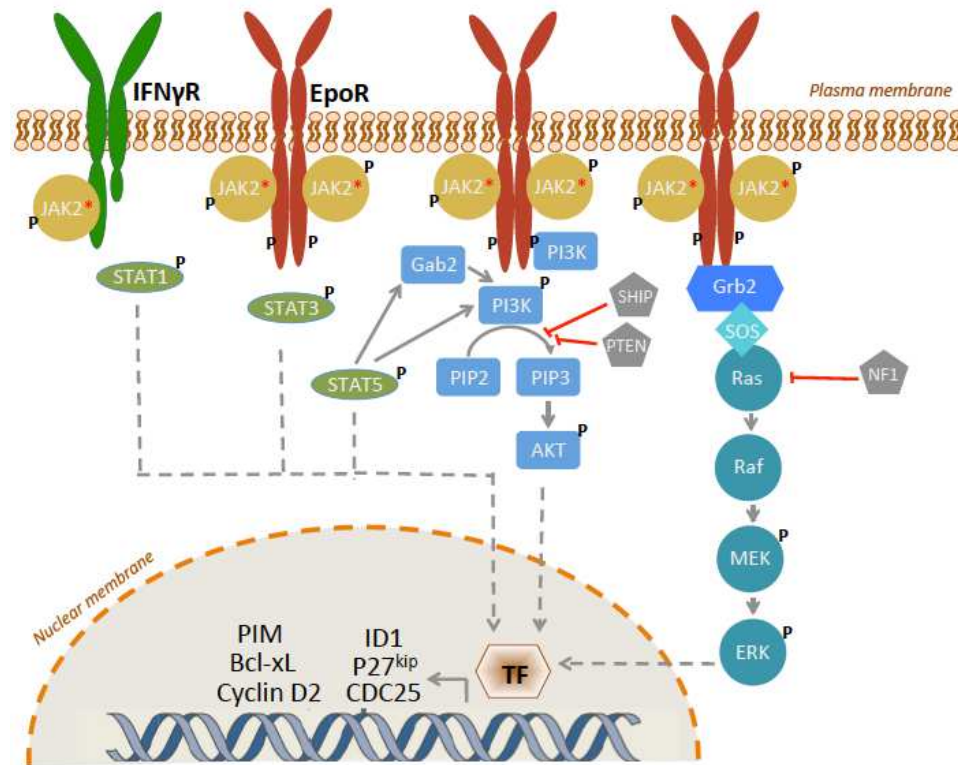
PIM, serine/threonine kinases, are the proteins that induce cell cycle progression and inhibits apoptosis. Activity of these kinases depends on the level of expression unlike other kinases, which are activated by phosphorylation. PIMs are transcriptional targets of STAT protein and lie upstream of anti-apoptotic BCL-2 family members and cell cycle regulators. JAK2<sup>V617F</sup> expressing BaF3/EpoR cell lines resulted in constitutive activation of JAK-STAT pathway and elevated levels of PIM1 and PIM2<sup>141</sup>. In another study using JAK2<sup>V617F</sup> positive human megakaryoblastic cell line, SET-2, it was shown JAK2<sup>V617F</sup> utilizes PIM/BAD/Bcl-xL pathways to promote cell survival<sup>142</sup>. Moreover, a recent study in a murine model KI for JAK2<sup>V617F</sup> demonstrated that treatment with vorinostat, a small-molecule inhibitor of histone deacetylase, normalized the peripheral blood counts and markedly reduced splenomegaly. In-vitro functional studies, treatment of JAK2<sup>V617F</sup>-expressing human erythroleukemia (HEL) cells with vorinostat inhibited proliferation and induced apoptosis by inhibiting JAK-STAT pathway and eventually decreasing PIM1 expression<sup>143</sup>. Further confirming the critical role of JAK-STAT pathway through PIM1 in the pathogenesis of MPN, two independent groups have demonstrated the significantly lower expression of PIM1 in JAK2<sup>V617F</sup> negative ET as compared to JAK2<sup>V617F</sup> positive ET<sup>144,145</sup>. Together these observations suggest PIM as an important downstream effector molecule of mutant kinase JAK2<sup>V617F</sup>.

ID1, a DNA binding inhibitor protein belonging to the basic helix loop helix (bHLH) family of transcription factors, is a new addition in JAK-STAT downstream effectors. ID1 acts a dominant negative transcription factor (TF) by binding to another TFs and inhibiting binding to DNA and eventually disrupting the expression of their targets. ID1 is known to be involved in various cell proliferation pathways as well as HSC self-renewal. A recent report by Wood and group has shown that in JAK2<sup>V617F</sup>-expressing HEL cell line, first, STAT5 directly binds to ID1 locus and regulates its expression. Second, inhibition of JAK2 reduces the expression of ID1 demonstrating it as a downstream target of JAK-STAT signaling. Furthermore, the expression of ID1 was increased in JAK2<sup>V617F</sup> positive BFU-E as compared to JAK2<sup>V617F</sup> negative BFU-E from PV patients and in-vitro functional studies demonstrated ID1 is a target of JAK2<sup>V617F</sup>-STAT signaling in erythroid progenitors<sup>146</sup>.

A recent study by A.R. Green's group provided the highlights on differential signaling of JAK2<sup>V617F</sup> by STAT1 pathway in ET and PV patients. They demonstrated that IFN- $\gamma$  pathway genes were upregulated due to remarkably significant pSTAT1 phosphorylation in JAK2<sup>V617F</sup> heterozygous erythroblasts in ET patents but not in PV patients. Furthermore, increased pSTAT1 in WT CD34+ progenitors favors ET-like phenotype while inhibition of STAT1 in JAK2<sup>V617F</sup> heterozygous CD34+ cells from PV patients represses erythropoiesis and favored megakaryopoiesis in-vitro. As JAK2 transmits signals through IFN- $\gamma$  receptor<sup>53</sup>, this led authors to conclude that JAK2<sup>V617F</sup> utilizes STAT1 differentially in ET and PV erythroblasts leading to phenotypic diversity<sup>147</sup>.

In a recent study by Gautier *et al.*, an interesting JAK2<sup>V617F</sup> dependent translational role of STAT5 in cell cycle regulation is dissected. CDC25A, an important cell cycle regulator, is shown to be upregulated in FDC-P1 EpoR-JAK2<sup>V617F</sup> cell lines and JAK2<sup>V617F</sup> expressing human and murine primary cells and its inhibition resulted in disrupted erythroid differentiation and blockade of EEC in PV patients but not in normal subjects. Authors have demonstrated that JAK2<sup>V617F</sup> does not regulate CDC25A transcription but translation. To investigate the pathway involved in JAK2<sup>V617F</sup> regulated CDC25A regulation in FDC-P1-EPOR-JAK2<sup>V617F</sup> cell line they opted

STAT5 as a qualified candidate downstream to JAK2. Inhibition of STAT5 resulted in decreased STAT5 phosphorylation, decreased CDC25 expression, and increased phosphorylation of e1F2 $\alpha$ , a transcription factor shown to regulate CDC25A protein levels in the same study. This study emphasizes on the fact pSTAT5 plays a role in erythroid proliferation in JAK2<sup>V617F</sup> patients and may exploit a newly defined pathway where constitutive JAK-STAT signaling targets cell cycle regulator CDC25A through e1F2 $\alpha$ <sup>148</sup>.



**Figure 12. JAK2<sup>V617F</sup> and aberrant downstream signaling pathways** The mutant JAK2 protein can bind to several JAK2-using cytokine receptors such as erythropoietin receptor (EpoR) and interferon gamma receptor (IFN $\gamma$ R), and constitutively activate multiple signaling cascades, such as JAK2-STAT, JAK2-AKT, JAK2-RAS-Raf-MEK-ERK, that result in altered expression of genes involved in cell proliferation, survival and differentiation of myeloid cells. Aberrant oncogenic STAT5 can also form complex with PI3K and Gab2 proteins and activate JAK2-AKT pathway. PTEN and SHIP phosphatase regulated level of PI3K-derived PIP species seem to play critical role in myeloid malignant phenotype in mouse models. NF1 counteracts RAS-Raf-MEK-ERK pathway and is found deleted in MPN patients. SHIP: SH2-containing 5' phosphoinositid phosphatase, PTEN: phosphatase with tensin homolog, NF1: Neurofibromatosis type 1 and TF: transcription factors.

The role of STAT5 in the pathogenesis of JAK2<sup>V617F</sup> positive MPNs was recently been elucidated in two studies using retroviral and KI JAK2<sup>V617F</sup> mouse models. Walz *et al.* transduced STAT5<sup>+/+</sup>, STAT5<sup>+/-</sup> and STAT5<sup>-/-</sup> mouse BM HSC cells with JAK2<sup>V617F</sup> expressing retrovirus and transplanted into lethally irradiated WT mice. Haplo-insufficient STAT5 mice developed JAK2<sup>V617F</sup> induced PV-like disease but leukocytosis was impaired. Mice homozygously deleted for STAT5 displayed no overt feature of JAK2<sup>V617F</sup> induced PV but were capable of developing MF-like disease. This suggest that STAT5 is essential for JAK2<sup>V617F</sup> induced PV pathogenesis but MF development is independent of STAT5 signaling<sup>149</sup>. The other group crossed KI JAK2<sup>V617F</sup> mice with STAT5 deficient mice and found that mice expressing JAK2<sup>V617F</sup> and deficient in STAT5 were unable to develop MPN phenotype and completely devoid of the EEC, a hallmark of PV, but interestingly when rescued with STAT5 they were capable of developing PV-like disease. However, in contrast to the previous study MF was not

observed in STAT5<sup>-/-</sup> mice<sup>150</sup>. Together these studies highlighted the importance of STAT5 in the pathogenesis of MPN **Figure 12**.

#### 3.1.4.2. PI3K/AKT pathway

The phosphoinositide 3-kinase (PI3K) plays a pivotal role in the mediation of cellular activities such as proliferation, differentiation, chemotaxis, survival, trafficking, and glucose homeostasis. This family of enzymes is recruited upon cytokine receptor activation and produces 3' phosphoinositide lipids (ex: phosphatidylinositol 3,4,5-trisphosphate, PIP3). The lipid products of PI3K act as second messengers by binding to and activating diverse cellular target proteins resulting in downstream signaling cascades. The importance of this signaling axis in the pathogenesis of myeloproliferative disorder was first illustrated in mouse models with the deficiency of two PIP3 phosphatases: SHIP (SH2-containing 5' phosphoinositid phosphatase) and *Pten* (phosphatase with tensin homolog), which developed multiple hematopoietic abnormalities including anemia, thrombocytopenia, leukocytosis, extra medullary hematopoiesis and impaired progenitor function<sup>151,152</sup>.

Given its implication in the crucial hematopoietic functions its deregulation in MPN is not surprising. Indeed, abnormally increased activation of PI3K-AKT pathway resulting in deregulated erythropoiesis and resistance to apoptosis is found to be implicated in the pathogenesis of PV<sup>153</sup>. In the same line of evidence, JAK2<sup>V617F</sup> is shown to activate PI3K/AKT pathways in BaF3, HeLa and FDCP-EpoR cell lines<sup>39,43,154</sup>. Moreover, this signaling pathway is also found constitutively activated in murine models of JAK2<sup>V617F</sup> positive MPN<sup>155,156</sup>. PI3K/Akt pathway is always activated by conventional cytokine/receptor activation but maybe activated by aberrant oncogenic STAT5. In a murine retroviral BM transplantation model, constitutively active mutant STAT5 formed a complex with catalytic p85 subunit of PI3K and the adapter protein Gab2, resulting in the leukemogenic activation of Akt/PKB, a crucial player of PI3K pathway<sup>157</sup>. All these observations strongly recommend JAK2/PI3K/AKT pathway as an important route in the pathogenesis of MPN **Figure 12**.

#### 3.1.4.3. RAS/RAF/MEK/ERK pathway

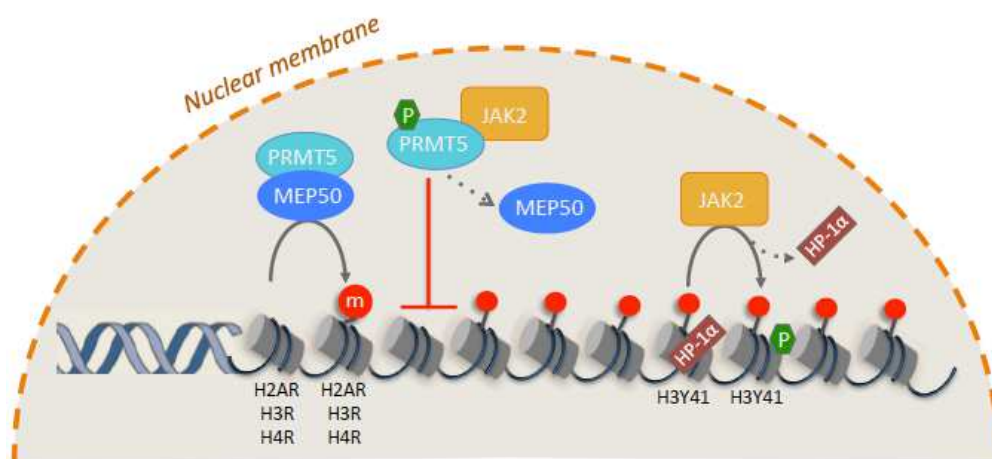
RAS/RAF/MEK/ERK or mitogen-activated protein kinase (MAPK) is another important pathway associated with transmission of extracellular stimuli into the cell through cytokines, growth factor and mitogens in order to activate a plethora of signaling pathways involved in proliferation, differentiation, and survival. Upon phosphorylation, cytokine receptor recruits docking protein containing SH2 domain like Growth factor receptor-bound protein 2 (GRB2). GRB2 forms a complex with SOS. Once this complex docks to activated receptor SOS becomes phosphorylated and in turn activates RAS. RAS, a small GTPase, act through three downstream signaling molecules RAF, MEK and ERK/MAPK.

Aberrant RAS pathway activation profile is associated with deregulated erythropoiesis in PV patients<sup>153</sup>. In fact, forced expression of constitutively active mutant of RAS resulted in abnormal erythroid differentiation and proliferation in in-vitro studies of mouse and human hematopoietic systems<sup>158,159</sup>. Further investigations have demonstrated that RAS targets MEK/ERK signaling axis in erythroid cells and that MEK inhibitor is sufficient to resume normal erythropoiesis<sup>160</sup>. In accordance with these data, expression of oncogenic KRAS in mice developed fetal myeloproliferative disorder with 100% penetrance characterized by tissue infiltration, hypersensitivity to growth factors, and myeloid hyper proliferation underlining its role in myeloid

leukemogenesis<sup>161</sup>. Oncogenic gain-of-function mutations in RAS are found in multiple myeloid malignancies. The differential activation of RAS by oncogenic tyrosine kinases is implied in the generation of multiple leukemic phenotypes. In support of this notion, Tokunaga and colleagues has recently shown that BCR-ABL, but not JAK2<sup>V617F</sup>, was able to suppress erythropoiesis when expressed into murine LSK cells, associated with stronger activation of RAS by BCR-ABL as compared to JAK2<sup>V617F</sup><sup>162</sup>. Moreover, RAS mutation is prevalent in around 7-13% of post-MPN AML patients<sup>77</sup>, suggesting its cooperation with other lesions in the promotion of MPN to AML by providing additional cell survival and proliferative signals. In addition to this, the importance of MAPK pathway in the pathogenesis of MPN is highlighted in studies of genetic profiling of patients. In two independent studies Nf1 (Neurofibromatosis type 1), a negative regulator of RAS, is found to be deleted or mutated in MPN patients especially in post ET/PV MF<sup>163,164</sup>. In conclusion, JAK2<sup>V617F</sup> signaling through MAPK pathway seems to play an important role in the pathogenesis of MPN **Figure 12**.

#### 3.1.4.4. JAK2 goes nuclear

The critical role of JAK2 as an initiator of cytoplasmic signaling cascades in normal and leukemogenic hematopoiesis is now well characterized. However, recent reports have unveiled its non-canonical nuclear functions that seem to add in its oncogenic capabilities. Dawson *et al.* using confocal immunofluorescent microscopy demonstrated that wild type as well as mutated JAK2<sup>V617F</sup> protein has its pool in the nucleus of hematopoietic cells. Moreover, JAK2 and specially activated JAK2 (K562, SET2, UKE-2 and HEL cell line) phosphorylate Tyr 41 (Y41) on histone H3 thereby inhibiting heterochromatin protein 1alpha (HP1 $\alpha$ ) that specifically binds to this region of H3. HP1 $\alpha$  is a known repressor of transcription. Chromatin immunoprecipitation assays in HEL cell line demonstrated that JAK2 regulates the expression of LMO2 oncogene by excluding HP1 $\alpha$ <sup>165</sup>. Thus, nuclear JAK2 not only contributes to leukemogenesis by expressing LMO2 oncogene but also over riding the tumor suppressor potential of HP1 $\alpha$  **Figure 13**.



**Figure 13. Nuclear role of JAK2** Activated JAK2 phosphorylates PRMT5 and disrupts the interaction with its co-factor MEP50, thereby, inhibiting PRMT5 dimethylation of histone proteins and may lead to gene expression. Activated JAK2 molecules can also phosphorylate histone H3Y41 and exclude HP-1 $\alpha$  from chromatin leading to heterochromatin derepression and increase gene expression, including the oncogene lmo2 expression. PRMT5: protein Arg methyltransferase 5, MEP50: methylome protein 50, HP-1 $\alpha$ : heterochromatin protein 1 alpha megakaryopoiesis: histone protein and m: methyl group.

JAK2<sup>V617F</sup> is also implicated in other oncogenic epigenetic modulations. Liu *et al.* have shown that JAK2<sup>V617F</sup> binds more strongly than wild type JAK2 to PRMT5, a methyltransferase that dimethylates arginine residue within histones H2A, H3, and H4. Moreover, this mutant kinase disrupts interaction of PRMT5 with its cofactor MEP50 and phosphorylates PRMT5 thereby impairing its ability to methylate its substrate. In accordance with these observations, elevated phosphoPRMT5 is detected in CD34+ cell from JAK2<sup>V617F</sup>-positive patients. Functional studies of PRMT5 knockdown in CD34+ cell resulted in enhanced erythroid colony formation while PRMT5 overexpression blocked this phenotype suggesting that JAK2<sup>V617F</sup> mediated abrogation of PRMT5 activity may induce myeloproliferative phenotype<sup>166</sup> **Figure 13.**

These findings suggest that JAK2<sup>V617F</sup> hijacks nuclear regulators to write its own epigenetic signature in order to induce and/or cooperate with leukemogenesis.

However, nuclear localization of JAK2<sup>V617F</sup>, using confocal imaging studies, remains controversial<sup>167</sup>.

#### **3.1.4.5. JAK2 as a chaperon**

Apart from triggering downstream signaling by cytokine receptors, JAK2 may fulfill a role of chaperone protein in trafficking EpoR and TpoR to cell surface.

Intact N-terminal domain of JAK2, binds to the erythropoietin receptor (EpoR) in the endoplasmic reticulum, facilitates its golgi-processed maturation and promotes its cell surface expression. Moreover, JAK2 kinase activity is not required for receptor transport to surface as JH3-JH7 domains are sufficient for receptor translocation<sup>93</sup>.

Likewise, JAK2 but also, to a lesser extent, TYK2 strongly promote cell surface localization of the TpoR by stimulating recycling and enhancing the protein stability of the mature, Golgi-processed form of the TpoR. In contrast to EpoR trafficking, JH1 kinase domain maybe involved in enhanced surface localization of TpoR. Moreover, FERM domain seems obligatory for this function<sup>92</sup>.

### **3.2. Effect of JAK2<sup>V617F</sup> mutation**

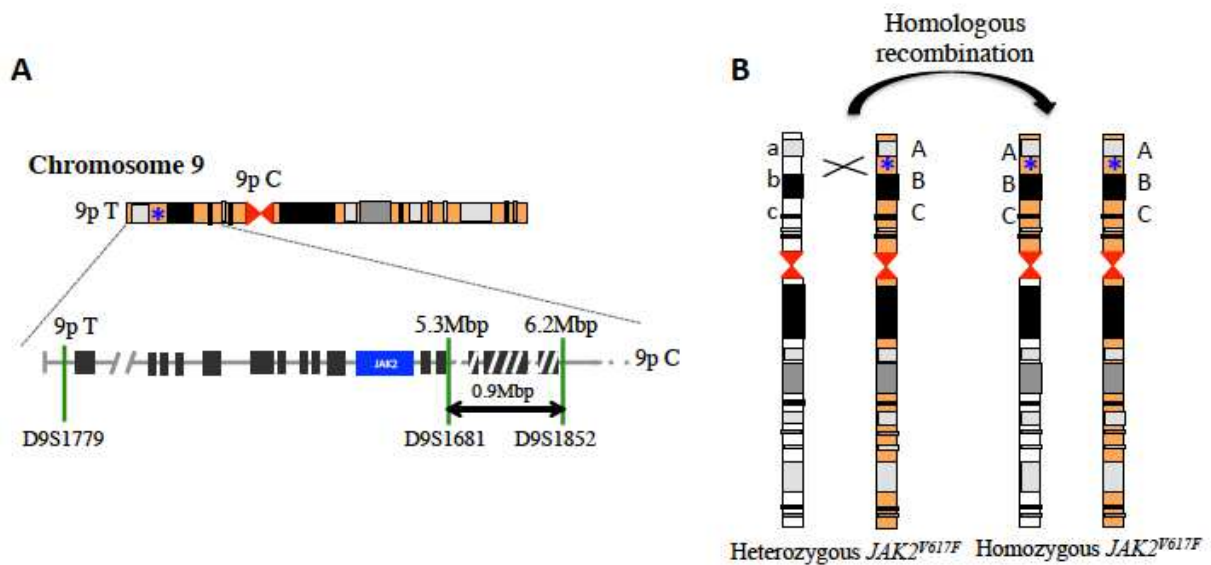
#### **3.2.1. JAK2<sup>V617F</sup> homozygosity: double sin and other stories**

Early studies of genome-wide screening for the loss of heterozygosity (LOH) loci in a cohort of 20 MPN patients revealed that 33% of PV patients but not ET patients are prone to acquire chromosome 9p LOH but also 10q and 11q in a very low frequency (5%). Further LOH analysis and X-inactivation pattern study in myeloid and lymphoid lineages from these patients demonstrated these LOHs are acquired HSC lesions<sup>168</sup>. JAK2<sup>V617F</sup> homozygosity is also present in granulocytes of ~30% of PV patients but rarely in ET<sup>39-42</sup>, which correlated with 9p LOH.

Refined microsatellite marker analysis in a cohort of 244 subjects showed that 9p LOH is present in ~34% PV, which confirmed the earlier finding<sup>168</sup>, in 22% PMF and in 3% ET patients but not in normal controls or CML and secondary erythrocytosis cases. Quantitative PCR in 9p LOH bearing patients revealed 2 copies of JAK2 in every patient suggesting LOH a product of mitotic recombination and not a chromosomal deletion. Mapping of 9p revealed that breakpoint for mitotic recombination between homologous chromatids might occur anywhere in a 0.9 Mbp long region on short arm of chromosome 9 between markers D9S1681 and D9S1852, implying that this whole region is fragile for homologous recombination (HR). HR results in different sizes of chromosome

regions, comprising of at least 85 genes and including JAK2, sharing a minimal common interval of 4.8 Mbp<sup>40</sup>

**Figure 14.**



**Figure 14. JAK2<sup>V617F</sup> homozygosity** A. Mitotic recombination occurs on short arm of chromosome 9 (9p) involving 6.2 mega base pairs (Mbp) at telomeric region (9p T). Black boxes indicate genes, green lines represent respective microsatellite markers, numbers indicate physical distance from 9p T in Mbp, blue asterisk and box indicate JAK2<sup>V617F</sup> mutation and JAK2 gene, respectively and 9p C denotes centromere. Breakpoint for homologous mitotic recombination can occur anywhere in 0.9Mbp dashed area between D9S1681 and D9S1852. B. JAK2<sup>V617F</sup> loss of heterozygosity (LOH) occurs due to homologous mitotic recombination on 9p. Figure is not drawn to scale. (Inspired from Kralovics et al. 2005)

Present circumstantial evidences strongly suggest that JAK2<sup>V617F</sup> homozygosity may play a role in the evolution of MPN by increased signaling and the expression of target genes<sup>169</sup> through double dose of JAK2<sup>V617F</sup> and also through probable loss of wild type JAK2. The functional consequences of JAK2<sup>V617F</sup> homozygosity are discussed in detail in section 3.2.2.1.

### 3.2.2. One mutation three phenotypes: a million dollar question

Although the discovery of the mutation JAK2<sup>V617F</sup> undoubtedly provided us with great insights into the molecular pathology of Ph- MPNs yet the mechanism of implication of one mutation in three correlated but distinct phenotypes (ET, PV and PMF) remains an enigma. Several theories have been stated to explain JAK2<sup>V617F</sup> pleiotropy including mutant allele dosage, cytokine receptor signalization and the role of additional genetic variations /alterations.

#### 3.2.2.1. JAK2<sup>V617F</sup> gene dosage theory

In mutant allele dosage theory it has been proposed that high JAK2<sup>V617F</sup> expression is correlated with PV phenotype whereas its low level expression develops ET. Cytogenetic and DNA sequencing analysis of blood mature myeloid compartment have demonstrated that JAK2<sup>V617F</sup> homozygosity is present in approximately 30% of PV patients due to 9pLOH in result of mitotic recombination<sup>39-42,168</sup>. JAK2<sup>V617F</sup> mutational analysis of individual myeloid progenitor colonies from ET and PV patients revealed that JAK2<sup>V617F</sup> homozygous clones are



present in almost all PV but are absent in ET patients. However, presence of two post-ET PV patients harboring JAK2<sup>V617F</sup> homozygous clones delineates the possibility that either these patients harbored minor homozygous sub-clones clones when presented with ET or the transformation of ET to PV phenotype is correlated with acquisition of JAK2<sup>V617F</sup> homozygosity<sup>75</sup>. Dupont *et al.* re-observed that majority of PV patients harbor homozygous JAK2<sup>V617F</sup> progenitors while ET is characterized by heterozygous JAK2<sup>V617F</sup> profile. In addition, high JAK2<sup>V617F</sup> allele burden in peripheral granulocytes correlated with high granulocyte count and hemoglobin levels in PV, and high granulocyte and platelet count in ET.

In-vitro methylcellulose erythroid colony-forming assay from CD34+CD38- progenitors has demonstrated that JAK2<sup>V617F</sup> triggers Epo hypersensitivity in mutant allele dose dependent manner and JAK2<sup>V617F</sup> homozygosity triggers Epo independence. Consequently, JAK2<sup>V617F</sup> rendered a selective proliferative advantage to erythroid cells in in-vitro cell culture assay from ET and PV CD34+CD38- progenitors with the enrichment of mutant allele in progeny<sup>170</sup>. These studies suggest that the phenotype of disease can be attributed to the level of JAK2<sup>V617F</sup>.

Currently, group led by A.R. Green presented another hypothesis regarding the prevalence of JAK2<sup>V617F</sup> homozygous clone in PV and ET. Studying mutational status in individual erythroid progenitors from 77 patients they found that JAK2<sup>V617F</sup> homozygous clones are prevalent in PV as previously reported but are also frequent in ET with relatively lower clone size. However, JAK2<sup>V617F</sup> homozygous clones have a selective proliferative advantage, due to an hypothetical alternative mechanism, in PV that is absent in ET and this expansion of JAK2<sup>V617F</sup> homozygous clone maybe correlated to erythrocytosis in these PV patients<sup>84</sup>. In the same line of evidence, JAK2<sup>V617F</sup> allele burden is shown to increase across the spectrum of Ph- MPN, being lowest in ET and highest in PMF<sup>86</sup>.

This JAK2<sup>V617F</sup> gene dosage to MPN phenotype relationship is also recapitulated in JAK2<sup>V617F</sup> mouse models. In a transgenic human JAK2<sup>V617F</sup> mouse model low human mutant to mouse wild type ratio (0.45) resulted in an ET like phenotype with marked thrombocytosis, slight neutrophilia and splenomegaly while high mutant to wild type ratio (1.0) resulted in a PV-like phenotype with marked erythrocytosis, increased thrombosis, marked neutrophilia, low serum Epo levels and severe splenomegaly<sup>171</sup>. Other transgenic<sup>155</sup> and retroviral transplantation<sup>172</sup> models also suggest that JAK2<sup>V617F</sup> level dictates MPN phenotype.

Recently, a KI mouse model for heterozygous and homozygous JAK2<sup>V617F</sup> expression under the control of endogenous promoter has been dissected to understand the role of JAK2<sup>V617F</sup> allele burden in MPN phenotypes. Although both heterozygous and homozygous mouse lines developed PV-like phenotype, the disease phenotype was more severe in homozygous mice with pronounced erythrocytosis, leukocytosis, thrombocytosis and splenomegaly. Moreover, JAK2<sup>V617F</sup> activated multiple downstream signaling pathways like JAK-STAT, AKT and ERK1/2 in a dose dependent manner<sup>156</sup>. However, human ET is recapitulated in an hJAK2<sup>V617F</sup> KI mouse model when human to mouse allele ratio was 1:1<sup>173</sup>. As compared to Akada's investigations this study indicates that human JAK2<sup>V617F</sup> might be less efficient in mouse biological system and therefore more expression levels are required to induce PV-like phenotype. Altogether, these lines of evidences strongly support the idea that level of JAK2<sup>V617F</sup> allele expression reflects MPN phenotype diversity **Figure 15**.

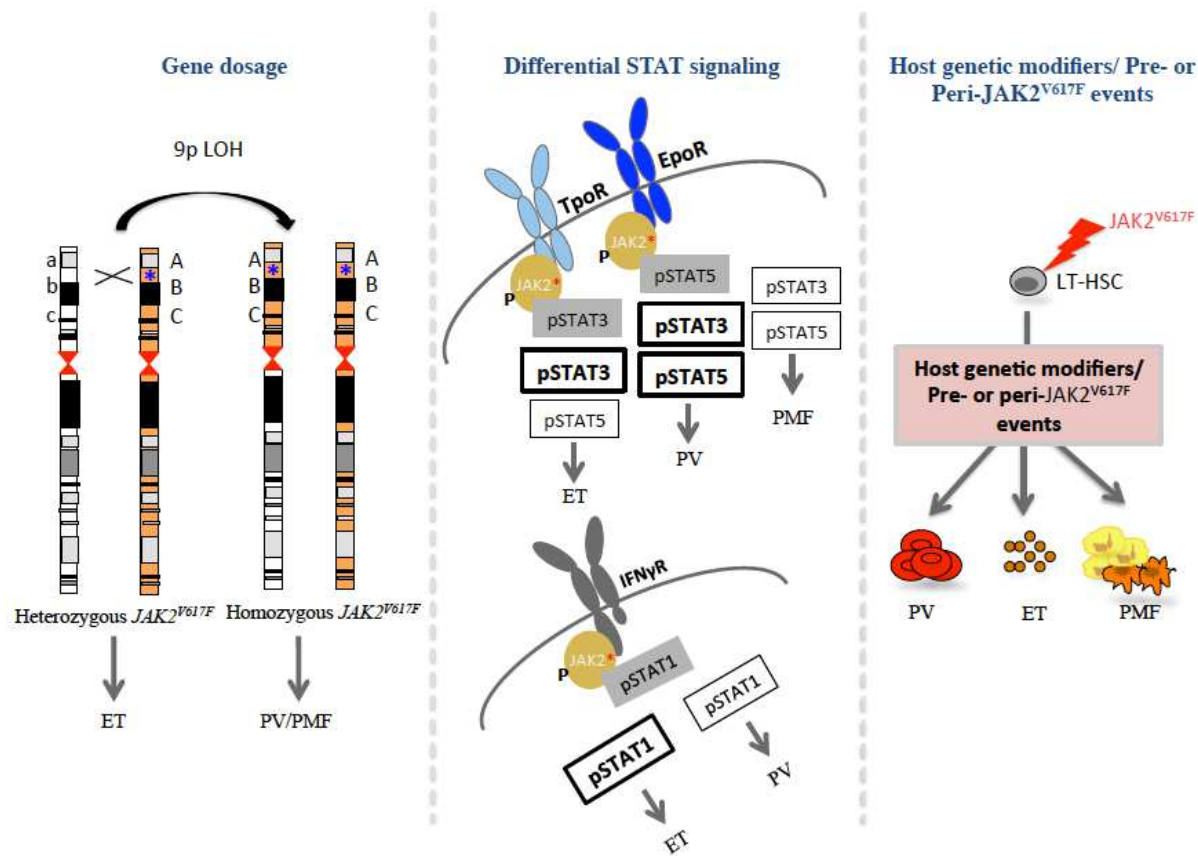
### 3.2.2.2. Differential STAT signaling

Differential activation thresholds of cytokine receptor may explain the involvement of different lineages in MPNs. A study of STAT phosphorylation status in ET, PV and PMF patients revealed specific STAT3/STAT5 phosphorylation patterns: slightly increased pSTAT-3 and reduced pSTAT-5 in ET, increased pSTAT-3 and pSTAT-5 in PV and reduced pSTAT-3 and pSTAT-5 in PIMF<sup>174</sup>. This first result is certainly surprising as it is expected that STAT signaling maybe more activated in PMF. Mpl signaling being more sensitive to low levels of JAK2<sup>V617F</sup> resulting in STAT3 phosphorylation and EpoR signaling being activated at higher levels of JAK2<sup>V617F</sup> inducing STAT5 phosphorylation could explain this differential phosphorylation to phenotype relationship. Particular biological characteristics of ET, PV and PMF can be attributed to this differential STAT phosphorylation. STAT3 plays an important role in megakaryopoiesis<sup>175</sup>. In contrast, pSTAT5 expression in PV can contribute to Bcl-xL upregulation in erythroid progenitor rendering them survival advantage<sup>131,132,176</sup>. Recent evidence demonstrates that JAK2<sup>V617F</sup> cannot induce a PV in STAT5 deficient mice, however the results on the development of myelofibrosis in STAT5 deficient mice are controversial between two different studies. STAT3 is known to be implicated in inflammatory responses especially in monocytes/macrophages and its activation maybe responsible for the storm of cytokines<sup>177,178</sup>. There is increasing evidence that STAT1 plays a central role in the development of disease phenotype. In a first study, JAK2<sup>V617F</sup> regulated differential STAT1 signaling is attributed to develop ET or PV phenotypes in patients. It was suggested that this was related to stimulation by interferon gamma. Although several studies show that EPO and TPO may induce STAT1 phosphorylation, some authors proposed that enhanced pSTAT1 response to JAK2<sup>V617F</sup> signaling constrains erythropoiesis in ET and induces megakaryocytic differentiation while reduced pSTAT1 signaling in PV promotes erythropoiesis and disrupts megakaryopoiesis<sup>147</sup>. In addition, in MPN patients the level of interferon gamma is elevated which maybe responsible for increased megakaryopoiesis at the expense of erythropoiesis. Another line of evidence that PV results due to increased JAK2<sup>V617F</sup> signaling is the presence of JAK2 exon 12 mutations exclusively in PV but not in ET and PMF. These mutations are shown to mediate strong downstream signaling as compared to JAK2<sup>V617F</sup><sup>128</sup> **Figure 15.**

### 3.2.2.3. Host genetic variation

Another explanation for a single allele causing three distinct disease entities could be due to the host-modifying influences. JAK2<sup>V617F</sup> activates type I cytokine receptor signaling in MPNs. Genotypic variation in JAK2 and/or receptors may influence their interaction and thus, could modify the disease phenotype. Genotyping of single nucleotide polymorphism (SNP) in genes implicated in JAK/STAT signaling pathway i.e., JAK2, Mpl, EpoR and GCSFR revealed some specific genotype-phenotype associations in MPN patients. Three JAK2 SNPs (rs7046736, rs10815148, and rs12342421) were associated with PV and ET but not PMF. Likewise, three JAK2 SNPs (rs10758669, rs3808850, and rs10974947) and an EpoR SNP (rs318699) were linked with PV but not ET and PMF. Furthermore, some JAK2 haplotypes were specific to PV. In conclusion, some JAK2 SNPs and haplotypes can be associated with specific MPN phenotypes but not type I cytokine receptors genetic variation except one EpoR SNP<sup>179</sup>. Host genetic factor collaboration to distinct MPN phenotypes is also supported in retroviral transplantation JAK2<sup>V617F</sup> mouse model. When JAK2<sup>V617F</sup> is expressed in Balb/c mice it induced erythrocytosis and marked leukocytosis with bone marrow fibrosis however, when expressed in C57Bl/6 it induced erythrocytosis with mild leukocytosis suggesting the strain specific contribution to disease

development<sup>180,181</sup>. Thus, the host genetic variation may play a dictating role in the disease pleiotropy while sharing a common disease allele. Recently, Godfrey *et al.* suggested that age promotes development or expansion of JAK2<sup>V617F</sup> homozygous-mutant clones and that gender modulates the phenotypic consequences of JAK2<sup>V617F</sup> homozygosity<sup>182</sup> **Figure 15**.



**Figure 15. JAK2<sup>V617F</sup> phenotypic pleiotropy hypotheses** Gene dosage: JAK2<sup>V617F</sup> heterozygous expression may lead to ET phenotype while chromosome 9 short arm loss of heterozygosity due to mitotic recombination resulting in JAK2<sup>V617F</sup> homozygosity and loss of WT JAK2 may lead to increased JAK2 signaling and increased disease phenotype severity such as PV and PMF. Differential STAT signaling: balance between STAT3, STAT5 and STAT1 activations by receptors that utilize JAK2 may lead to specific blood lineage hyper proliferation producing different disease phenotypes. Finally, host genetic modifiers: host genetic variations, such as JAK2 haplotypes and receptor specific SNPs or an additional pre- or per-JAK2<sup>V617F</sup> event, may contribute to myeloproliferative disease diversity in the presence JAK2<sup>V617F</sup> hit.

#### 3.2.2.4. Other cell-intrinsic and extrinsic factors

How the presence of JAK2<sup>V617F</sup> might contribute to the expansion of one myeloid lineage is unclear. One probable proposition is the induction of lineage specific switch of hematopoietic multipotent progenitor. Geron *et al.* demonstrated enhanced erythroid differentiation and engraftment of human cord blood progenitors from PV patient as compared to normal subjects and attributed this to JAK2<sup>V617F</sup>. JAK2<sup>V617F</sup> expressing progenitors expressed increased GATA-1 (erythroid transcription factor) and decreased FOG-1 (megakaryocytic transcription factor) transcripts, skewing transcriptome profile towards erythroid lineage and this could be evidenced by increased levels pGATA-1, pAkt and pSTAT protein levels. Interestingly, a selective JAK2 inhibitor, TG101348, could be able to handicap this erythroid differentiation and engraftment capability of hematopoietic progenitor with selective decrease in GATA-1 and increase in FOG-1 transcripts. Thus, JAK2<sup>V617F</sup> driven GATA-1, Akt and STAT5 pathways may provide a rationale of marked erythroid expansion in PV<sup>183</sup>.

### 3.2.2.5. Pre- or Peri-JAK2<sup>V617F</sup> events

One way to explain that how a unique mutation can give rise to three phenotypes is that it may collaborate with an additional pre- or peri- JAK2<sup>V617F</sup> event. This event would be specific depending on each phenotype. There are several lines of evidence to support this argument. Very first evidence is the presence of clonal hematopoiesis in some PV and PMF patients but in many ET patients in the absence of JAK2<sup>V617F</sup> clonality<sup>52,184,185</sup>. These studies suggest that an additional event is responsible for the clonal expansion. Similarly, the presence of JAK2<sup>V617F</sup>-negative EEC in JAK2<sup>V617F</sup>-positive patients<sup>186</sup>, transformation of JAK2<sup>V617F</sup>-positive MPN into JAK2<sup>V617F</sup>-negative AML<sup>187</sup> and development of different myeloid malignancies with or without JAK2<sup>V617F</sup> in same family<sup>188</sup> provides additional support to the acquisition of an event before JAK2<sup>V617F</sup>, which may derive different phenotypes in collaboration of JAK2<sup>V617F</sup>. All these evidences are discussed in detail in section 3.4 **Figure 15**.

### 3.2.3. JAK2<sup>V617F</sup> in HSC compartment

The fact that Ph- MPNs are clonal hematopoietic stem cell disorders was already evidenced by X-linked inactivation studies in PV and ET patients long before the discovery of the mutation JAK2<sup>V617F</sup><sup>45,46,64,65</sup>. Soon after the discovery of JAK2<sup>V617F</sup>, hematopoietic compartments were investigated to identify the hematopoietic differentiation stage at which the disease is acquired. Two teams have demonstrated the presence of JAK2<sup>V617F</sup> in HSC (CD34+CD38-Lin-CD90+)<sup>49</sup> and LTC-ICs (long term culture initiating cells)<sup>170</sup> from PV patients. Moreover, flow cytometric analysis of HSC and progenitors (CMP, GMP and MEP) demonstrated expansion of these compartments in PV patients<sup>49</sup>.

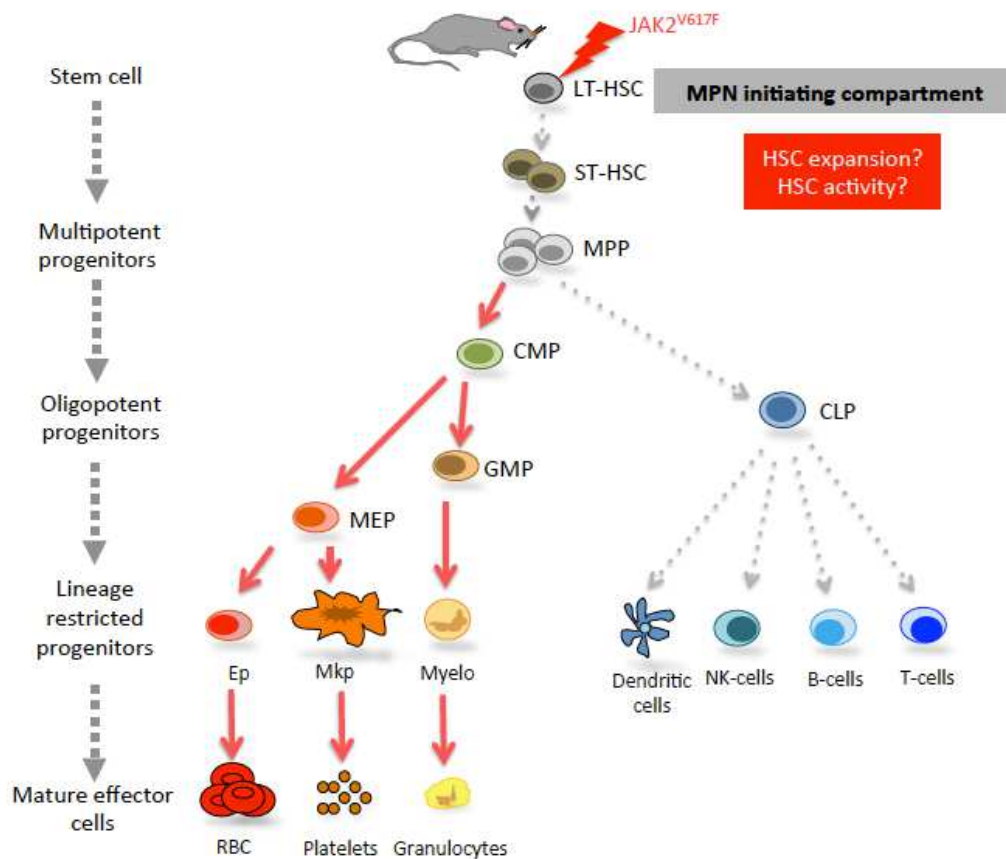
In a recent study, Anand S. *et al.* investigated the effect of JAK2<sup>V617F</sup> on HSC pool size and its *in vivo* myeloid differentiation potential in ET, PV and PMF patients. JAK2<sup>V617F</sup> did not increase ET and PV HSC pool size (in contrast to PV patients studied by Jamieson<sup>49</sup> *et al.*) but PMF HSC and GMP compartments. Also, JAK2<sup>V617F</sup> increased *in vivo* selective terminal myeloid differentiation potential of HSC from ET, PV and PMF. This study also indicated the marked JAK2<sup>V617F</sup> homozygous clonal expansion as compared to heterozygous clones in erythroid as well as granulocytic differentiation in PV patients<sup>189</sup>.

The presence of the mutation in myeloid and lymphoid mature lineages in PV and PMF demonstrated that JAK2<sup>V617F</sup> bearing progenitor is capable of generating normal hematopoietic hierarchy<sup>50,51,190</sup>. However, detection of JAK2<sup>V617F</sup> in B/NK/myeloid progenitors in much higher frequency from PMF patients as compared to PV patients points towards the different allele burden in HSC compartment in PV and PMF<sup>50</sup>.

*In vivo* studies, using CD34+ cell engraftment from PV and PMF patients in non-obese diabetic-severe combined immunodeficient (NOD/SCID) mice, demonstrated that PV and PMF patients harbor JAK2<sup>V617F</sup> positive SCID repopulating cells (SRCs) and that can be concluded by the presence of human CD45+ cells in multiple myeloid and lymphoid compartments even after three months post graft, further emphasizing that PV and PMF patients harbor JAK2<sup>V617F</sup> mutation in a cell of apex in hematopoietic pyramid and is capable of long-term multilineage differentiation<sup>191,192</sup>. Also, there are functional differences between HSC compartment of PV and PMF patients. First, although majority of LT-SRCs were positive for JAK2<sup>V617F</sup> from PMF patients, majority of LT-SRCs were wild type for JAK2 from PV patients, pointing towards different HSC compartment compositions of PV and PMF but with no differential proliferative or self renewal property and second, HSC

differentiation program of PV and PMF were different with PMF clearly skewed towards myeloid differentiation<sup>192</sup>.

An interesting human allogeneic stem cell transplant (SCT) case study has been reported in 2008 in which donor JAK2<sup>V617F</sup> cells were present just after transplantation in recipient but disappeared overtime. Seven years after transplantation 1% JAK2<sup>V617F</sup> donor cells were still detectable in recipient blood suggesting that JAK2<sup>V617F</sup> mutation appeared in a cell with self-renewal capacity but it did not give a proliferative advantage to mutated cells. Moreover, only JAK2<sup>V617F</sup> was not sufficient to develop disease at least in this case<sup>193</sup>.



**Figure 16. Effect of JAK2<sup>V617F</sup> on hematopoietic cell populations** In mouse models JAK2<sup>V617F</sup> expression in hematopoietic stem cell (HSC) results in myeloproliferative neoplasm (MPN) development and only long-term hematopoietic stem cell (LT-HSC) compartment contains MPN initiating cells. The effects of JAK2<sup>V617F</sup> on HSC expansion and activity remains a controversy, however, its expression expands the myeloid oligopotent progenitors and their progeny. Red arrows represent hyperproliferation of respective cell type. ST-HSC: short term-hematopoietic stem cell, MPP: multipotent progenitor, CMP: Common myeloid progenitor, GMP: granulocyte-macrophage progenitors, MEP: megakaryocyte-erythroid progenitors, Ep: erythroid progenitor, Mkp: megakaryocytic progenitor, myeloid progenitor and RBC: red blood cells.

Overall, human studies have allowed identifying the hematopoietic stage at which JAK2<sup>V617F</sup> is acquired but the impact of this mutation in HSC activity is still largely unknown.

To date, several JAK2<sup>V617F</sup> retroviral (RV)<sup>39,154,172,180,181</sup>, transgenic (TG)<sup>155,171,194</sup> and Knock-In (KI)<sup>156,173,195</sup> mouse models have been studied to unravel the functional consequences of JAK2<sup>V617F</sup> in the initiation and development of MPN. All models developed MPN phenotypes of varying degrees claiming that JAK2<sup>V617F</sup> alone is sufficient

to induce MPN. Moreover, these models displayed an expansion of erythroid and myeloid mature and progenitor compartments recapitulating human MPNs. Detailed analyses to understand the impact of JAK2<sup>V617F</sup> on HSC compartment in KI mouse models underlined its controversial consequences. Using BMT assays from highly purified LT-HSC, ST-HSC and MPP, Mullally *et al.* have found that only LT-HSC compartment carries the JAK2<sup>V617F</sup> disease initiating population and consequently; JAK2<sup>V617F</sup> positive GMPs and MEPs are unable to initiate an MPN<sup>195,196</sup>. Akada *et al.* have demonstrated an expansion of LT-HSC and ST-HSC compartments in their JAK2<sup>V617F</sup> KI mouse model<sup>150,156</sup>. However, Mullally *et al.*<sup>195</sup> have demonstrated normal LSK pool and Li *et al.*<sup>173</sup> have demonstrated a reduced pool of LSK cells at later stage of disease.

In Mullally's model JAK2<sup>V617F</sup> has rendered a strong proliferative advantage to HSC in BMT competitive settings while HSC repopulating function was found impaired in Li's KI model. Functional consequences of JAK2<sup>V617F</sup> on LSK cell cycle status demonstrated no effect<sup>195</sup> or reduced cycling<sup>173</sup>. JAK2<sup>V617F</sup> also reduced apoptosis in the later KI mouse model thus providing a way to the accumulate DNA damage resulting in deregulated HSC function<sup>173</sup>.

Overall, these mouse models have provided profound but contradictory insights into the functional consequences of JAK2<sup>V617F</sup> in HSC activity. Thus the effect of JAK2<sup>V617F</sup> in HSC compartment remains a matter of controversy<sup>197</sup> **Figure 16.**

### 3.3. JAK2<sup>V617F</sup> mutation: *Of Mice and MPN*

Nothing beats the idea of purposely-constructed animal models to explore and understand the pathophysiology of an illness and to test a therapeutic intervention for the same. Since the discovery of JAK2<sup>V617F</sup> mutation, many *in vitro* and *in vivo* studies have focused on elucidating the role of JAK2<sup>V617F</sup> in the initiation, development, diversity, complexity and evolution of disease. To date, three different strategies have been utilized to generate JAK2<sup>V617F</sup> mouse models i.e., retroviral transduced bone marrow transplantation (BMT), mouse transgenesis (TG) and gene Knock-In (KI). All three types of JAK2<sup>V617F</sup> models mimicked human MPN-like features providing crucial insights into the molecular pathogenesis of Ph- MPNs and further allowing to exploit these models to test novel therapies and the role of additional event in the progression and/or transformation of disease.

#### 3.3.1. Retroviral BMT mouse models

BMT of retrovirally-transduced cell is the rapid way of expressing desired gene in a selected cell population *in vivo*. Several teams have opted this gene transfer technique to study the impact of JAK2<sup>V617F</sup> on hematopoiesis.

**1. James *et al.***<sup>39</sup> have reported, for the first time, JAK2<sup>V617F</sup> mutation to induce erythrocytosis in C57Bl6 mice. These mice have developed a hematocrit level of 60% in contrast to 40% in control mice, though detailed phenotypic analyses was not provided.

**2. Wernig and colleagues**<sup>181</sup> have provided first detailed characterization of JAK2<sup>V617F</sup> induced MPN in C57Bl/6 and Balb/c mouse strains. Mice developed PV with elevated hematocrits low serum Epo levels, marked leukocytosis but thrombocytosis was evidenced only in Balb/c strain. Moreover, BM fibrosis is evidenced in

Balb/c but not in C57Bl/6 mice. Disease transplantability assays using total BM or splenocytes from C57Bl/6 mice into lethally irradiated recipients were failed. This model provided some interesting insights into the role of host genetic variation in the phenotype of disease. First, although significant leukocytosis is observed in C57Bl/6 mice, leukocytosis was much more pronounced in Balb/c strain. Second, slightly increased platelet counts were observed in Balb/c mice whereas thrombocytosis was not observed in C57Bl/6 mice. Finally, diffused reticulin fiber indicating fibrosis was only evidenced in BM of Balb/c mice while C57Bl/6 mice lacked this feature. All these observations compel to belief that host specific modifiers may contribute to the disease phenotype.

**3. Lacout *et al.***<sup>172</sup> developed a JAK2<sup>V617F</sup> mouse model in C56BL/6J background with low and high expresser groups. Both groups developed high hematocrits with increased hemoglobin and RBC numbers, leukocytosis specifically due to neutrophilia but lacking thrombocytosis. In later stages of disease BM cellularity was decreased with the accumulation of heavy deposits of reticulin fibers while neutrophilia was still evident and was associated with anemia. Disease from both groups was transplantable to secondary recipients. Interestingly, recipients from high expresser group developed severe leukocytosis and recipients from low expresser group showed an episode of transient thrombocytosis. Overall, this model mimicked human PV-like disorders with its transformation into myelofibrosis and indicates the contribution of JAK2<sup>V617F</sup> expression level in disease phenotype.

**4. Zaleskas and co-workers**<sup>180</sup> were next to dissecting the molecular pathogenesis of JAK2<sup>V617F</sup> in Balb/c and C57Bl/6 hosts. Both strains developed PV with elevated hemoglobin, reticulocytes, RBC mass, hematocrit along with low serum Epo levels. Leukocytosis was mild in C57Bl/6 mice but strikingly pronounced in Balb/c specifically due to neutrophilia. However, none of the mouse strains showed signs of thrombocytosis despite functional abnormalities in platelets. Autopsy revealed significant splenomegaly, particularly in Balb/c mice. Later stages of disease PV was declined with the development of anemia in some animals along with appearance of fibrotic mass in BM and spleen, thus PV transformed into ‘spent phase’ resembling human MF. Interestingly, transplanted mice with BM from the mice featuring MF developed PV and not the MF suggesting that the problem with fibrosis was the “the seed not the soil”<sup>198,199</sup>. In conclusion, both strains faithfully modeled human PV-like disease with its transformation to myelofibrosis.

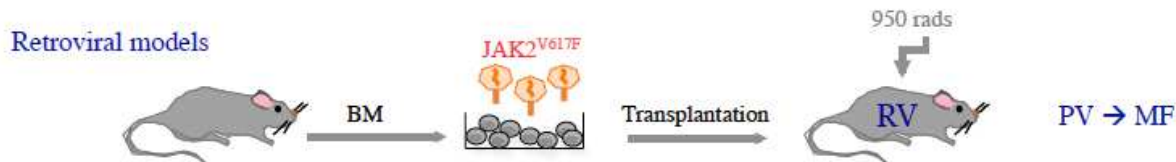
**5. Bumm *et al.***<sup>154</sup> also used the same strategy of gene transfer for the generation of a mouse model in Balb/c strain to understand the functional consequences of JAK2<sup>V617F</sup> in MPN development and to get further insights of its impact on hematopoietic stem and progenitor compartment (HSPC). These mice featured PV with elevated levels of hematocrit, low serum Epo levels and red cell abnormalities, normal to elevated leukocytosis but no increase in platelets. JAK2<sup>V617F</sup> animals displayed significant splenomegaly and accumulation of reticulin fibers BM, depicting post-PV ‘spent phase’ towards MF. A detailed analysis of hematopoietic stem and progenitor cell compartment using flow technique revealed expansion of stem (Lin-ckit+) and progenitor (CMP, GMP and MEP) compartments as a functional consequence JAK2<sup>V617F</sup> mutation. Further FACS analyses have demonstrated an increase in late erythroid (Lin/CD117<sup>+</sup>/CD71<sup>low</sup>) and megakaryocyte (Lin/CD117<sup>+</sup>/CD9<sup>+</sup>/FcγR<sup>low</sup>/CD41<sup>+</sup>) progenitors. Somewhat striking, 5 months after primary transplantation these mice showed no clinical symptoms of MPN with very low numbers of transduced cell detected in peripheral blood and reversal of blood parameters to normal values depicting the resumed normal hematopoiesis. Secondary transplantations to lethally irradiated mice over all failed with the exception of a minor sub group where it provoked a transient MPN phenotype that was resolved in 30 days. This model closely recapitulated human MPN but emphasized that although

JAK2<sup>V617F</sup> expression renders a proliferative advantage to HSPC compartment it hardly provides an additional self-renewal capacity to these cells.

All these models claimed the responsibility of sole JAK2<sup>V617F</sup> mutation in the induction of MPN. However, the reason why JAK2<sup>V617F</sup> always resulted in a PV and not an ET, despite of JAK2<sup>V617F</sup> expression and functional abnormalities in megakaryocytic lineage still remains enigmatic. One possible explanation is the acquisition of an additional event in ET that may modulate platelet proliferation resulting in thrombocytosis. Host genetic modifiers that might play a role in the variation of disease phenotype can be the other possibility. Finally, the level of mutated kinase also seems important in the development of an ET or a PV in these mouse models as proposed in human disease.

However, retroviral mouse models have some shortcomings like transgene over-expression and un-predicted site integration. .





**Figure 17. Lesson from JAK2<sup>V617F</sup> retroviral BMT mouse models** The mutation JAK2<sup>V617F</sup> is solely responsible to induce MPN

**Table 1: JAK2<sup>V617F</sup> retroviral mouse models**

	JAK2 <sup>V617F</sup> gene source	Mouse genetic background	Hematocrit (%)	WBC (10 <sup>9</sup> /L)	Platelets (10 <sup>9</sup> /L)	Phenotype
1	Murine	C57/B16	60 Vs. 40	NA	NA	PV
2	Murine	C57/B16 Balb/c	72.5 Vs. 45 73.7 Vs. 50	20 Vs. 10 112 Vs. 6.5	524 Vs. 576 591 Vs. 263	PV PV
3	Murine	C57/B16J (low expresser group)	61	18 Vs. 12	900 Vs. 900	PV
		C57/B16J (high expresser group)	57	20 Vs. 12	900 Vs. 900	PV
4	Murine	C57/B16 Balb/c	65 Vs. 40 70 Vs. 40	18 Vs. 10 55 Vs. 10	1000 Vs. 1100 600 Vs. 600	PV PV
5	Murine	Balb/c	83 Vs. 50	40 Vs. 10	800 Vs. 800	PV

**Table 1: JAK2<sup>V617F</sup> retroviral mouse models, continued**

	Spleen (g)	MF	Effect on lineages	Colony formation	HSPC	Reference
1	NA	NA	NA	NA	NA	James et al., 2005
2	0.4 Vs. 0.1	No	Ter119+CD71+: 5 X ↑ SPI	↑ BFU-E, CFU-G, CFU-M, CFU-GM, GEMM, EEC	NA	Wernig et al., 2006
	1 Vs. 0.1	Yes	Ter119+CD71+: 10 X ↑ SPI Gr1+Mac1+: 10 X ↑ SPI, 3 X ↑ BM	↑ BFU-E, CFU-G, CFU-M, CFU-GM, GEMM, EEC	NA	
3	0.36 Vs. 0.1	Yes	Erythroid precursor: ↑ SPI, ↓ BM Myeloid precursors: ↑ SPI, ↑ BM	↑ BFU-E, CFU-E CFU-GM, EGMC, EEC	NA	Lacout et al., 2006
	0.36 Vs. 0.1	Yes	Erythroid precursor: ↑ SPI, ↓ BM Myeloid precursors: ↑ SPI, ↑ BM	↑ BFU-E, CFU-E CFU-GM, EGMC, EEC	NA	
4	↑ (Values NA)	Yes	Ter119+CD71+: 3.5 X ↑ SPI Myeloid cells: ↑ BM	↑ CFU-E, EEC	NA	Zaleskas et al., 2006
	0.58 Vs. 0.08	Yes	Ter119+CD71+: 3.5 X ↑ SPI Myeloid cells: ↑ BM	↑ CFU-E, EEC	NA	
5	0.97 Vs. 0.1	Yes	Erythroid precursor: ↑ SPI, ↑ BM Myeloid precursors: ↑ SPI, ↑ BM	↑ CFU	↑CMP, GMP, MEP, LK	Bumm et al., 2006

### 3.3.2. Transgenic mouse models

The next wave of mouse models exploited transgenesis as the technique to transfer JAK2<sup>V617F</sup> cDNA to mouse oocytes for the generation of stable transgenic lines.

**1. Shide and al.**<sup>155</sup> were the first to use this approach for expressing murine JAK2<sup>V617F</sup> under the control of H2k-b promoter for generating two transgenic mouse lines. JAK2<sup>V617F</sup> low expresser group (JAK2<sup>V617F</sup>/JAK2<sup>WT</sup> mRNA expression=0.45) was quite heterogeneous with respect to disease phenotype. Among 49% of animals that developed the disease some showed PV (20% of total) with or without thrombocytosis, some developed ET (30% of total) and some mice developed just elevated leukocyte counts. BM histology revealed hyper cellular BM with trilineage hyperplasia and neither traces of reticulin fiber nor the evidence of neo bone formation were found. JAK2<sup>V617F</sup> induced significant splenomegaly in animals featuring who have developed the disease. In JAK2<sup>V617F</sup> high expresser group (JAK2<sup>V617F</sup>/JAK2<sup>WT</sup> mRNA expression=1.35) all animals developed elevated leukocytes and platelets and low hemoglobin levels as early as one month after the birth. As the disease progressed WBC and platelet count declined to reach near normal values by 9 months and this was accompanied by decrease in hematocrit from 42% at 1 month to 32% at 6 months after birth. Mild to severe reticulin fiber accumulation along with neo bone formation was observed over time. Splenomegaly was even more pronounced in this group. The frequencies of erythroid and myeloid progenitor in BM and spleen of JAK2<sup>V617F</sup> expressing mice did not vary as compared to their wild type counter-parts; however, endogenous erythroid growth is observed in both groups.

This exogenous JAK2<sup>V617F</sup> mouse model presented a complete spectrum of MPN phenotypes including ET, PV and PMF while emphasizing on the level of JAK2<sup>V617F</sup> expression as an important modulator of disease phenotype. Mice in low expresser group expressing JAK2<sup>V617F</sup> approximately half of WT JAK2 developed an ET or PV like phenotype. Even this intergroup variation is found to be correlated with T (mutant)/G (WT) ratios derived by sequencing of PCR products of JAK2 cDNA with low T/G ratios found in ET-like and higher T/G ratio in PV-like phenotypes. In agreement with this notion, high expresser group expressing JAK2<sup>V617F</sup> 3 times more than former group developed PMF in later stages of disease.

**2. Xing et al.**<sup>194</sup> generated two TG mouse lines using Vav gene promoter to control human JAK2<sup>V617F</sup> expression in hematopoietic cells. High JAK2<sup>V617F</sup> -expresser group (hJAK2<sup>V617F</sup> to mJAK2 mRNA ratio ~10:1) of mice displayed markedly increased counts of white cells, red cells, hemoglobin, hematocrit, and platelets as compared to low-expresser group (hJAK2<sup>V617F</sup> to mJAK2 mRNA ratio ~1:1). Within high expresser group 75% mice developed strong elevations in the platelets (usually 3000 X 10<sup>9</sup>/L) but with normal or slightly increased counts in white cells and red cells. The phenotype of these mice resembles that of human ET patients. The rest 25% mice displayed abnormally high levels of red blood cells and white blood cells as well as platelet and thus, recapitulated human PV-like disease. All mice in this group developed splenomegaly and mice that developed severe ET or PV displayed myelofibrosis. Interestingly in low-expresser group mice just displayed moderately increased blood cell counts not enough to be characterized as an ET or a PV-like phenotype. Overall, in this study TG mice showed ET or PV phenotype evolving into myelofibrosis. Moreover, it indicated a direct relation of JAK2<sup>V617F</sup> expression with disease development. However, the finding of JAK2<sup>V617F</sup> expression leading to two different MPN phenotypes complicated the JAK2<sup>V617F</sup> dosage theory.

**3. Tiedt *et al.***<sup>200</sup> were next to generate JAK2<sup>V617F</sup> TG mouse model by employing an innovative approach for the inducible expression of JAK2<sup>V617F</sup>. They inserted 9 bacterial artificial chromosomes (BAC) containing exon 1-12 of human *JAK2* followed by *JAK2* mutated cDNA (exons 13-25) in inversed orientation and flanked by antiparallel lox sites. Crossing of these mice with Cre TG mice resulted in a number of transgene activation and copy number reduction. These mouse lines expressed different JAK2<sup>V617F</sup> levels under the control of minimal human *JAK2* promoter depending upon the exposition of Cre recombinase activity, the more the exposition the less JAK2<sup>V617F</sup> expression.

Mice expressing low JAK2<sup>V617F</sup> level in hematopoietic cells induced by permanent expression of cre under the Vav-Cre showed a JAK2<sup>V617F</sup> mutant to WT mRNA ratio of 0.45:1. 20 weeks after birth they developed an ET phenotype with remarkable increase in platelets, slight decrease in hemoglobin, mild neutrophilia and eventual development of myelofibrosis. When JAK2<sup>V617F</sup> TG mice were crossed with Mx-Cre TG mice and induced with low number of pIpC injections they showed a higher JAK2<sup>V617F</sup> mutant to WT mRNA ratio (1:1). 20 weeks after induction these animals displayed a PV-like disease with increased hemoglobin, thrombocytosis and neutrophilia eventually developing myelofibrosis. Splenomegaly was more severe and serum Epo levels were suppressed in JAK2<sup>V617F</sup> Mx-Cre mice as compared to JAK2<sup>V617F</sup> Vav-Cre mice. Notably, a considerable phenotypic variability, particularly concerning thrombocytosis and granulocytosis, within JAK2<sup>V617F</sup> Mx-Cre mice was evidenced depending upon the level of JAK2<sup>V617F</sup> expression. These TG mice developed a full spectrum of MPN i.e., ET, PV and MF. Of note, in this study is the development of ET-like disease with low levels of JAK2<sup>V617F</sup> expression and PV-like disease with high levels of JAK2<sup>V617F</sup> expression. This study authentically demonstrates that the level of JAK2<sup>V617F</sup> expression is a crucial factor of disease phenotype development.

In a recent investigation same team has demonstrated that JAK2<sup>V617F</sup> expression increased the LSK pool in Vav-Cre and Mx-Cre but also SCI-Cre transgenic mice. Moreover, JAK2<sup>V617F</sup> also rendered a competitive advantage to peripheral blood myeloid and LSK cells. To study the impact of WT *JAK2* on disease phenotype, SCI-Cre JAK2<sup>V617F</sup> transgenic mice, displaying PV-like disease, were crossed with conditional *JAK2* knockout mice. Interestingly, insufficiency of WT *JAK2* in SCI-Cre JAK2<sup>V617F</sup> JAK<sup>fl/+</sup> or SCI-Cre JAK2<sup>V617F</sup> JAK<sup>fl/fl</sup> mice resulted in higher hemaoglobin and erythrocyte numbers but no effect on platelet or granulocyte count was observed. These double transgenic *JAK2* KO mice also displayed a drastic reduction in survival. These observations point towards dominant negative role of WT *JAK2* in the responsiveness of erythropoiesis to the JAK2<sup>V617F</sup><sup>201</sup>.

Overall, all TG models faithfully displayed human MPN and clearly demonstrated that the level of JAK2<sup>V617F</sup> expression modulates MPN phenotype. Low level of JAK2<sup>V617F</sup> expression compared to its WT counterpart lead to an ET-like disease and high level of JAK2<sup>V617F</sup> expression resulted in PV-like disease. Furthermore, these models displayed an increased clonogenic myeloid hematopoietic progenitor cell pool along with endogenous erythroid colonies (EEC), a hallmark of PV. Notably, as suggested by retroviral models<sup>180,202</sup> that host genetic background plays a role in phenotypic development of disease, Tiedt *et al.* indicated that within same genetic background different JAK2<sup>V617F</sup> expression levels can produce different disease phenotypes. It is difficult to compare results among different TG mouse models generated to date because of different promoters and numbers of integrated transgene. Similarly, these models do not recapitulate human MPN at genetic level due to number and position of transgenes integrated in these mice models although they serve the purpose adding useful insight to disease pathogenesis.

### Transgenic models



**Figure 18. Lesson from JAK2<sup>V617F</sup> transgenic mouse models** The level of JAK2<sup>V617F</sup> expression is crucial for MPN phenotype

**Table 2: JAK2<sup>V617F</sup> transgenic mouse models**

	JAK2 <sup>V617F</sup> gene source	Promoter	Activation	Mouse genetic background	Hematocrit (%)	WBC (10 <sup>9</sup> /L)	Platelets (10 <sup>9</sup> /L)
1	Murine	H-2Kb	Constitutive	BDF1 (low expresser group)	49.9 Vs. 50	20	1400
				BDF1 (high expresser group)	42 Vs. 50	30	2500
2	Human	Vav	Constitutive	C57/B16 (low expresser group)	42 Vs. 45	9.1 Vs. 8.3	1278 Vs. 968
				C57/B16 (high expresser group)	50 Vs. 45	11.8 Vs. 8.3	2708 Vs. 968
3	Human	Vav-Cre	Inducible	C57/B16	47 Vs. 46	13 Vs. 11	3710 Vs. 1409
		Mx-Cre	Inducible pIpC (6X)		49 Vs. 46	28 Vs. 11	8883 Vs. 1409
			Inducible pIpC (3X)		51 Vs. 46	14 Vs. 11	2792 Vs. 1409
			Inducible pIpC (1X)		61 Vs. 46	34 Vs. 11	5316 Vs. 1409

**Table 2: JAK2<sup>V617F</sup> transgenic mouse models, continued**

	Phenotype	Spleen (g)	MF	Effect on lineages	Colony formation	Reference
1	ET, PV	1.0 Vs. 0.1	No	Ter119+CD71+: ↑ SPI Gr1+Mac1+: 10 X ↑ SPI, ↑ BM	↑ EEC	Shide et al., 2008
	MF	1.57 Vs. 0.1	Yes	Ter119+CD71+: ↓ BM, SPI Gr1+Mac1+: 10 X ↑ SPI, BM	↑ EEC	
2	-	0.08 Vs. 0.09	No	Ter119+CD71+: ↑ SPI GR1: ↑ SPI, ↑ BM	NA	Xing et al., 2008
	ET, PV	0.18 Vs. 0.09	Yes	Ter119+CD71+: ↑ SPI GR1: ↑ SPI, ↑ BM	↑ BFU-E, CFU-E CFU-GM, CFU-GEMM, ECFU-Mk, EEC	
3	ET	0.35 Vs. 0.1	Yes	Ter119+CD71+: ↑ SPI, ↓ BM Myeloid cells: ↑ SPI, BM	↑ CFU-GM, CFU-GEMM, CFU-G, BFU-E, CFU-MK	Tiedt et al., 2008
	ET	-	Yes		↑ CFU-GM, CFU-GEMM, CFU-G, BFU-E, CFU-MK	
	PV	0.75 Vs. 0.1	Yes	Ter119+CD71+: ↑ SPI, ↓ BM Myeloid cells: ↑ SPI, BM	↑ CFU-GM, CFU-GEMM, CFU-G, BFU-E, CFU-MK	
	PV	-	Yes		↑ CFU-GM, CFU-GEMM, CFU-G, BFU-E, CFU-MK	

### 3.3.3. KI mouse models

Clinical evidences suggest that JAK2<sup>V617F</sup> heterozygosity is associated with ET and JAK2<sup>V617F</sup> homozygosity is associated with PV<sup>75,203</sup>. Moreover, RV BMT and TG mouse models have clearly demonstrated the crucial role of JAK2<sup>V617F</sup> expression on MPN phenotype. Therefore, the expression of JAK2<sup>V617F</sup> at physiological levels was mandatory to precisely dissect this hypothesis. This was achieved in various JAK2<sup>V617F</sup> KI mouse models in which JAK2<sup>V617F</sup> is expressed under the control of endogenous promoter.

**1. Akada *et al.***<sup>156</sup> were first to utilize this approach to generate JAK2<sup>V617F</sup> KI heterozygous and homozygous conditional mice. JAK2<sup>V617F</sup> is introduced in mouse gene and a floxed mouse *JAK2* cDNA containing 13-24 exons followed by PGK-Neo-stop cassette is inserted in intron 12. Removal of this WT JAK2 to express JAK2<sup>V617F</sup> is induced at adult age by pIpC injections. Strikingly, pIpC injection in heterozygous floxed mice, crossed with MxCre Tg mice, resulted in a non-heterozygous JAK2<sup>V617F</sup> mice with only 50% JAK2<sup>V617F</sup> mRNA as compared to WT mRNA. These mice developed increased hematocrits, WBC, platelet counts, low serum Epo levels, splenomegaly and mild BM fibrosis hence recapitulating human-PV like disease with its evolution into secondary myelofibrosis. Interestingly, homozygous expression of JAK2<sup>V617F</sup> also resulted in PV-like phenotype of severe nature as compared to heterozygous mice with pronounced blood parameters, splenomegaly and BM fibrosis. Notably, hemoglobin levels were found to be lower in homozygous mice as compared to heterozygous mice. Moreover, BM cellularity is also decreased with the increase of JAK2<sup>V617F</sup> allele burden. The JAK2 downstream signaling was more enhanced in homozygous mice than in heterozygous mice. Interestingly, LSK and myeloid progenitors (CMP, GMP and MEP) populations were significantly increased in the BM and spleen of these KI mice along with increase of BFU-E, CFU-E and CFU-GEMM and EEC.

Overall, heterozygous JAK2<sup>V617F</sup> expression in these mice did not result in an ET-like disease as expected from patients' studies. Expression of JAK2<sup>V617F</sup> increased HSPC pool in these mice.

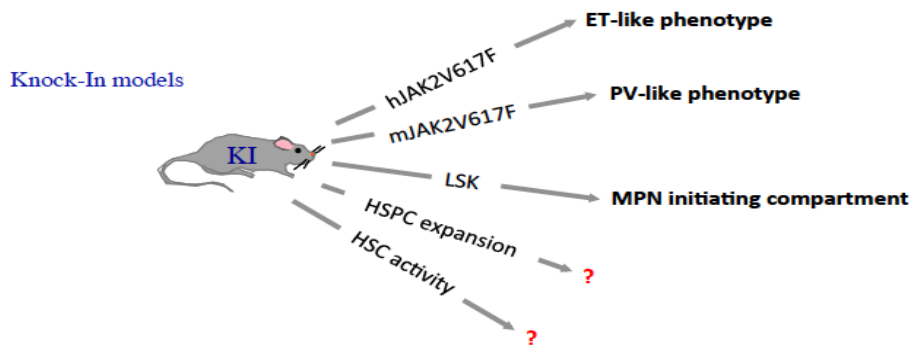
**2. Mullally *et al.***<sup>195,196</sup> were next in exploring the role of JAK2<sup>V617F</sup> in MPN development. They developed a conditional heterozygous JAK2<sup>V617F</sup> KI mouse model using the flex switch technology in intron 13. These floxed heterozygous mice were then crossed with E2ACre transgenic mice to induce germline JAK2<sup>V617F</sup> expression. These mice displayed elevated hematocrits, WBC count and splenomegaly. BM and spleen histopathology revealed marked erythroid and mild megakaryocytic hyperplasia. Thus, these mice featured human PV-like disease with reduced survival. Intriguingly, despite the mild increase in megakaryocytes in spleen and CD41+ cells in BM no increase in megakaryocyte ploidy or platelet numbers was observed. Moreover, increased WBC counts did not increase the Gr1Mac1 compartment size in these mice. Reticulin fibers were absent in spleen and BM of these animals, even in older mice (>6 months of age). Immuno-phenotypic analysis revealed no quantitative increase in LSK (Lin<sup>-</sup>Sca1<sup>+</sup>cKit<sup>+</sup>), as opposed to Akada *et al.*, and LT-HSC (LSK CD150<sup>+</sup>CD48<sup>-</sup>) but an increase in myeloid progenitors (Lin<sup>-</sup>Sca1<sup>-</sup>cKit<sup>+</sup>) mainly as a result of expansion in MEP (Lin<sup>-</sup>Sca1<sup>-</sup>cKit<sup>+</sup>FcγR<sup>-</sup>CD34<sup>+</sup>), Pre-CFU-E (Lin<sup>-</sup>cKit<sup>+</sup>CD150<sup>+</sup>CD41<sup>-</sup>CD105<sup>+</sup>) and Pro-erythroblast (CD71<sup>+</sup>Ter119<sup>+</sup>) populations. These results demonstrated that JAK2<sup>V617F</sup> resulted in marked erythroid skewing of hematopoietic progenitors. Interestingly, JAK2<sup>V617F</sup> rendered these expanded erythroid progenitors Epo hypersensitive but they did not show Epo independent growth. Fractionated BM transplantation experiments demonstrated that MPN initiating population resides within LSK compartment and that JAK2<sup>V617F</sup> did not transform MEP or GMP populations.

Competitive BM transplantation assay revealed that JAK2<sup>V617F</sup> gives a competitive advantage to early hematopoietic and late myeloid cellular stages.

**3. Li and colleagues**<sup>173</sup> generated a conditional heterozygous JAK2<sup>V617F</sup> KI mouse model that expressed human JAK2<sup>V617F</sup> allele under the control of mouse regulatory elements. A floxed PGK-Neo-poly(A) transcriptional cassette followed by human JAK2<sup>V617F</sup> cDNA and a SV40-poly(A) were introduced into the translation start of exon 2 of mouse JAK2 and targeted to mouse ES. These floxed mice were then bred with Mx1Cre transgenic mice and progeny was injected with pIpC at 6 weeks of age to excise PGK-Neo-Poly(A) cassette and to induce heterozygous expression of human JAK2<sup>V617F</sup>. Of 6-week pIpC injection, JAK2<sup>V617F</sup> expression resulted in significantly increased platelet counts and mildly elevated hematocrit but not in consistent significant increase in WBC. No change in plasma Epo levels was observed even after 26 weeks of pIpC injection. BM histology revealed megakaryocyte hyperplasia with clustered hyperlobated megakaryocytes. No increase in reticulin fibers was observed even 26 weeks after pIpC injection. Spleen histology was normal at 6 and 26 weeks after pIpC injection. These features demonstrated that heterozygous hJAK2<sup>V617F</sup> expression resulted in an ET-like disease in these mice.

Interestingly, in later stages of disease (>26 weeks after pIpC injection) ~10% animals developed PV with elevated hematocrit, splenomegaly and a drop in platelet counts with trilineage hyperplasia in BM and spleen. Animals showing a PV-like disease did not show an increase in reticulin fibers. However, one mouse in this group displayed MF-like features with the development of anemia, leukopenia, splenomegaly and accumulation of reticulin fibers in BM. Expression of JAK2<sup>V617F</sup> resulted in increased pools of CFU-Mk, CFU-GM, CFU-M and BFU-E at early stages of disease (6 weeks after pIpC injection) with sustained increase in erythroid progenitor pool even late after disease induction. Interestingly, in contrast to what was observed in previous two JAK2<sup>V617F</sup> KI mouse models, expression of human JAK2<sup>V617F</sup> had deleterious effects on HSPC compartment. These mice showed no quantitative change in HSPC compartment (LSK, CMP, GMP and MEP) on initial stages of disease but reduced numbers of LSK and progenitors 26 weeks after induction of disease. LSK cells demonstrated reduced cell cycle, increased DNA damage and reduced apoptosis. Furthermore, competitive BM transplantation assay from these KI mice demonstrated that JAK2<sup>V617F</sup> impaired competitive capacity of BM cells. Secondary transplantation further emphasized on compromised self-renewal capacity of HSC compartment.

Overall, these JAK2<sup>V617F</sup> KI mouse models have profoundly increased our knowledge about the pathophysiology of MPN especially the functional consequences of JAK2<sup>V617F</sup> on HSPC compartment. Each model developed a transplantable MPN. Surprisingly, in contrast to what was expected from clinical studies, mouse JAK2<sup>V617F</sup> heterozygous expression did not result in an ET phenotype, which is observed only in Li's model where human JAK2<sup>V617F</sup> is used. This phenotypic difference points towards the probable inherent differences in human and mouse genes, difference in mouse and human environment and the targeting strategies. Mullally and co-workers demonstrated for the first time that MPN initiating cells reside in LSK compartment. Although, JAK2<sup>V617F</sup> is found in LT-HSC in MPN patients<sup>49</sup> and expected to give a subtle advantage to HSC in order to sustain life long disease, all three models demonstrated contradictory functional consequences of JAK2<sup>V617F</sup> on HSPC. Whether JAK2<sup>V617F</sup> expands the HSC pool and renders them a competitive advantage or impairs their HSC activity remains the open question.<sup>197</sup>



**Figure 19. Lessons from JAK2<sup>V617F</sup> Knock-In mouse models** Mouse JAK2<sup>V617F</sup> expression resulted in PV-like phenotype while human JAK2<sup>V617F</sup> expression resulted in an ET-like phenotype. MPN initiating cells reside in LSK compartment. The effect of JAK2<sup>V617F</sup> on HSC compartment is yet to be revealed.

**Table 3: JAK2<sup>V617F</sup> KI mouse models**

	JAK2 <sup>V617F</sup> gene source	Activation	Zygoty	Allele expression ratio (VF: WT)	Hematocrit (%)	WBC (10 <sup>9</sup> /L)	Platelets (10 <sup>12</sup> /L)
1	Mouse	Conditional pIpC Inducible	Heterozygous Homozygous	0.53:1 -	80 Vs. 50 70 Vs. 50	40 Vs.10 58 Vs.10	1.5 Vs. 0.8 3.5 Vs. 0.8
2	Mouse	Conditional Constitutive	Heterozygous	VF<WT	80 Vs. 45	17 Vs. 10	No significant increase
3	Human	Conditional pIpC Inducible	Heterozygous	1:1	60 Vs. 55	10 Vs. 8	1.7 Vs. 1.1

**Table 3: JAK2<sup>V617F</sup> KI mouse models, continued**

	Phenotype	Spleen (g)	MF	Effect on cell lineages	Colony formation	Effect on HSPC	Reference
1	PV	0.7 Vs. 0.1	Yes	Ter119+CD71+: ↑10X SPI Gr1+Mac1+: ↑SPI	↑CFU-GM, CFU-GEMM, BFU-E, EEC	↑LSK, CMP, GMP, MEP	Akada <i>et al.</i> , 2010
	PV	1.8 Vs. 0.1	Yes	Ter119+CD71+: ↑20X SPI Gr1+Mac1+: ↑SPI	↑CFU-GM, CFU-GEMM, ↑↑BFU-E, EEC	↑LSK, CMP, GMP, MEP	
2	PV	0.08 Vs. 3.1	No	Ter119+CD71+: ↑SPI, BM CD41+: ↑BM	↑BFU-E	↑LK, MEP, Pre-CFU-E	Mullally <i>et al.</i> , 2010
3	ET (10% PV)	Normal	Yes (In 1 mouse)	Ter119+CD71+: ↑SPI, BM Gr1Mac1: ↑SPI, BM CD41+: ↑BM	↑CFU-GM, CFU-M, CFU-G, BFU-E, EEC	↓LSK, CMP, GMP, MEP	Li <i>et al.</i> , 2010

### 3.4. Pre- JAK2<sup>V617F</sup> events: *back to square one?*

Although the presence of JAK2<sup>V617F</sup> in majority of MPNs patients and the development of MPNs via JAK2<sup>V617F</sup> expression in mouse models endorse the fact that JAK2<sup>V617F</sup> is an important pathologic event to induce the disease, yet there exist other observations which compel to believe that probably a pre-JAK2 event might occur that provides a fertile ground for the development and/or progression of the disease.

The very first evidence came from X-inactivation clonality and quantitative JAK2<sup>V617F</sup> assay studies in 2006. It has been demonstrated that clonal granulopoiesis is correlated with JAK2<sup>V617F</sup> allele ratio in most PV but not in ET and PMF patients suggesting that JAK2<sup>V617F</sup> is sufficient to derive clonal hematopoiesis in PV but not in ET and PMF. Some patients with ET and PV with clonal granulopoiesis harbored a minor sub-clone positive for JAK2<sup>V617F</sup> suggesting that JAK2<sup>V617F</sup> is acquired on a clonal background. Moreover, some ET and PMF patients having clonal hematopoiesis were negative for JAK2<sup>V617F</sup> pointing towards another genetic alteration that phenocopy JAK2<sup>V617F</sup> <sup>52,184-186</sup>. These studies suggest that there must be another genetic factor involved in the disease clonal emergence and JAK2<sup>V617F</sup> is a late genetic event acquired during disease progression.

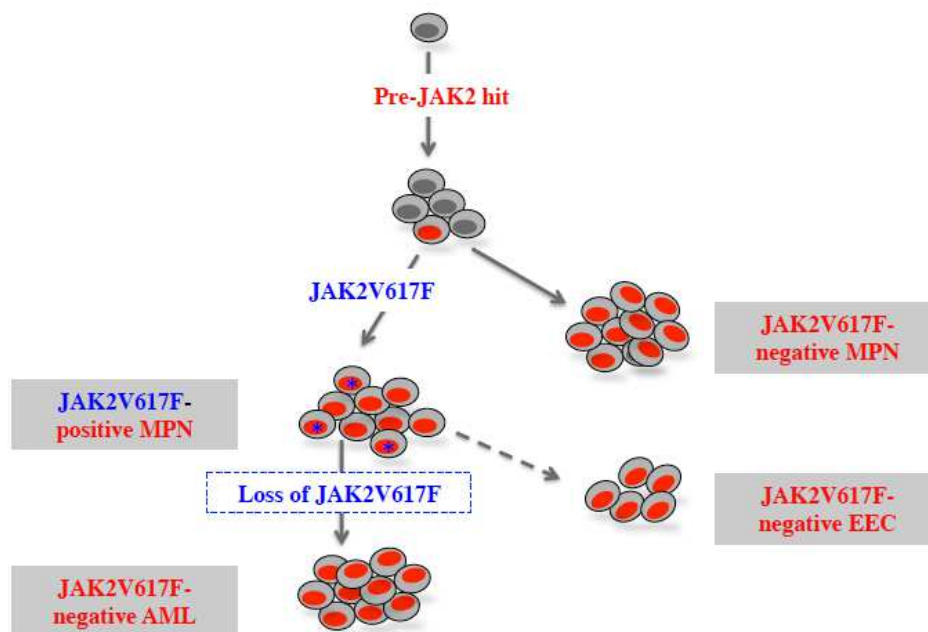
Another evidence in the favor of the idea that acquisition of JAK2<sup>V617F</sup> is preceded by a yet-undefined event is transformation of JAK2<sup>V617F</sup> positive MPNs to JAK2<sup>V617F</sup> negative AMLs. In a study of 17 JAK2<sup>V617F</sup> positive MPN patients only 5 MPNs transformed into JAK2<sup>V617F</sup> positive AML while 9 progressed into JAK2<sup>V617F</sup> negative AML. Clonality study demonstrated the common ancestor of JAK2<sup>V617F</sup> positive MPN and JAK2<sup>V617F</sup> negative AML clones suggesting that additional genetic event may have preceded JAK2<sup>V617F</sup> acquisition and that the sub-clone harboring JAK2<sup>V617F</sup> disappeared at the time of transformation <sup>187</sup>.

Erythropoietin independent growth of erythroid progenitor is considered to be the hallmark of PV and this cytokine independence is attributed to JAK2<sup>V617F</sup>. However, presence of wild type EECs in PV patients compels us to believe a molecular lesion other than JAK2<sup>V617F</sup> that have rendered cytokines independence to erythroid progenitor before the acquisition of JAK2<sup>V617F</sup> <sup>186</sup>.

Studies of familial MPN cases present the most pronouncing clues to the pre-JAK2 events. First, the presence of JAK2<sup>V617F</sup>-positive ET, JAK2<sup>V617F</sup>-positive PV, JAK2<sup>V617F</sup>-positive PMF, Bcr-Abl-positive CML and JAK2 WT mastocytosis in one family suggests a common molecular lesion that predisposed the family to multiple MPNs. Second, the presence of JAK2<sup>V617F</sup> heterozygous mutation in 1<sup>st</sup> and 3<sup>rd</sup> generations while homozygous wild type 2<sup>nd</sup> generation, indicating the sporadic acquisition of JAK2<sup>V617F</sup> mutation in familial cases, point towards a masked factor that facilitate the acquisition of JAK2<sup>V617F</sup>.

Also, absence of JAK2<sup>V617F</sup> mutation in B and T-cells of these patients emphasizes on somatic acquisition of mutation like in sporadic MPNs and not the germ line transmission. And last, the increased risk of ET, PV and PMF development in first-degree relatives suggest a common shared susceptibility factor that predispose the family to the development of MPNs <sup>188,204,205</sup>.





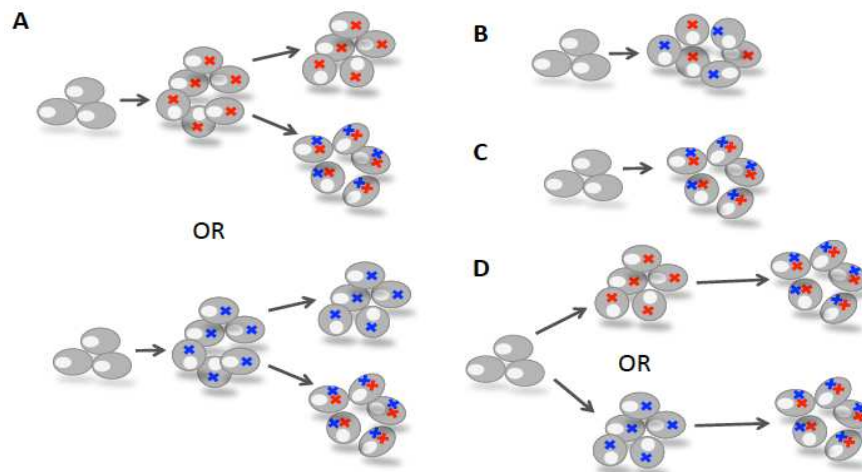
**Figure 20. Pre-JAK2<sup>V617F</sup> hits** Evidences suggest that the JAK2<sup>V617F</sup> mutation can be preceded by another not-yet-identified event that may not only phenocopy it but may also favors its acquisition. Red filling denotes ‘not-yet-identified pre-JAK2 hit’ and blue asterisk denotes ‘JAK2<sup>V617F</sup> mutation’. JAK2<sup>V617F</sup> negative MPN patients with clonal hematopoiesis are observed. In some other cases MPN patients with JAK2<sup>V617F</sup> mutation evolve into JAK2<sup>V617F</sup> mutation negative AML. Clonality studies demonstrate that JAK2<sup>V617F</sup> positive MPN and JAK2<sup>V617F</sup> negative acute myeloid leukemia (AML) originated from same ancestor clone nominating JAK2<sup>V617F</sup> a passenger mutation on the background of a pre-JAK2 event. JAK2<sup>V617F</sup> positive MPN cases with JAK2<sup>V617F</sup> negative endogenous erythroid colonies (EEC) are also reported suggesting an alteration that could be responsible for autonomous growth in these patients different from JAK2<sup>V617F</sup>.

In 2009, three teams reported a JAK2 haplotype, 46/1, as a predisposing factor to MPN. 46/1 haplotype is a 280 Kb long nucleotide sequence that consists of three genes: JAK2, INSL6 and INSL4 on chromosome 9p. It is also called ‘GGCC’ haplotype because of these four SNPs (rs3780367, rs10974944, rs12343867 and rs1159782), located in JAK2 gene, in complete linkage disequilibrium and therefore, tends to be inherited together. Genome wide SNP and chromosome 9 haplotype analyses revealed that first, the haplotype 46/1 is more prevalent in MPNs than in normal subjects and secondly, JAK2<sup>V617F</sup> mutation is acquired preferentially in cis to this haplotype<sup>206–208</sup>. Interestingly, this haplotype is demonstrated to be associated to the predisposition of ET and PMF regardless of the JAK2<sup>V617F</sup> mutation<sup>209,210</sup>. Altogether, these observations suggest that 46/1 haplotype can possibly predispose to MPNs independent of JAK2<sup>V617F</sup> and confers a favorable environment for the acquisition of JAK2<sup>V617F</sup> **Figure 20.**

### 3.5. JAK2<sup>V617F</sup> clonal diversity: *chaos is a friend of mine*

MPNs are clonal stem cell disorders driven by activated kinases like BCR-ABL1 in CML and JAK2<sup>V617F</sup> in Ph-MPNs. Given the essential role of JAK2 in myelopoiesis and overwhelming presence of JAK2<sup>V617F</sup> mutation in ET, PV and PMF, JAK2<sup>V617F</sup> alone is expected to initiate and derive MPN progression in a classic model. However, there exist deviations to this classic model with the presence of double JAK2 mutant clones, JAK2 mutant clones with other mutations or cytogenetic abnormality and different mutations in independent clones. Also, no strict temporal order in the acquisition of these mutations is followed **Figure 21**.

Several JAK2 mutations other than JAK2<sup>V617F</sup> are found to be present in the same or independent clones along with JAK2<sup>V617F</sup> in MPNs. Grunebach and colleagues found the JAK2 mutation ‘V617FD620E’ in the peripheral blood of a young PV patient. Sequence analysis of blood cell subsets revealed that while D620E is present in all the subsets, V617F is just present in myeloid lineage. These findings suggested that D620E is harbored at the apex of hematopoietic hierarchy and that V617F is acquired later with its skewed differentiation towards myeloid lineage<sup>211</sup>. Interestingly, this and another mutation E270E were also reported in 2 MPN patients negative for JAK2<sup>V617F</sup><sup>212</sup>. Other JAK2 mutations C618R and C616Y are reported to coexist with V617F in two different PV patients<sup>213,214</sup>. JAK2 exon 12 mutations are reported to be present in JAK2<sup>V617F</sup> negative PV patients<sup>128</sup> but it is also reported to be present in an independent clone in JAK2<sup>V617F</sup> positive PV patient<sup>215</sup>. JAK2<sup>V617F</sup> is also reported to be present concurrently with other tyrosine kinase abnormality, like BCR-ABL translocations, in PV and PMF patients<sup>216,217</sup>. Beer and colleagues studied different genetic lesions simultaneously acquired in MPN and their clonal relationship in a group of 13 MPN patients already screened for harboring atleast 2 lesions in peripheral blood samples.



**Figure. 21: Schematic representation of JAK2<sup>V617F</sup> clonal diversity** Different models to explain the pattern of existence and the order of acquisition of JAK2<sup>V617F</sup> and another genetic alteration, such as a mutation or cytogenic abnormality, in same patient. Red and blue crosses denote JAK2<sup>V617F</sup> and any other genetic alteration ‘X’, respectively. **A.** JAK2 mutation can be acquired first and X can co-exist with JAK2<sup>V617F</sup> as a sub-set or vice versa. **B.** JAK2<sup>V617F</sup> mutation can be acquired along with X in two separate clones. **C.** JAK2<sup>V617F</sup> and X can be acquired in same clone. **D.** X can antedate JAK2<sup>V617F</sup> or vice versa. Different orders and patterns of JAK2<sup>V617F</sup> have put it in question as a disease-initiating and/or -driving lesion.

Six patients co-harbored a cytogenetic alteration and JAK2<sup>V617F</sup> mutation. Quantitative pyrosequencing showed that three patients harbored JAK2<sup>V617F</sup> along with trisomy 9 and two patients with trisomy 8.

20q loss of heterozygosity analysis demonstrated that 20q del preceded BCR-ABL1 in 1 CML patient, it is co-harbored with JAK2<sup>V617F</sup> in 1 PMF patient and it is present in an independent clone in one JAK2<sup>V617F</sup> positive ET patient.

Six patients with JAK2<sup>V617F</sup> also carried another tyrosine kinase pathway mutation (MPL W515L, KIT D816V and JAK2 E543-D544del). Analysis of individual colonies revealed that all of these mutations were present in independent clones. X-chromosome inactivation pattern revealed that these clones may share common founder clone or may arise independently<sup>218</sup>.

In another study, del 20q is observed before as well as after the acquisition of JAK2<sup>V617F</sup> in MPN patients suggesting a lack of temporal order in its acquisition<sup>219</sup>.

Mutations in TET2 (ten eleven translocation) gene have been recently reported in MPNs. Hematopoietic clonal analysis from MPN patients demonstrated that TET2 mutations can be present in same or independent clones in JAK2<sup>V617F</sup> patients. Moreover, these mutations may follow or precede JAK2<sup>V617F</sup> mutations<sup>220-222</sup>.

Altogether, these findings suggest that JAK2<sup>V617F</sup> may collaborate with other cytogenetic abnormalities and mutations in MPN evolution and that other mutations maybe acquired independent of JAK2<sup>V617F</sup> positive clone while sharing the founder clone or not. These deviations have put JAK2<sup>V617F</sup> in question as a standalone disease-initiating and -driving lesion..

## 4. Other molecular abnormalities in MPN – *it's complicated!*

*'I think the next century will be the century of complexity.'*

-Dr. Stephen Hawking, January 2000

**A**lthough, since its discovery, JAK2<sup>V617F</sup> is monopolizing the status of the most frequently associated molecular lesion in classical non BCR-ABL MPNs (~60%), several other molecular abnormalities are also found to be present in minor subsets of these disease entities. The most relevant genetic aberrations identified till date can be categorized loosely in five groups: mutations in genes involved in intracellular signaling (*JAK2* exon 12, *MPL*, *CBL*, *LNK*, *SOCS*), mutations in epigenetic regulators (*TET2*, *ASXL1*, *DNMT3A*, *PCR2*), mutations affecting RNA splicing machinery (*SF3B1*, *SRSF2*, *SF3A1*, *U2AF1*, *ZRSR2*) and mutations involved in leukemic transformation (*IDH1/2*, *IKZF1*, *NRAS/KRAS*, *TP53*, *RUNX1*). These alterations can be mutually exclusive or can be traced back to the same ancestor founder clone devoid of specific temporal order of their acquisition.

### 4.1. Genes involved in intracellular signaling

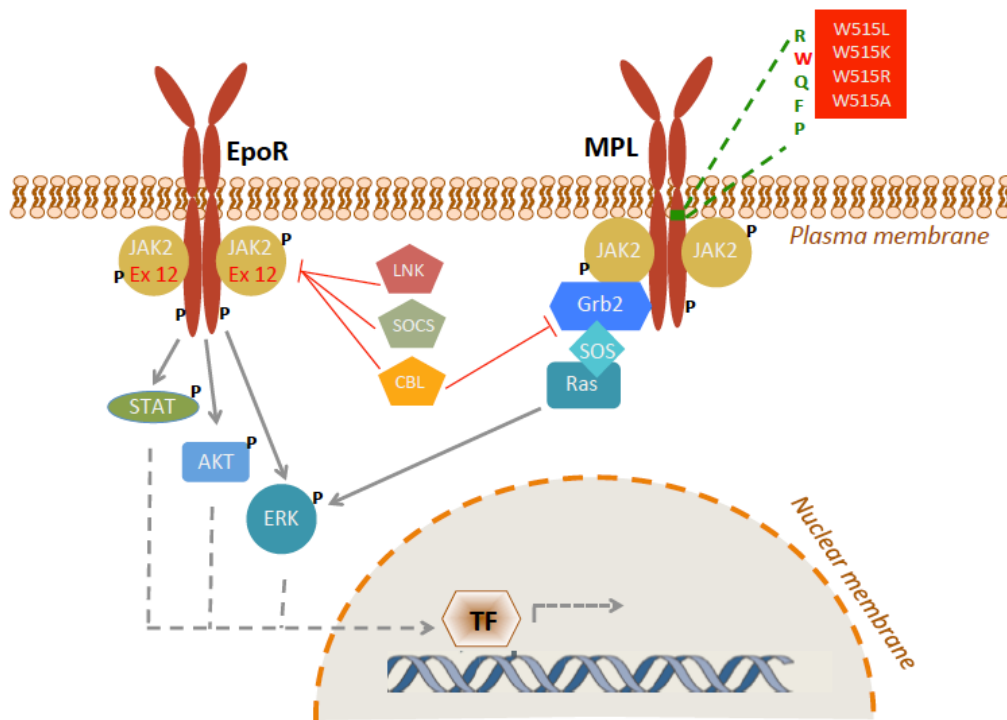
#### a) Mutations in *JAK2* exon 12

Several mutations were discovered in exon 12 of *JAK2* specifically in a small subset of JAK2<sup>V617F</sup> negative PV but not in ET and PMF, comprising around 3% of all PV patients<sup>128</sup>. However, these exon 12 mutation positive PV may transform into secondary myelofibrosis<sup>223</sup>. These mutations are found in region between SH2 and JH2 domains and are thought to modify JH2 structure in similar manner as JAK2<sup>V617F</sup> rendering the JAK2 constitutively active. These mutations induce cytokine independence in erythroid progenitors from PV patients and one of these mutations (JAK2K539L) has shown to cause myeloproliferative phenotype in retroviral BM transplantation mouse model<sup>128</sup>. Although *JAK2* exon 12 mutations are more frequent in JAK2<sup>V617F</sup> negative PV, it is reported to be present in an independent clone in JAK2<sup>V617F</sup> positive patient as well<sup>215</sup> **Figure 22 and 24.**

#### b) Mutations in thrombopoietin receptor gene *MPL*

Myeloproliferative leukemia virus (*MPL*) thrombopoietin receptor regulates the differentiation and proliferation of megakaryocytes and development of platelets via signaling through JAK-STAT pathway. Several gain-of-function somatic mutations were discovered in exon 10 of *MPL* gene resulting in the substitution of tryptophan at codon 515 with leucine (W515L), arginine (W515R), alanine (W515A) and lysine (W515K) in JAK2<sup>V617F</sup> negative ET and PMF but not PV patients<sup>66,68,224,225</sup>. W515 is a crucial amino acid of the 5 amino-acid amphipathic domain (KWQFP and RWQFP in mouse and human, respectively) located in the transmembrane-cytoplasmic junction of MPL receptor. These five amino acids play a major role in the cytosolic confirmation of MPL and regulate the spontaneous activation of the receptor<sup>226,227</sup>. In addition, three other mutations (S505N, A506T and L510P)<sup>228</sup> are also reported in the transmembrane (TM) domain of MPL receptor that may influence the receptor confirmation whereas mutation S505N has been also described in familial ET cases<sup>229</sup>. These mutations are present not only in up to 15% of JAK2<sup>V617F</sup> negative ET and PMF patients<sup>230</sup> but also extremely rarely with JAK2<sup>V617F</sup><sup>228,230</sup>. Clonal analysis of individual hematopoietic colonies from MPN patients revealed

that MPLW515L/K mutations are acquired at an early hematopoietic stage in a lympho-myeloid progenitor cell<sup>231</sup>. Mutation W515L is the most frequently associated mutation (~9%) in among all *MPL* exon 10 mutations and was shown to lead cytokine independent growth and thrombopoietin hypersensitivity in 32D, UT7 and Ba/F3 cell lines due to constitutive phosphorylation of JAK2, STAT3, STAT5, AKT, and ERK. When expressed in murine BM transplantation model it induced thrombocytosis, leukocytosis and splenomegaly with marked fibrosis<sup>224</sup> **Figure 22 and 24**.



**Figure 22. Signaling pathway defects involved in pathogenesis of MPN.** Mutations in exon 12 of JAK2 and thrombopoietin receptor (*Mpl*) can constitutively activate JAK2-STAT, JAK2-AKT and JAK2-MAPK pathways leading to myeloid cell survival, proliferation and differentiation. Red box indicates substitution of tryptophan (megakaryopoiesis) at codon 515 with leucine (W515L), arginine (W515R), alanine (W515A) and lysine (W515K) in amphipathic domain of *MPL* leading to spontaneous activation of the receptor. LNK, SOCS and CBL proteins negatively regulate these pathways by inhibiting or degrading cytokine receptors (like, *EpoR*, *TpoR*), tyrosine kinases (JAK2) and signaling adaptors (like GRB2) and mutations in these molecules can abolish this negative feedback mechanism that is normally supposed to constrain MPN phenotype.

### c) Mutations in *LNK* gene

*LNK*, also known as *SH2B3*, is a member of SH2B family of adapter proteins implicated in the hematopoiesis by negatively regulating JAK2 activation through its SH2 domain, thus controlling *Epo-R* and *MPL* signaling<sup>100,101</sup>. *LNK*<sup>-/-</sup> mice developed an MPN-like disorder with thrombocytosis, leukocytosis, splenomegaly, fibrosis and extensive extra medullary hematopoiesis with enhanced B-cell production<sup>232</sup>. Moreover, *LNK* deficiency also resulted in increased early hematopoietic progenitor pool as well as HSC pool with enhanced self-renewal and quiescence<sup>232,233</sup>. Seita J. and colleagues have demonstrated *LNK* as a negative regulator of HSC self-renewal through modified thrombopoietin (TPO)-mediated signal transduction in *LNK*<sup>-/-</sup> HSC<sup>234</sup>. In the same line, *MPL* signaling is shown to play a critical role in adult HSC quiescence<sup>235,236</sup>. *LNK* mutations were first described in exon 2 of *LNK* in a minor subset (~6%) of *JAK2*<sup>V617F</sup> negative ET, PMF and erythrocytosis patients resulting in

the absence of SH2 and PH domains, consequently abolishing LNK-negative feedback function completely or partially<sup>237,238</sup>. However, these mutations are found in much greater frequency (~13%) in post-MPN AML. Moreover, chronic-blast phase paired sample mutation analyses have shown that some of these mutations are only the late genetic events as they are present in blast crisis but not in chronic phase of MPN. These mutations are found to be associated with JAK2<sup>V617F</sup> and IDH2R140Q mutations in rare cases suggesting that LNK mutations are not mutually exclusive events. However it is yet to be revealed if they cohabitate in same clone or not<sup>239</sup>. These findings suggest that LNK induced phenotype may depend on additional events.

Sigal Gery and team has demonstrated that LNK inhibits JAK2<sup>V617F</sup> and MPLW515L mediated constitutively activated signaling pathways<sup>240,241</sup>. Recently, LNK deficiency was shown to participate in the acceleration of the PV phenotype in BM retroviral transduction murine model<sup>242</sup>. LNK was also shown to be over expressed in JAK2<sup>V617F</sup> positive patients<sup>241,243</sup> modulating MPN proliferative process<sup>243</sup>. Very recently, a missense mutation in *SH2B2* (second member of SH2B family, third being SH2B1) was also reported in one post-MPN leukemic patient<sup>244</sup> **Figure 22 and 24**.

#### **d) Mutations in *CBL* gene**

The CBL are E3 ubiquitin ligases and multifunctional adaptor proteins that are implicated in signal transduction in response to different stimuli. They are involved in negative regulation of receptor tyrosine kinases and can target cytokine receptors (EpoR, TpoR), tyrosine kinases (JAK2) and signaling adaptors (GRB2) in order to promote their proteasomal degradation through ubiquitination. So far, three family members of CBL are identified in humans: c-CBL, CBL-b and CBL-c<sup>102</sup>. c-CBL is implicated in negative regulation of HSC self-renewal and differentiation pathways as c-CBL deficient mice developed enhanced HSC pool, self-renewal capacity and hypersensitivity to Tpo. Likewise, CBL-b deficiency also recapitulated the same defects pointing towards the redundant functions of these proteins in HSC regulation. In contrast, double knockouts of CBL, lacking c-CBL and CBL-b, developed fatal myeloproliferative disorder with HSC defects demonstrating that they cooperate to generate a myeloproliferative phenotype<sup>245,246</sup>. c-CBL mutations are present in multiple myeloid malignancies with its frequency of 6% in PMF patients where its is very rare in ET and PV patients and are strongly associated with 11q acquired UPD<sup>247</sup>. These mutations abrogate c-CBL ubiquitin ligase activity and confer a proliferative advantage to 32D cells overexpressing FLT3. Moreover, mutated c-CBL doesn't only lose its catalytic activity but it also acts as dominant negative protein and inhibits its counterparts: CBL-b and CBL-c<sup>248</sup>. Finally, mutated c-CBL has shown to develop myeloproliferative disorder with mastocytosis in BMT mouse model, validating the role of these mutations in the pathogenic process of myeloid malignancies<sup>249</sup>. CBL mutations are also reported in upto 13% post-MPN AML patients suggesting its potential contribution to leukemic transformation<sup>77,250</sup>. In one case, *CBL* mutation is reported after the acquisition of JAK2<sup>V617F</sup> but it over rode JAK2<sup>V617F</sup> over disease evolution showing that both mutations occurred in different clones and also that CBL mutation gave the dominating advantage to cells as compared to JAK2<sup>V617F</sup><sup>247</sup>. In the same line, in another case CBL mutation was detected in a patient with JAK2<sup>V617F</sup> PMF and it persisted after leukemic transformation whereas JAK2<sup>V617F</sup> clone disappeared<sup>77</sup>. These observations demonstrate that *CBL* mutations are particularly associated with aggressive phases of MPN like PMF and secondary AML attributing its role in disease progression rather than initiation **Figure 22 and 24**.

#### e) Mutations or hyper methylation in *SOCS* gene

SOCS family of proteins consist of eight members SOCS1-7 and CIS, all containing C-terminal SOCS box and a central SH2 domain with which they bind to cytokine receptors in order to mediate negative regulation of cytokine signaling. CIS, SOCS1-3 are expressed in response to variety of cytokine signals and their over-expression results in inhibition of signaling through conventional negative feedback loop. Moreover, analysis of SOCS3 overexpressing transgenic and *SOC3*<sup>-/-</sup> mouse embryogenesis revealed that SOCS3 negatively regulates fetal liver erythropoiesis. SOCS3 TG mice phenocopied *JAK2*<sup>-/-</sup> mice mentioning that maybe SOCS3 regulates erythropoiesis through *JAK2* activity<sup>251</sup>. *SOCS* mutations are very rare in MPN (*SOCS3*<sup>F136L</sup> in PV)<sup>252</sup>; however, decrease in SOCS1 and SOCS3 expression due to hyper methylation of CpG islands is reported in *JAK2*<sup>V617F</sup> positive ET and PV and *JAK2*<sup>V617F</sup> and MPLW515 negative ET<sup>253,254</sup>. Hyper methylation in *SOCS2* is also reported in MPN patients and *in vitro* functional studies in *JAK2*<sup>V617F</sup> positive cell lines provided the evidence that *SOCS2* gene silencing maybe a step towards cytokine independence in MPN pathogenesis<sup>255</sup>. Whether SOCS collaborates with *JAK2*<sup>V617F</sup> in the pathogenesis of MPN is controversial. Initially, Hookham and colleagues demonstrated that *JAK2*<sup>V617F</sup> acquires the ability to hyper phosphorylate the SOCS3, rendering it unable to inhibit mutant *JAK2*, thus it overcomes normal SOCS3 regulation<sup>256</sup>. Afterwards, Hann and group provided the evidence that SOCS proteins preferentially degrade *JAK2*<sup>V617F</sup> protein and potentially hinder *JAK2*<sup>V617F</sup> driven cytokine independence to cells<sup>257</sup>. Thus, the exact position of SOCS proteins in the story of MPN pathogenesis is yet to be defined **Figure 22 and 24**.

### 4.2. Genes involved in leukemic transformation

#### a) *RUNX1*

The transcription factor *RUNX1* (also called AML1 or CBFA2) along with its heterodimeric partner core-binding factor beta (CBF $\beta$ ) comprise core binding factor (CBF) which is essential for definitive hematopoiesis and functional deregulation of *RUNX1* leading to leukemia<sup>258,259</sup>. In the same line several acquired genetic alterations of *RUNX1* are frequently found in AML and MDS<sup>260-262</sup>. It has also been reported that C-terminal deleted mutant of *RUNX1* attenuates the DNA-damage response in HSC thus proving a window for the accumulation of additional events required for multistep leukemogenesis<sup>263</sup>. *RUNX1* mutations are rare in MPN<sup>261,264</sup> but are far more pertinently associated with MPN progression towards AML (27-37%)<sup>77,264</sup>. Transduction of *RUNX1* mutant, AML1D171N, to CD34+ cells from chronic phase MPN patient resulted in the proliferation of immature myeloid cells, enhanced self-renewal capacity, and proliferation of primitive progenitors<sup>264</sup>. These observations strongly recommend *RUNX1* mutations as the potential leukemogenic marker in MPN stem cells and that they may induce MPN leukemic transformation **Figure 23 and 24**.

#### b) *IKZF1*

*IKZF1* codes for a lymphoid transcription factor IKAROS known for its implication in chromatin remodeling associated with normal lymphopoiesis<sup>265</sup>. Mouse models for IKAROS deficiency displayed multiple severe lymphoid defects leading to lymphoma and lymphoid leukemia<sup>266,267</sup>. Moreover, its role in myelopoiesis and HSC activity has also been established<sup>268-270</sup>. *IKZF1* mutations are particularly frequent in BCR-ABL1 positive B-progenitors acute lymphoblastic leukemia (B-ALL)<sup>120</sup>. In a recent study of a cohort of MPN patients it has

been found that *IKZF1* deletions, as a part of common deleted region of del 7p are present in <1% and 20% of chronic phase MPN and post-MPN AML patients, respectively. Clonal analysis of mutational events shows that *IKZF1* deletion is a late genetic event after the acquisition of *JAK2*<sup>V617F</sup> and del13q, suggesting it a potential driver of leukemogenesis<sup>271</sup> **Figure 23 and 24.**

#### c) *IDH1/2*

*IDH1* and *2* encode enzymes iso-citrate dehydrogenase1 and 2 that participate in the catalysis of oxidative decarboxylation of iso-citrate to  $\alpha$ -ketoglutarate ( $\alpha$ -KG) and its activity is necessary for cellular protection from oxidative stress<sup>272</sup>. *IDH1/2* mutational screening in two independent cohorts revealed several point mutations in chronic phase ET, PV and PMF (0.8%, 1.9% and 4%, respectively) and in blast phase MPN (21%) regardless of *JAK2*, *MPL* and *TET2* mutations<sup>273,274</sup>. Recently, *IDH1/2* mutations are found to be associated to lower leukemia-free survival in *JAK2*<sup>V617F</sup> positive PMF patients. Moreover, chronic-blast phase paired analysis revealed *IDH* and *JAK2* mutations at both time points suggesting *IDH* as a surrogate marker for AML progression and a collaborator of *JAK2*<sup>V617F</sup><sup>275</sup>. Mutated *IDH1/2* enzymes have reduced affinity to iso-citrate but have neomorphic catalyzing activity towards  $\alpha$ -KG into 2-hydroxyglutarate (2-HG) resulting in the net decreased supply of  $\alpha$ -KG<sup>276-278</sup>, thus disrupting the enzymatic activity of an  $\alpha$ -KG dependent enzyme TET-2. In same line of evidence, Figueroa M and team have demonstrated that *IDH2* mutants impaired the TET2 catalytic function in cells and that these mutations are mutually exclusive in a large cohort of AML patients. Moreover, depletion of *IDH1/2* or TET2 impaired hematopoietic differentiation and increased stem/progenitor cell marker expression suggesting both mutations sharing same route to leukemia<sup>279</sup>. It has also been demonstrated that 2-HG is also an antagonist of  $\alpha$ -KG and contributes to genome-wide histone and DNA-methylation alterations<sup>280</sup>. These results suggest a common mechanism of leukemogenesis by *IDH1/2* (decreasing TET2 activity) and TET2, and may explain low incidence of *IDH1/2* and *TET2* concurrent mutations<sup>274,279,281</sup> **Figure 23 and 24.**

#### d) *TP53*

*TP53* encodes a tumor suppressor protein P53 that is implicated in DNA repair mechanisms, cell cycle arrest, apoptosis and senescence after genomic damage and is a frequently altered gene in multiple cancers. *TP53* mutation is present in ~ 5-10% cases of *de novo* AML<sup>250,282</sup>. Although, *TP53* mutations are rarely associated to chronic phase MPN (3.1%), they are strongly associated to transformation of MPN to AML (20-27%)<sup>77,283</sup> suggesting *TP53* mutations as potential AML transformation predictor. These mutations mostly target both alleles. Moreover, duplication of 1q is reported in 18.18% of post-MPN AML cases while it is present in only 0.32% of chronic MPN cases<sup>283</sup>. The minimal amplified region on 1q harbor *MDM4*, a potent inhibitor of P53 often amplified in several types of cancers<sup>284,285</sup>. 1q amplification and *TP53* mutations are mutually exclusive events pointing towards a common role of both anomalies in the transformation of MPN to AML. In one interesting case of post-ET AML, transformation occurred with the loss of wild type *TP53* on the background of *MPL*, *TET2* and multiple *P53* mutations in an oligo clonal hematopoietic system<sup>286</sup>. All these observations compel to believe that *TP53* plays an important role in MPN leukemic transformation **Figure 23 and 24.**



#### e) *NRAS/KRAS*

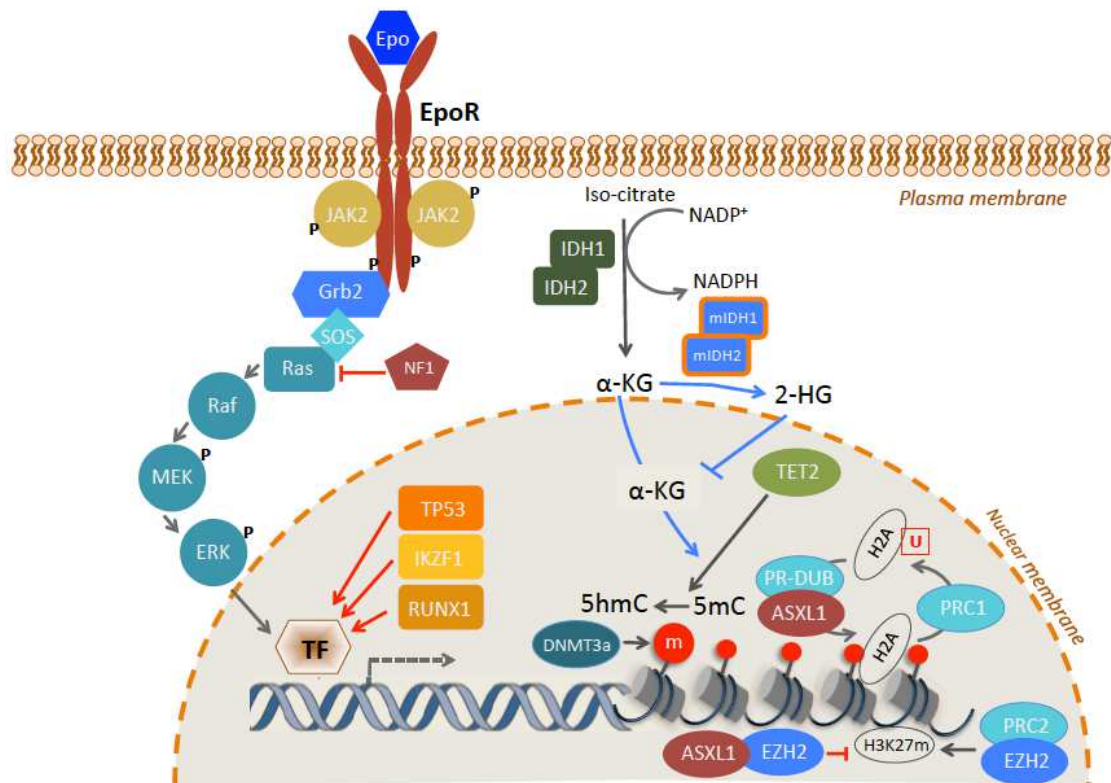
RAS, a small GTPase, act through three downstream signaling molecules RAF, MEK and ERK/MAPK for the transmission of cytokine signals to the nucleus. In mammals there are three homologs of RAS: HRAS, KRAS and NRAS. The most frequent mutation is *KRAS* and *NRAS* occur at codon 12, 13 and 61 leading to inhibition of their activity resulting in uncontrolled activation of RAS downstream pathways. RAS mutations are found in approximately 13% of post-MPN AML patients pointing towards its contribution in the progression of MPN into AML. However, there is evidence of this mutation in the chronic phase of MPN of an AML patient raising the question of its contribution in the initiation of disease<sup>77</sup>. NF1 (neurofibromin1), a negative regulator of RAS is also found mutated in MPNs, mostly in MF and rarely in ET and PV<sup>163,164</sup> **Figure 23 and 24**.

### 4.3. Genes involved in epigenetic regulation

#### a) *TET2*

The TET family (*TET1*, *TET2* and *TET3*) encodes for a  $\alpha$ -ketoglutarate dependent methylcytosine dioxygenase that converts 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5-hmC)<sup>287,288</sup>. 5-hmC is widely distributed in all tissues but its function is not known<sup>289</sup>. Mutations in *TET2* are frequent in multiple myeloid malignancies and are associated with compromised catalytic activity. Patients with mutated *TET2* show low global level of 5hmC than healthy controls<sup>290,291</sup>. *TET2* mutations can be missense or nonsense substitutions, splice site, and insertions or deletions resulting in frame shift or stop codon that are sufficient to impair its function placing it in the category of tumor suppressor genes. In-vitro studies implying *TET2* knock-down in human cord blood CD34+ cell demonstrated that its disruption affects 5-hmC levels in human myeloid cells and enhances monocytic differentiation on the expense of lymphoid and erythroid lineage thereby contributing in the pathogenesis of myeloid malignancies<sup>291</sup>.

Recently, several *TET2* deficient murine models provided evidence that *TET2* disruption decreases the level of genomic 5hmC, enhanced lymphopoiesis and myelopoiesis, extra medullary hematopoiesis, splenomegaly, increases progenitors and boosted HSC pool, self-renewing potential and repopulating capacity in competitive transplantation assays<sup>292-295</sup>. These findings clearly suggest its role as a tumor suppressor whose deregulation may contribute to the pathogenesis of myeloid malignancies. Indeed, *TET2* mutations are found in 4-11% ET, 15% PV, 19% PMF and 26% post-MPN AML<sup>296</sup>. *TET2* mutations are present in HSC of patients and clonal studies showed that it lacks temporal order of acquisition, say; it can precede *JAK2*<sup>V617F</sup>, maybe acquired as a late genetic event after *JAK2*<sup>V617F</sup> or can be present in independent clones in same patients<sup>220-222</sup>. Moreover, *JAK2*<sup>V617F</sup> and *TET2* mutations seem to play a synergistic role in erythroid clonal dominance<sup>222</sup>. A recent study highlighted the role of *TET2* as an age associated myeloid malignancies initiator. In a cohort of elderly individuals (>65 years) without hematological malignancies mutated *TET2* was present in ~5% cases associated with clonal hematopoiesis. In a 5-year follow-up of these cases one patient developed *JAK2*<sup>V617F</sup> positive ET<sup>297</sup>. This study revealed an ideal disease-initiating role of *TET2* in old-age associated MPN. One case of germline *TET2* mutation along with *JAK2*<sup>V617F</sup> mutation is also reported in an MPN patient<sup>220</sup> **Figure 23 and 24**.



**Figure 23: Genes involved in leukemic transformation and epigenetic modulations** RAS activating mutations are frequently found during leukemic transformations leading to uncontrolled activation of JAK2-MAPK pathway. NF1, a negative regulator of RAS, is also found mutated in MPN patients thus providing an additional way to the activation of MAPK signaling. Mutations in RUNX1 and TP53 and deletions in IKZF1 are also reported to be strongly associated with post-MPN leukemic transformation. IDH1/2 catalyze oxidative decarboxylation of iso-citrate to  $\alpha$ -ketoglutarate ( $\alpha$ -KG) and protects the cell against oxidative stress. IDH1/1 mutations are frequently associated with leukemic transformation. TET2 converts 5mC to 5hmC and needs  $\alpha$ -KG to accomplish this reaction. Mutations in TET2 reduce levels of 5hmC in cell. IDH1/2 mutation may also indirectly participate in epigenetic regulation through TET2 signaling (indicated by blue lines). Mutant IDH1/2 converts  $\alpha$ -KG into 2-hydroxyglutarate (2-HG) resulting in  $\alpha$ -KG reserve depletion and reduced 5hmC. 2-HG may also antagonize  $\alpha$ -KG. Interestingly, gain-of-function mutations of IDH1/2 (mIDH1/2) and loss of function of Tet2 are mutually exclusive in MPN. DNMT3a maintains CpG dinucleotides in methylated state and mutations in DNMT3a may interrupt its gene silencing potential. EZH2, component of the polycomb complex PRC2 with SUZ12, catalyzes the trimethylation of lysine 27 of histone H3 (H3K27me3) repressive mark and loss of function alterations in EZH2 or ASXL1 (that interacts with PRC2 components), later resulting in failure of EZH2 recruitment to its loci, may disrupt its methyl transferase and associated repressive activity. ASXL1 may form a complex with a deubiquitinase (like PR-DUB in drosophila melanogaster) and counteract Histone 2A (H2A) ubiquitination by PRC1 complex. Epigenetic mutations may lead to aberrant gene expression, gene activation or failure of repression. Red filled circles represent methyl groups, U represent ubiquitin molecule and TF means transcription factor.

#### b) PRC2

Polycomb repressive complex (PRC) is a protein complex that plays an essential role in development, cell fate decision and proliferation, and is recognized as global epigenetic transcriptional repressors. Two main families of PRC are identified in mammals, namely PRC1 and PRC2. The catalytic core of PRC2 is composed of SUZ12, EED, RBAP46/48 and EZH1 or EZH2 while other members being AEBP2, JARID2, PHF1, PHF19, and MTF2. EZH (enhancer of zeste homolog) catalyze the trimethylation of lysine 27 of histone H3 (H3K27me3)<sup>298</sup>. EZH2 deletions and truncating loss-of-function mutations are described in multiple myeloid malignancies with its frequency of 3% in PV and 13% in PMF<sup>299-301</sup>. These findings suggest the abrogation of histone methyltransferase activity of EZH2 as a potential mechanism of leukemogenesis and point towards its role as a

tumor suppressor. EZH2 can be involved indirectly to hematopoietic abnormalities. Moreover, somatic mutations and deletions affecting other members of PRC2, like SUZ12, JARID2 and AEBP2, are also observed recently in PV and other MPN/MDS patients<sup>302,303</sup>. These observations clearly demonstrate that PRC2 function maybe compromised in myeloid disorders by mutation of distinct genes **Figure 23 and 24**.

### c) *ASXL1*

ASXL1 (Additional sex comb like 1) is a nuclear polycomb protein involved in transcriptional repression and activation via its N-terminal region by modulation of histone 3 (H3). *Asx*, homolog of ASXL1 in drosophila, form a complex with polycomb repressive proteins called Polycomb repressive deubiquitinase (PR-DUB). Loss of *Asx* resulted in deubiquitination of H2Aub1 and repression of *HOX* genes. PRC1 is shown to be associated with monoubiquitination of histone H2A through its E3 ligase activity. Thus, polycomb gene repression is a result of dynamic balance between H2A ubiquitination by PRC1 and H2A deubiquitination by PR-DUB<sup>304</sup>. Mutations in *ASXL1* are found in an important subset of MDS and CMML patients highlighting its role in myeloid malignancies<sup>305</sup>. However, ASXL1 deficient mouse model did not recapitulate myeloid disease apart from mild splenomegaly and slight augmentation in myeloid compartment<sup>306</sup>. *N/K-RAS* mutations occur concomitantly with ASXL1 in chronic myelomonocytic leukemia<sup>307</sup>. Mouse model of ASXL1 deficiency expressing a mutant of N-RAS, NRASG12D, demonstrated marked increase in *HoxA9* and *HoxA10* expression in bone marrow nucleated cells accompanied with the increased myeloid components. As *HOX* genes are the targets of ASXL1, these results suggest that loss of ASXL1 cooperates with oncogenic RAS to develop myeloid malignancies<sup>308</sup>. Knock down of ASXL1, a member of the polycomb group of proteins involved in transcriptional repression and activation, in BaF3 cells resulted in decreased genome-wide H3K27me3 despite the preserved expression of the core PRC2 members. ASXL1 physically binds to EZH2 and therefore, loss of function mutations in ASXL1 may prevent recruitment of EZH2 to its target loci<sup>308</sup>.

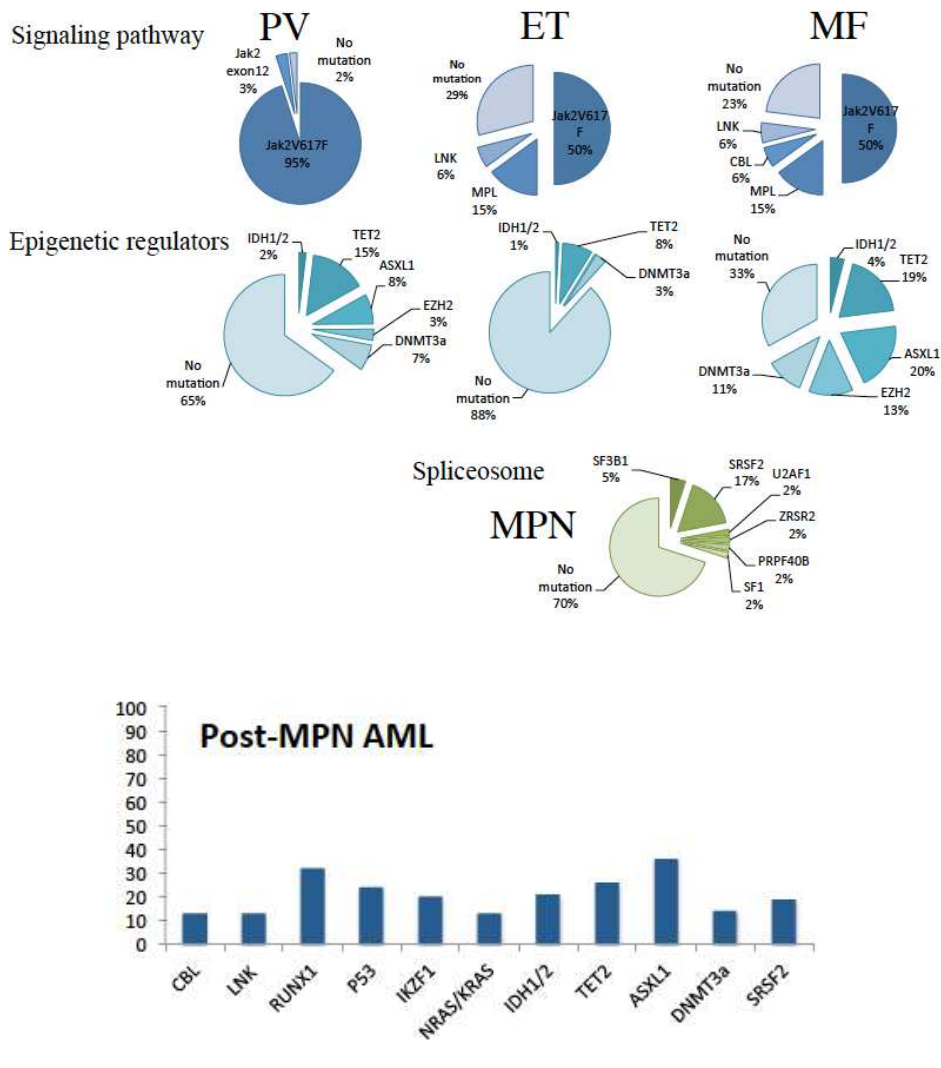
*ASXL1* mutations are very rare in ET and PMF (<8%) but they more frequent in PMF (13-26%). Moreover, 22-38% post-MF AML patients also harbor this mutation including it in a class of mutations that might participate in leukemic transformation<sup>307,309,310</sup> **Figure 23 and 24**.

### c) *DNMT3a*

DNMT3a belongs to a methyltransferase family of proteins responsible for *de novo* addition of methyl group to the cytosine within CpG dinucleotides. Recently, Challen *et al.* explored the role of DNMT3a in mouse model of DNMT3a deficiency. DNMT3a loss expanded HSC pool in BM of serial transplant model while decreasing their differentiating potential thereby upregulating HSC multipotency genes and down regulating differentiation factors, establishing DNMT3a a critical epigenetic silencer of HSC-specific genes to regulate efficient differentiation<sup>311</sup>. *DNMT3a* mutations are frequently found across the spectrum of myeloid malignancies with its frequency of 3% in ET and 7% in PV, 7-15% in PMF and 14% in post-MPN AML<sup>312,313</sup>. In all cases these mutations are heterozygous implying that just the haploinsufficiency of DNMT3a is sufficient for oncogenic phenotype. Moreover, its acquisition concomitant with other oncogenic mutations does not rule out the possibility that it collaborates with other lesions to induce leukemogenesis **Figure 23 and 24**.

#### 4.4. Genes involved in RNA splice machinery

RNA splicing is a highly orchestrated process of recruitment, rearrangement and disengagement of small nuclear ribonucleoprotein (snRNP) and other protein components in order to tailor pre-mRNAs into translatable mRNAs. Mutations in these components are found in 9.4% of MPN patients<sup>314</sup>. SF3B1, a core protein of spliceosome, is found mutated in spectrum of myeloid neoplasms especially in myelodysplastic syndrome. Mutations in *SF3B1* are also noted in 3% ET<sup>315</sup> and 4-6.5% PMF<sup>315,316</sup> patients. No association between *SF3B1* mutations and thrombosis was noted. Furthermore, *SF3B1* mutations did not influence survival. However, they are found to be strongly associated with BM ring sideroblast<sup>316</sup>. Mutations in *SRSF2*, serine/arginine-rich splicing factor 2, are found in 17% of PMF patients and 18.9% of AML patients derived from MPN. This mutation is associated with poor over-all and leukemia-free survival. *SRSF2* mutations are found to be significantly associated with *IDH* mutations<sup>317,318</sup>. These observations suggest that *SRSF2* mutations may contribute to leukemic transformation of MPNs. Mutations in other genes of splicing machinery such as *U2AF1*, *ZRSR2*, *PRPF40B* and *SF1*, are found in less than 2% of MPN patients<sup>314</sup>. The functional consequences of these mutations in MPN pathogenesis are yet to be revealed **Figure 24**.



**Figure 24.** Approximate frequencies of recurrent mutations found in MPN and post-MPN AML

## 5. Treatments of MPN

We have come a long way in the understanding of Ph- MPN from Dameshek's speculations to its present complex molecular definition, where it is established that MPN are driven by constitutive JAK2 downstream signaling through JAK-STAT, PI3K and MAPK pathways, and of-course the biggest challenge to-date is to find a way to its complete cure. Although, the discovery of JAK2<sup>V617F</sup> have accelerated the investigations of new JAK2-inhibitor molecules but, till yet, none of them have proven to be curative for ET, PV or PMF letting the place to conventional therapy with the primary goals to prevent thrombohemorrhagic events in ET and PV, and to alleviate anemia, splenomegaly and constitutional symptoms in PMF or post-ET/PV myelofibrosis (MF). Although interferon alpha treatment is associated with same complete remission in PV patients, allogeneic stem-cell transplantation (Allo-SCT) remains the only potential curative approach restricted to high risk PMF or post-ET/PV MF because of its high morbidity and mortality consequences.

### 5.1. Conventional approaches in management of MPN

#### 5.1.1. Essential thrombocythemia

The primary goal of traditional treatments is normalization of platelet counts and resolution of thrombocytosis related symptoms. Aspirin or acetylsalicylic acid (ASA) inhibits thromboxane that in normal circumstances helps aggregate platelets. At low doses (81mg/day, range 40–100 mg/day), as an antiplatelet therapy, aspirin is recommended in all ET patients as it has shown beneficial effects against thrombosis and microvascular disturbances in ET patients<sup>319,320</sup> and especially in patients with JAK2<sup>V617F</sup> or cardiovascular risk factors<sup>321</sup>. However, in low risk ET 'wait and watch' strategy is suggested as compared to low-dose aspirin as primary prophylaxis of thrombosis<sup>321</sup>. High-risk ET patients with age over 60 years, platelets > 600 X 10<sup>9</sup>/L and bearing cardiovascular risk factors are the recommended to switch to cytoreductive therapies. Hydroxyurea (HU) or hydroxycarbamide, an alkylating antineoplastic agent, is a drug of choice in first-line therapy in such ET patients to prevent thrombosis<sup>322</sup>. The aim of HU treatment is to keep platelets lower than 400 X 10<sup>9</sup>/L. Patients who do not tolerate or are resistant to HU should be moved to a second-line therapy like anagrelide (Ana) and interferon- $\alpha$  (IFN- $\alpha$ ). Ana, a drug implicated in reducing number of platelets by interfering in the maturation of megakaryocytes<sup>323</sup>, is a current second-line drug of choice recommended in such situations<sup>324</sup>. JAK2<sup>V617F</sup> positive ET patients are shown to be more sensitive to HU as compared to Ana treatment<sup>83</sup>. Furthermore, in a comparative study, Ana treatment in ET patients was shown to be associated with decreased hemoglobin levels and increased BM fibrosis<sup>325</sup>. In another study of combination drug therapy using low doses of aspirin either with HU or Ana in high risk ET patients, aspirin plus Ana was shown inferior as compared to aspirin plus HU with associated increased rates of arterial thrombosis, hemorrhage and transformation to myelofibrosis<sup>326</sup>. Thus, special precaution is required while prescribing this drug and because of the associated side effects many a times, patients are withdrawn from Ana treatment.

IFN- $\alpha$  is a cytokine naturally released by several types of cells in response to pathogenic invasion into our body like viruses, bacteria and tumor cells. Ana- and HU-treated non-responding ET patients can be treated with recombinant IFN- $\alpha$  (subcutaneously 90  $\mu$ g/week), which is found to be effective in decreasing platelet counts,

and  $JAK2^{V617}$  allele burden. In a phase II study of pegylated (PEG) IFN- $\alpha$ 2a in a cohort of 39 ET patients, 81%, 34% and 6% patients achieved overall hematologic, molecular and complete molecular (undetectable  $JAK2^{V617F}$ ) responses, respectively. The reduction of  $JAK2^{V617}$  allele burden upon IFN- $\alpha$  suggests its selective targeting of malignant cells. However, almost all patients suffered its adverse effects and at least 10% discontinued the treatment<sup>327</sup>. Busulfan, an alkylating agent, is recommended to elderly patients as compared to IFN- $\alpha$  which is recommended to patients <65 years<sup>319</sup>. Busulfan (Bfn) is administered 4 mg/day initially and then 2mg/day in order to achieve a hematological response (platelet count < 400 X 10<sup>9</sup>/L). In a study including 36 ET patients, all the patients responded to Bfn therapy with the disappearance of thrombocytosis-related symptoms<sup>328</sup>. Other cytoreductive agents include radiophosphorus (P<sup>32</sup>) and pipobroman, a piperazine derivative, which are effective in reducing platelet counts but are associated with myelofibrotic and leukemic transformation and are preferably recommended to high-risk elderly patients (age > 75 years)<sup>324</sup>.

### 5.1.2. Polycythemia vera

The main targets of PV treatments are to maintain a safe hematocrit (Hct) level and to reduce the risks of morbidity. Phlebotomy is the first measure taken to achieve and maintain Hct<45% in all PV patients<sup>78,329</sup>. However, this non-invasive treatment does not restrain the hyper myeloproliferation. Low dose ASA is recommended in all PV patients to prevent thrombotic complications<sup>330</sup>. Hematocrit target of less than 45% had a significantly lower rate death due to cardiovascular causes and major thrombosis than did those with a hematocrit target of 45 to 50%<sup>331</sup>. High-risk PV patients are recommended to also start first-line cytoreductive therapy like HU with a target to maintain Platelet lower than 400 X 10<sup>9</sup> / L and WBC higher than 2 X 10<sup>9</sup> / L<sup>319</sup>. Patients intolerant or resistant to HU should be switched to second-line strategies like IFN- $\alpha$  or Bfn treatment. In a study, PEG-IFN- $\alpha$  2a treatment in PV patients yielded 94.6% complete hematologic response and 17.5% complete molecular ( $JAK2^{V617F}$  not detectable) response but 5% of patients had to stop treatment due to its toxic effects<sup>332</sup>. Another study also confirmed efficient results of PEG-IFN- $\alpha$  2a treatment in  $JAK2^{V617F}$  positive PV patients with a tolerability at a dose of 90 $\mu$ g/week<sup>327</sup>. P<sup>32</sup> and Bfn are the other options of treatment in PV but their use is currently restricted to PV patients aged over 65 years. Bfn is shown to induce longer remission and a longer overall survival as compared to P<sup>32</sup> in a comparative study in PV patients<sup>333</sup>. Pipobroman is also used as a second-line therapeutic agent in PV. In a comparative study of Pipobroman versus HU as a first-line therapy, former was associated with increased incidence of AML/MDS and later with myelofibrotic transformation<sup>334</sup>.

### 5.1.3. Primary myelofibrosis

Wait and watch strategy is advised in asymptomatic PMF patients. The clinical indications for treatment in PMF or post ET/PV MF are anemia and splenomegaly.

Anemia can be treated with androgen, prednisone, danazol, thalidomide, lenalidomide and erythropoiesis-stimulating drugs. Prednisone, danazol and androgens are used to treat anemia with a response rate of 30-40%<sup>335</sup>. Other immunomodulatory and antiangiogenic drugs like thalidomide is effective in anemia in 20% patients and prednisone in combination with low-dose thalidomide is effective in reducing adverse effect of the latter<sup>336</sup>. Prednisone can also be used in combination with lenalidomide, a derivative of thalidomide, with a response rate of 19-30% but myelosuppression is a major limiting factor<sup>319</sup>. Lenalidomide alone is shown effective in

decreasing *JAK2*<sup>V617F</sup> allele burden in some patients but its particularly effective del (5q13) positive myelofibrotic patients<sup>337,338</sup>. Epo is not recommended to treat anemia due to its aggravating effects on splenomegaly and ineffectiveness in blood-transfusion dependent patients<sup>319</sup>.

HU is the first-line therapy for symptomatic splenomegaly with its effectiveness in reducing spleen volume in up to 40% of patients<sup>339</sup>. In absence of response to HU and indications of symptomatic portal hypertension and rapid blood fusions, patients are advised for splenectomy. However, around 50% of patients suffer post-splenectomy complications like bleeding, thrombocytosis, hepatomegaly, thrombosis and increased circulating blasts<sup>319</sup>. Over all perioperative mortality rate in splenectomy is 10%. Radiotherapy is also an option for treating hepatosplenic and non-hepatosplenic extra medullary erythropoiesis but remissions are often transient<sup>340</sup>.

Allo-SCT is the only curative therapy in myelofibrotic patients but it is restricted to high-risk patients due to high rate of morbidity and mortality<sup>319</sup>.

Ruxolitinib is the first JAK1/2 inhibitor that has recently (November 2011) been approved by FDA for the treatment of high and intermediate risk MF. In COntrolled MyeloFibrosis Study with ORal JAK inhibitor Treatment (COMFORT)-1 and COMFORT-2 trials, ruxolitinib resulted in resolution of constitutional symptoms probably by major reduction in pro inflammatory cytokines and splenomegaly in >40% of patients and its effects were independent of the presence of *JAK2*<sup>V617F</sup><sup>341</sup>. However, thrombocytopenia and anemia are the worst side effects. It is also shown to improve survival in myelofibrosis patients<sup>342</sup>.

Ruxolitinib is currently under trial for PV and ET. It is resulted in the attenuation of splenomegaly and constitutional symptoms like MF and erythrocytosis but less effective in thrombocytosis in these patients<sup>319</sup>.

## **5.2. Investigational drugs in management of MPN**

Despite the beneficial effects of conventional therapy the cure of MPN remains an uphill task due to their non-specificity, adverse effects and low response rate. Several drugs including Pomalidomide, JAK inhibitors and histone deacetylase inhibitors are in clinical trials for MPN treatment.

### **5.2.1. JAK2 inhibitors**

#### **5.2.1.1. SAR302503 (TG101348)**

SAR302503 is the only JAK2 inhibitor that has undergone phase III clinical trial in MF patients. 39% of MF patients have responded palpable splenomegaly and constitutional symptoms up to 50%. It stood promising in effective correction of leukocytosis and thrombocytosis in more than 70% of patients. Moreover, in 45% of patients, especially in a group with high allele burden, SAR302503 treatment resulted in significant and durable decrease in *JAK2*<sup>V617F</sup> allele burden up to 50%<sup>343</sup>. However, no effect or an increase was also observed in some patients. It was also evaluated in *JAK2*<sup>V617F</sup> BMT PV murine model and shown to decrease hematocrit, endogenous colony formation, organomegaly, reticulin fibrosis, STAT5 phosphorylation and *JAK2*<sup>V617F</sup> allele burden<sup>344</sup>.

#### **5.2.1.2. CYT387**

In a phase 1/2 trial CYT387, a JAK1 and 2 inhibitor, has shown a response rate of >45% in anemia, splenomegaly and constitutional symptoms in intermediate and high risk PMF and post PV/ET MF patients.

Patients previously non-responsive to ruxolitinib, Pomalidomide or SAR302503 also responded to CYT387. Moreover, around 58% of transfusion dependent patients developed transfusion independency<sup>345</sup>. In a murine MPN model CYT387 normalized white cell counts, hematocrit, spleen size, restored physiologic levels of inflammatory cytokines and reduced *JAK2*<sup>V617F</sup> allele burden<sup>346</sup>.

### **5.2.1.3. LY2784544**

LY2784544 is a JAK2 inhibitor. In a phase 1 study including ET, PV and PMF patients it has been shown to reduce spleen size in PV and MF patients. However, no reduction in *JAK2*<sup>V617F</sup> allele burden was observed in patients<sup>341</sup>. In contrast to patient studies, recently it has been shown to specifically reduce Ba/F3-*JAK2*<sup>V617F</sup>-GFP tumor burden in the Ba/F3-*JAK2*<sup>V617F</sup>-induced SCID mouse MPN model. Moreover, LY2784544 potently inhibited STAT5 phosphorylation in Ba/F3-*JAK2*<sup>V617F</sup>-GFP tumor cells but was much less potent at inhibiting interleukin-3-stimulated WT JAK2-mediated signaling and cell proliferation<sup>347</sup>. These results suggest LY2784544 as a promising targeted agent against *JAK2*<sup>V617F</sup> for the suppression of *JAK2*<sup>V617F</sup>-induced MPN pathogenesis.

Other JAK inhibitors include lestaurtinib (CEP-701), pacritinib (SB1518), NS018 BMS911543, ITF2357, MK-0683 and XL019 and to date no impressive effect on *JAK2*<sup>V617F</sup> allele burden is achieved<sup>341</sup>.

## **5.2.2. Other drugs**

### **5.2.2.1. Pomalidomide**

It's a 2<sup>nd</sup> generation immunomodulatory drug currently in phase II trial and resulted in 25% response as a single-agent or in-combination with prednisone in MF patients. However, it is found effective in anemia response but not in the correction of splenomegaly<sup>348,349</sup>.

### **5.2.2.2. HDAC inhibitor**

Histone deacetylase (HDAC) inhibitors are also explored in Ph- MPN treatment. In an open-label pilot study Givinostat, an oral HDAC inhibitor, exposure resulted in reduction of spleen size and pruritus along with freedom from phlebotomy in a subset of patients. A modest reduction in *JAK2*<sup>V617F</sup> allele burden is also observed in ET and PV patients but not in MF patients<sup>350</sup>. Recently, it has been proposed that Givinostat acts on *JAK2*<sup>V617F</sup> positive MPN cells by down modulating hematopoietic transcription factors NFE2 and C-MYB<sup>351</sup>. These observations suggest epigenetic modulation as a promising therapeutic approach.

### **5.2.2.3. HSP90 inhibitor**

HSP90 is a chaperone protein that binds to and stabilizes proteins. *JAK2*<sup>V617F</sup> is shown to be its preferred client. PU-H71, a potent HSP90 inhibitor is shown to bind and degrade JAK2 in mutant cells, resulting in the disruption of JAK-STAT signaling. PU-H71 treatment in MPN mouse model resulted in normalization of peripheral blood cell count, splenomegaly and reduction of *JAK2*<sup>V617F</sup> allele burden<sup>352</sup>. These results provided a rationale to test HSP90 inhibitors in the treatment of JAK2-dependent MPN. In the same line, HSP90 inhibitor are shown to be potent in degrading JAK2 and blocking JAK2/STAT5, MAP kinase, and AKT signaling in cells bearing JAK2



mutations that are resistant to JAK2 inhibitors. Thus, HSP90 proves itself a promising therapeutic target in JAK2-driven cancers, including those with genetic resistance to JAK enzymatic inhibitors<sup>353</sup>.

A successful therapeutic approach signifies a specific mutation or pathway to be targeted for the correction of disease. The underpinnings of Ph- MPNs are poorly understood but still JAK-STAT pathway seems a promising target. Most of MPN patients harbor JAK-STAT activating mutations like *JAK2*, *MPL* and *LNK* and JAK-STAT axis is a crucial component in cytokine expression. In general, JAK2 inhibition is associated with abrogation of STAT3/5, Akt, and ERK phosphorylation and induction of apoptotic cell death. Apart from these mutations other mutations are also found in MPNs especially in MF and AML transition phase. These mutations are not specific to a certain MPN or mutually exclusive. To add in confusion they don't have a strict temporal order and some of them are not initiators but are drivers of clonal dominance. Finally, most mutations being loss of function they are difficult to target with drugs. Specific JAK2<sup>V617F</sup> targeting might help avoid cytokine rebound syndrome (as a result of JAK1 targeting) and myelosuppression (as a result of JAK2 targeting) but may eradicate a sub clone of disease leaving behind disease initiating or driving culprit clone. In conclusion, given such a complex situation the question why current JAK2 inhibitors have limited therapeutic outcomes in histologic, cytogenetic and molecular remissions is not surprising.

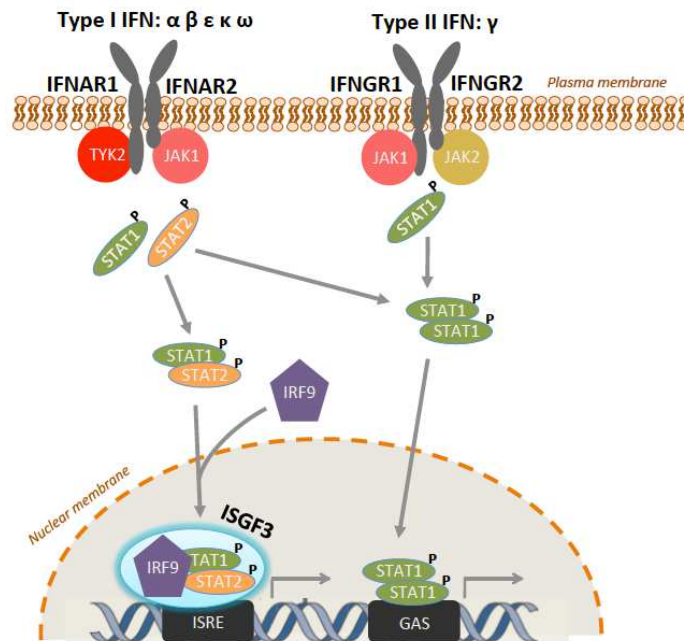
### **5.3. Interferon-alpha (IFN- $\alpha$ )**

Interferons (IFN) are widely expressed cytokines that act as first line of defense against viral infections and play an important role in adaptive immunity for the surveillance of malignant cells. Classically, IFN family includes two main classes: type I IFNs and type II IFNs. In humans, type I IFNs has 5 structural homologs IFN- $\alpha$  (which can be further subdivided into 13 different subtypes, IFN- $\alpha$ 1, - $\alpha$ 2, - $\alpha$ 4, - $\alpha$ 5, - $\alpha$ 6, - $\alpha$ 7, - $\alpha$ 8, - $\alpha$ 10, - $\alpha$ 13, - $\alpha$ 14, - $\alpha$ 16, - $\alpha$ 17 and - $\alpha$ 21), IFN- $\beta$ , IFN- $\delta$ , IFN- $\epsilon$  and IFN- $\kappa$ , which are secreted by several cell types upon viral infection. Type II IFN has only one member, IFN- $\gamma$ , secreted by immune cells exclusively. A new class of IFN is also introduced called IFN- $\lambda$  with three members IFN- $\lambda$ 1, - $\lambda$ 2 and - $\lambda$ 3 sharing the same antiviral activity as type I IFN but structurally different from them. Among all these IFNs, only IFN-  $\alpha$ 2 is approved to date for its therapeutic use in myeloid malignancies.

#### **5.3.1. Interferon mediated signaling**

IFN- $\alpha$  binds to its cognate type 1 IFN $\alpha$  receptor (IFNAR) that comprises of two chains IFNAR1 and IFNAR2. IFNAR2 has three isoforms IFNAR2-a, -b and -c. IFNAR2-a is involved in trans signaling and IFNAR2-b is short dominant negative isoform. Conventional IFNAR complex comprises of IFNAR1 and IFNAR2-c (the long transmembrane isoform) and constitutively binds to TYK2 and JAK1, respectively. IFN- $\gamma$  binds to IFN- $\gamma$  receptor, which is also composed of two subunits, IFNGR1 and IFNGR2, which are associated with JAK1 and JAK2, respectively. IFN- $\alpha$  binding to IFNAR complex induces their dimerization and auto phosphorylation that in turn activates the associated JAK family members. Activated JAK members provide the docking sites for STAT protein that form homodimers and heterodimers. STAT1-STAT2 heterodimer binds to IFN regulatory factor 9 (IRF9) to form a complex termed IFN stimulated gene (ISG) factor 3 (ISGF3) that translocate into

nucleus to bind specific elements called IFN stimulated response element (ISRE) in promoter regions of IFN regulatory genes leading to their transcription. Other STAT homo and heterodimers translocate to nucleus and bind to another type of element – IFN- $\gamma$  activated site (GAS) element- that is present in promoter region of ISG to conduct transcription. IFNAR signaling diversity is achieved in part through different STAT dimers and in part by other downstream signal transmitter molecules including MAPK, PI3-K and others.



**Figure 25. Interferon mediated signaling** Type I Interferons ( $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\epsilon$  and  $\kappa$ ) bind to IFNAR, which are associated with JAK2 and TYK2 while type II interferon ( $\gamma$ ) binds to IFNGR, which is associated with JAK1 and JAK2. Ligand binding to receptor results in activation of associated JAK members that in turn phosphorylate STAT1 and/or STAT2 molecules. Activated STATs lead to the formation of STAT1-STAT2 heterodimer that binds to IRF9 complexes, which are known as ISGF3 complexes. These complexes translocate to the nucleus and bind to ISREs in DNA to initiate gene transcription. Both type I and type II IFNs also induce the formation of STAT1-STAT1 homodimers that translocate and bind to GAS elements that are present in the promoter of certain ISGs, thereby initiating the transcription of these genes. IFAR: interferon- $\alpha$  receptor, IFGR: interferon- $\gamma$  receptor, STAT: Signaling activator and transducer molecule, IRF9: IFN-regulatory factor 9, ISGF3: IFN-stimulated gene factor 3, ISRE: IFN-stimulated response elements GAS: IFN- $\gamma$ -activated site

### 5.3.2. Molecular mechanisms of IFN- $\alpha$ action

IFN- $\alpha$  is implicated in wide range of biological activities like immune modulation, antiproliferative actions, apoptosis and anti-angiogenesis.

#### 5.3.2.1. IFN- $\alpha$ in immunomodulation

IFN- $\alpha$  may stimulate the cyto-toxic activity of T-cells and macrophage in order to target malignant cells. IFN-alpha is shown to induce the rapid differentiation of monocytes into highly activated dendritic cells (DCs) that are particularly effective in taking up complex antigens and inducing T- and B-cell immunity. In CML patients, IFN- $\alpha$  exposure resulted in DC differentiation from progenitor cells both *in vitro* and *in vivo*<sup>354</sup>. These DC cells may serve as antigen presenting cells for CML specific peptides. Moreover, these DC cells bear the capacity to promote CD8+ T-cells cross priming.

HLA-A2-restricted PR1 peptide is derived from proteinase 3 (P3) and neutrophil elastase. P3 is over-expressed

in a variety of myeloid leukemias, including 75% of CML patients<sup>355</sup>. PR1-specific cytotoxic T-cells (PR-CTLs) are increased and contribute to complete cytogenetic remission (CCR) in CML patients receiving IFN, but they are not detected in patients at relapse despite continuous treatment with IFN<sup>356</sup>. It is also proposed that IFN- $\alpha$  elicits CML-specific central memory CTL that may contribute to continuous CCR after IFN withdrawal suggesting it a therapy to prolong CR in CML<sup>357</sup>.

Several novel antigens, like PV65, PV13 and MPD5, have been identified in a subset of IFN- $\alpha$  treated PV patients. Upregulation of these self-antigen in response to IFN- $\alpha$  might enhance their abilities in elicitation of immune reactions in patients<sup>358,359</sup>. Prchal and co-worker have proposed another mechanism to eliminate malignant clone in JAK2<sup>V617F</sup> positive PV upon IFN- $\alpha$  treatment. They proposed that IFN- $\alpha$  mobilizes regulatory T-cells (T-regs) from BM to peripheral blood thereby reducing their immunosuppressive and tumor promoting in BM microenvironment. Suggesting that T-regs may, at least in part, be associated with suppression of malignant clones<sup>360</sup>.

The ensemble of these observations suggests that IFN- $\alpha$  can play a role in the generation of DC driven antitumor activity and T-cell immunity in MPN patients, pointing out its therapeutic potentials that could be successfully used in strategies of cancer immunotherapy.

#### 5.3.2.2. Effect of IFN- $\alpha$ on cell proliferation

Cell growth inhibition is one of the most known effects of IFN- $\alpha$ . Passage of cell cycle is dependent on highly orchestrated formation and activation of cyclin dependent kinase (CDK) and their cyclin protein complexes. In a mouse macrophage cell line IFN- $\alpha$  treatment was resulted in G1 arrest due to upregulation of p19 and p21, CDK inhibitors (CDKi). This upregulation of CDKi was accompanied by diminution of cyclin D1/CDK4- and cyclin E/CDK2-associated kinase activities. Likewise, IFN- $\alpha$  exposure to human lymphoid cell lines Daudi and U266 arrested them on G1 phase by up regulating p15 and p21 proteins. However, same study demonstrated that cells might escape IFN- $\alpha$  induced growth inhibition by skipping CDKi mRNA translation as IFN- $\alpha$  treatment in H9 cell line resulted in high levels of p21 mRNA but not protein<sup>361</sup>.

IFN- $\alpha$  may use other pathways to inhibit cell growth. CrkL belongs to a group of proteins that also includes c-Crk, Grb-2, and Nck. These proteins function as adapters, linking tyrosine-phosphorylated receptors or their substrates to downstream signaling elements. IFN- $\alpha$  stimulated TYK2 tyrosine phosphorylates CrkL that constitutively interacts with C3G, a guanine exchange factor for Rap-1 in U-266 and Daudi cell lines. Rap-1 is a small GTPase that antagonizes RAS signaling pathway. Thus, CrkL links the functional Type I IFNAR complex to the C3G-Rap-1-RAS growth inhibitory pathway<sup>362</sup>. In the same line, addition of CrkL antisense oligonucleotide reverted the inhibitory effects of IFN- $\alpha$  on BFU-E and CFU-GM formation from human CD34+ cells suggesting a role of CrkL in antiproliferative effect of IFN- $\alpha$ <sup>363</sup>. Of note, CrkL is a major tyrosine-phosphorylated protein in the peripheral blood of CML patients<sup>364</sup>. IFN- $\alpha$  has proven remarkable clinical activity in CML treatment. Therefore, it is worth hypothesizing that IFN- $\alpha$  implies CrkL to induce cell growth inhibitory effects.

In early studies, IFN- $\alpha$  has shown to inhibit erythroid, granulocytic and megakaryocytic progenitors growth from PV and PMF patients *in vitro*<sup>365,366</sup>. Recently, team led by Hoffman demonstrated that *in vitro* IFN- $\alpha$  treatment more effectively inhibited CFU-GM and BFU-E formation from CD34+ cells of JAK2<sup>V617F</sup> positive PV patients than controls and this inhibition is more frequently associated with JAK2<sup>V617F</sup> homozygous clones (97% of cases)

than heterozygous clones (53% of cases) suggesting a selective and JAK2<sup>V617F</sup> dose-dependent role of IFN- $\alpha$  in MPN treatment<sup>367</sup>.

Tpo induced megakaryopoiesis from human lineage depleted CD41+ (Lin-CD41+) megakaryocytic progenitors is repressed upon exposure of IFN- $\alpha$ . IFN- $\alpha$  induces SOCS-1 (suppressor of cytokines signaling-1) expression to shunt Tpo induced Mpl-JAK2-STAT3 signaling resulting in repression of megakaryopoiesis<sup>99</sup>. Furthermore, IFN- $\alpha$  does not alter human CD34+ derived megakaryocytic progenitor proliferation but intervenes in proplatelets formation and decreases the number of culture-derived platelets from human megakaryocytes in result of deregulated expression of transcription factors involved in late-stage megakaryopoiesis like GATA-1, VWF, NF-E2 and MafG. This study explains the thrombocytopenia in IFN- $\alpha$  treated patients<sup>368</sup>. Moreover, myelosuppressive effects of IFN- $\alpha$  can be justified by the fact that IFN- $\alpha$  exposure to human bone marrow stromal cells strongly down regulates the production of growth factors like GM-CSF and IL-1 and also counteracts IL-1 by inducing IL-1 receptor antagonist (IL-1RA) thereby reducing its hematopoietic activity<sup>369</sup>.

However, recently two teams have demonstrated a non-conventional effect of IFN- $\alpha$  in HSC proliferation in mouse models. Both teams demonstrated that short term IFN- $\alpha$  exposure amplifies HSC compartment and long-term treatment confers a differentiation-coupled but not self-renewal coupled proliferation hence, compromising the re-constitutive capacity of this amplified cell pool and finally exhausting HSC<sup>370,371</sup>. Esser *et al.* demonstrated that IFN- $\alpha$  treatment mediated HSC proliferation through increased STAT1 signaling that in turn upregulated SCA-1. Another pathway, possibly involved HSC activation is P13-k signaling as IFN- $\alpha$  repressed PTEN, a negative regulator of P13-k signaling, and increased phosphorylation of AKT. However, STAT1<sup>-/-</sup> mice expressed less phosphorylated AKT upon IFN- $\alpha$  treatment suggesting, atleast partially, PI3-K is downstream of IFNAR-STAT1 signaling. Consistent with these observations, DNA microarray analysis in SLAM cells revealed that IFN- $\alpha$  treatment increased the expression of cell cycle regulators CDK7 and Ccnb2 and decreased the expression of Elavl1, repressor of CDKi p21<sup>370</sup>. Likewise, Sato and group demonstrated increased expression of p21 and decreased expression of p27 and p57 CDKis in HSC of IFN- $\alpha$  treated mice. Moreover in HSC, IFN- $\alpha$  down regulated mRNA expression of c-Mpl, encoding for Tpo receptor implicated in quiescence of HSC, suggesting that loss of HSC pool is due to reduced Tpo responsiveness<sup>371</sup>.

In conclusion, IFN- $\alpha$  is found to activate several signaling pathways to inhibit or induce cell cycling and proliferation in non-exclusive manner.

### 5.3.2.3. Anti-angiogenic effect of IFN- $\alpha$

IFN- $\alpha$  has been identified as a powerful angiogenic repressor. It has shown to inhibit basic fibroblast growth factor (bFGF), interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF) gene expression. Moreover, gene expression profile of endothelial cells (EC) also demonstrated IFN- $\alpha$  induced up regulation of anti-angiogenic factors like CXCL9, CXCL10 and CXCL11. In addition, IFN- $\alpha$  induced impairment in EC proliferation and migration can be attributed to its anti-angiogenic potential<sup>372</sup>.

Patients with MPN present increased angiogenic activity with upregulated circulating serum level of VEGF. IFN- $\alpha$  treated CML patients have lower VEGF level than HU- or non-treated patients, nominating IFN- $\alpha$  as a potent anti-angiogenic drug<sup>373</sup>.

#### 5.3.2.4. Effect of IFN- $\alpha$ on cellular apoptosis

IFN- $\alpha$  may act either by inducing or protecting against apoptosis, a programmed cell death. In B chronic lymphoblastic leukemia (B-CLL) cells IFN- $\alpha$  treatment upregulated Bcl-2 expression and thereby protected the cells from apoptotic death<sup>374</sup>. Conversely, in squamous cell skin cancer (SCSC) cell line IFN- $\alpha$  induced apoptosis partially independent of Bcl-2 expression, as overexpression of Bcl-2 in IFN- $\alpha$  exposed SCSC cells conferred only partial resistance to apoptosis<sup>375</sup>. However, the reason for disparity in IFN- $\alpha$  choice of action is largely unknown and can be attributed to cell type, environment or factors secreted by cell. Microarray analysis have identified several directly and indirectly induced ISGs implicated in apoptosis. These include caspase-4, -8, tumor necrosis-related apoptosis-inducing ligand (TRAIL), Fas/FasL, death-activating protein kinase (DAP kinase), IFN regulatory factors (IRFs), X-linked inhibitor of apoptosis (XIAP), dsRNA-activated protein kinase, PML (acute promyelocytic leukemia gene) and regulator of IFN induced death (RID)<sup>376</sup>.

Biochemical studies demonstrated that hematopoietic progenitor cell (HPC) growth inhibition was accompanied with P38-MAPK phosphorylation in CD34+ cell from PV and blockade of P38-MAPK pathway restored IFN- $\alpha$  inhibited CFU-GM and BFU-E proliferation<sup>367</sup>. IFN- $\alpha$  is also shown to induce apoptosis in U266 multiple myeloma cell line through PI3K/mTOR pathway<sup>377</sup>.

These pathways can act in a non-mutually exclusive manner to induce apoptotic cell death in response to IFN- $\alpha$  exposure.

#### 5.3.3. IFN- $\alpha$ in MPN treatment

In 1987, Gutterman *et al.* were first to communicate the IFN- $\alpha$  treatment in CML and observed complete hematological response in 71% of patients in trial with impressive cytogenetic remission<sup>378</sup> and in 1990s, it was established as a better treatment choice associated with longer overall survival and delayed disease progression than other conventional therapies available<sup>379</sup>. However, patients under IFN- $\alpha$  treatment often encountered significant toxicities including depression, gastrointestinal problems and neurologic disturbance. Advent of imatinib, a selective inhibitor of BCR-ABL tyrosine kinase, has evolved the CML therapy and was found to be superior in achieving hematological and molecular response as compared to IFN- $\alpha$ <sup>380</sup>, however, long term follow-up studies shown that not all patients remain in complete molecular remission and often patients become resistant to tyrosine kinase inhibitors (TKI)<sup>381</sup>. Since then, IFN- $\alpha$  is reincorporated in CML treatment trials as a combination drug with other tyrosine kinase inhibitors, as a maintenance strategy after TKI therapy and during pregnancy. Imatinib with pegylated IFN- $\alpha$ -2a was associated with significantly higher complete molecular response than imatinib alone in a recent trial of CML patients<sup>382</sup>. Moreover, imatinib and IFN- $\alpha$ -2a combination therapy followed by IFN- $\alpha$ -2a as a maintenance strategy resulted in 75% of cases that remained in complete molecular remission even 2.4 years after discontinuation of imatinib<sup>383</sup>.

Silver, Bellucci and Giles were the pioneers in studying the effect of IFN- $\alpha$  treatment in PV and ET patients showing the suppression of myeloproliferation and correction of pruritus and other constitutional symptoms<sup>384-386</sup>. Since then, many initiatives have been taken to test IFN- $\alpha$  in clinical settings of MPN treatment using two available commercial forms, IFN- $\alpha$ -2a and IFN- $\alpha$ -2b, with no biological evidence of better efficacy of one over other. In almost all ET and PV trials, IFN- $\alpha$  treatment was resulted in rapid normalization of platelet counts, erythrocytosis, leukocytosis and splenomegaly with the achievement of objective hematological response in around 80% of patients. Around 60% of PV patients had reduced phlebotomy requirements or became

phlebotomy-free. Moreover, IFN- $\alpha$  was able to resolve the constitutional symptoms including pruritus. However, toxicity including gastrointestinal problems, musculoskeletal pains, fatigue, depression and flu-like symptoms resulted in the discontinuation of IFN- $\alpha$  therapy in around 25% of patients in trials.

In contrast, IFN- $\alpha$  response rate in MF patients are quite disappointing. Only 30% could have positive effects on splenomegaly. Of note, IFN- $\alpha$  toxicity in PMF patients was far more important than ET and PV patients and that resulted in IFN- $\alpha$  treatment withdrawal in around 50% of cases. The frequent side effect was the worsening of cytopenias. Although, these results strongly recommend IFN- $\alpha$  as a potential drug to treat ET and PV, like for any other myeloid malignancy, question arises if IFN- $\alpha$  is capable to eradicate the malignant clone.

MPN are HSC disorders and therefore, mutated HSC are the ultimate target of any therapy to completely eradicate the disease. Initial studies of IFN- $\alpha$  treatment in PV patients have demonstrated that IFN- $\alpha$  is capable of reverting monoclonal hematopoiesis into polyclonal one (results based on X-linked inactivation studies)<sup>387</sup>. Moreover, in certain patients IFN- $\alpha$  treatment achieved the hematological response with the disappearance of cytogenetic markers<sup>388-390</sup>. These studies indicate a selective action of IFN- $\alpha$  on malignant clone.

Discovery of JAK2<sup>V617F</sup> provided an excellent tool to measure the IFN- $\alpha$  activity on JAK2<sup>V617F</sup> clones in Ph-MPN. Recently, two groups<sup>327,332</sup> have published their investigations of ET and PV treatment using pegylated IFN- $\alpha$ -2a. They achieved impressive hematologic responses comparable to standard IFN- $\alpha$ -2a but with less toxicity and therefore, better patient compliance. Kiladjian *et al.* demonstrated complete hematological response (CHR) in 89% of patients and complete molecular response (CMR), based on JAK2<sup>V617F</sup> quantification in peripheral granulocytes, in 24% of patients<sup>332</sup>. However, this eradication of mutant clone doesn't mean complete eradication of malignant clone as erythroid progenitor assay after 18-40 months of discontinuation of treatment from BM of 4 patients who have achieved CMR and were still in CHR demonstrated the growth of EEC and residual JAK2<sup>V617F</sup> positive PV clones<sup>391</sup>. Cardama *et al.* have reported CHR in 38% and 54% of ET and PV patients, respectively. Moreover, CMR, based on BM mononuclear cells, was achieved in 6% and 14% of ET and PV patients, respectively<sup>327</sup>. Both of the studies indicated the selective action of IFN- $\alpha$  treatment in the eradication of JAK2<sup>V617F</sup> clones. Of note, hematologic response in both studies was achieved in few weeks after treatment whereas CMR needed at least 12 months of IFN- $\alpha$  exposure indicating towards a long-term treatment schedule to achieve a CMR.

Although, this reduction and elimination of JAK2<sup>V617F</sup> clone justifies the hematologic responses in ET and PV patients but does not exactly mean the cure to disease<sup>392</sup>. JAK2<sup>V617F</sup> can be a second genetic event<sup>184,186</sup> and IFN- $\alpha$  could have left behind the pre-existing or co-existing abnormalities. In the same line, in a patient with two molecular abnormalities, TET2 and JAK2<sup>V617F</sup>, IFN- $\alpha$  treatment could only eliminate the JAK2<sup>V617F</sup> clone without effecting TET2 clone<sup>391</sup>. Nevertheless, such results suggest that even if the malignant clone is not eradicated long term IFN- $\alpha$  treatment is sufficient to achieve and sustain CHR for long time after the discontinuation of disease, thus modifying disease natural history.

Recently, peg-IFN- $\alpha$ -2a has shown promising results in a small cohort of primary and secondary myelofibrotic patients regardless of JAK2<sup>V617F</sup> mutation. Peg-IFN- $\alpha$ -2a rapidly corrected thrombocytosis and leukocytosis (3 months) and anemia (6 months) in 80% of patients. Remarkably, 6 out of 18 patients achieved complete hematologic response and toxicity was tolerable with just 2 patients discontinuing the treatment out of 18<sup>393</sup>. Interestingly, low dose IFN- $\alpha$ -2b extended treatment shown to alter the BM histology with resumed BM architecture, improved dysmegakaryopoiesis, and disappearance of reticulin and collagen fibers in early primary

myelofibrotic patients<sup>394</sup>.

Although HU remains the first choice in ET and PV treatment but the leukemogenic risk associated with it and other cytoreductive agents like pipobroman is much important<sup>395</sup> and therefore, IFN- $\alpha$  is recommended in younger patients as a first line therapy arguing that it is a non-leukemogenic drug<sup>396</sup>.

Thus, the low dose extended IFN- $\alpha$  exposure may result in the high rates of molecular remissions in ET and PV patients, which were never reported before with any other therapy in MPN. In addition, IFN- $\alpha$  treatment yields high hematologic and histologic response with tolerable toxicity. All these characteristics nominate IFN- $\alpha$  as potential non-leukemogenic drug of choice in MPN patients.

## **RESULTS**



## **Result 1**

**JAK2<sup>V617F</sup>-positive MPN: KI mouse models and IFN- $\alpha$  therapy**

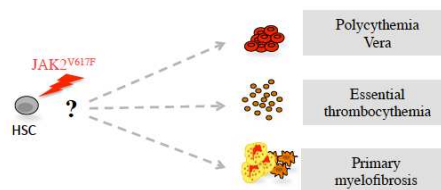
## Approach

The level of  $JAK2^{V617F}$  in retroviral BMT and TG murine models is ectopic and subjected to the positional effects of transgene integration site as well as the number of copies, that have been integrated. To better understand the pathogenesis of MPNs, it was necessary to express  $JAK2^{V617F}$  at physiological levels. Therefore, we have generated and characterized  $JAK2^{V617F}$  knock-in (KI) murine model in which mouse  $JAK2^{V617F}$  is constitutively expressed under the control of the endogenous mouse  $JAK2$  promoter.

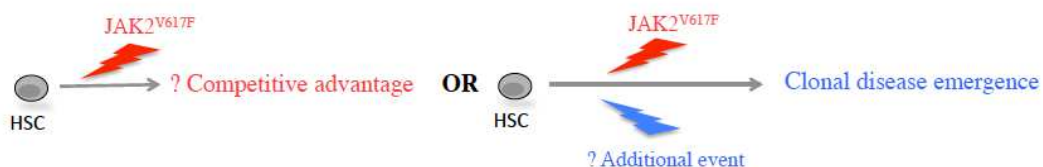
We have developed two  $JAK2^{V617F}$  KI models. The first one is constitutive (Article 1).  $JAK2^{V617F}$  is expressed in all cell types expressing  $JAK2$ , and the second one is conditional (Article 2),  $JAK2^{V617F}$  is expressed in restricted types of cells, depending upon the tissue-specific Cre recombinase expression. For the second model, we crossed the conditional  $JAK2^{V617F}$  KI mice with transgenic mice expressing Cre under a vav promoter (vav-Cre TG) in order to target the expression of  $JAK2^{V617F}$  in hematopoietic cells. In fact, vav-Cre TG mice also target expression of  $JAK2^{V617F}$  in some endothelial and germinal cells, allowing male vav-Cre/  $JAK2^{V617F}$  KI mice to be used to generate  $JAK2^{V617F}$  constitutive KI progenies.

## Objectives

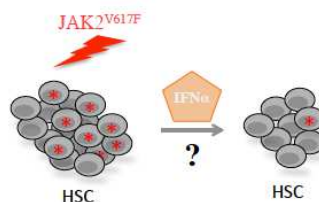
- A) To characterize the phenotype of  $JAK2^{V617F}$  KI mice



- B) To re-evaluate the effect of  $JAK2^{V617F}$  in HSC activity



- C) To assess the therapeutic potential of interferon- $\alpha$  in MPN treatment



**Figure 26. Objectives to study  $JAK2^{V617F}$  KI mouse model**

In (Article 1 and 2), we described the phenotype of  $JAK2^{V617F}$  KI. In (Article 2) we analyzed the properties of  $JAK2^{V617F}$  KI early hematopoietic cells, with a special interest for the role of  $JAK2^{V617F}$  in clonal dominance, and the effect of IFN $\alpha$  therapy on  $JAK2^{V617F}$  KI phenotype and  $JAK2^{V617F}$  disease initiating cells.

## A) Phenotype of JAK2<sup>V617F</sup> KI mice

### Context

Although, the discovery of JAK2<sup>V617F</sup> have provided us a great deal of insights into the pathogenesis of MPNs, phenotypic pleiotropy of JAK2<sup>V617F</sup> still remains an enigma. In patients, JAK2<sup>V617F</sup> heterozygosity is associated with ET and homozygosity, due to mitotic recombination, with PV. We<sup>172</sup> and others<sup>155,171</sup> have previously demonstrated that the expression of JAK2<sup>V617F</sup> in retroviral bone marrow transplantation (BMT) and transgenic (TG) murine models is sufficient to develop human MPN-like disease and, interestingly as expected from patient studies, different levels of JAK2<sup>V617F</sup> expression resulted into different disease phenotypes i.e., JAK2<sup>V617F</sup>/JAK2<sup>WT</sup> <0.5 causes ET and JAK2<sup>V617F</sup>/JAK2<sup>WT</sup> >0.5 causes PV. These observations strongly suggest that the level of JAK2<sup>V617F</sup> expression is crucial in the development of disease phenotypes.

### Results

Constitutive STAT5 and ERK1/2 phosphorylations were demonstrated in BM cells from constitutive JAK2<sup>V617F</sup> KI mice. Cloning assays showed  $\approx$  45% of endogenous erythroid colonies (EEC) in BM and spleen from constitutive or conditional vavCre / JAK2<sup>V617F</sup> KI mice (CFU-E growing after 2 days of culture without added erythropoietin). These results demonstrate that JAK2<sup>V617F</sup> in our KI mice induced constitutive activation of JAK2 downstream signaling and rendered cells independence from cytokines.

We brought strong evidence in our JAK2<sup>V617F</sup> heterozygous KI mice, using quantitative allele specific QRT-PCR on spleen and BM cells from KI mice that each allele was equally transcriptionally efficient. Indeed we observed an equal amount of JAK2<sup>V617F</sup> and JAK2<sup>WT</sup> mRNA expression, validating the correct functionality of JAK2<sup>V617F</sup> KI allele. Western blot analysis from BM and spleen cells demonstrated the translation of comparable amount of JAK2 protein in WT and KI mice, but we could not distinguish JAK2<sup>WT</sup> from JAK2<sup>V617F</sup> in KI mice due to unavailability of JAK2<sup>V617F</sup> specific antibody. In the conditional vavCre / JAK2<sup>V617F</sup> KI mice (Article 2) and SCLCreERT / JAK2<sup>V617F</sup> KI mice after tamoxifen induction (data not shown) we also demonstrated an equal amount of JAK2<sup>V617F</sup> and JAK2<sup>WT</sup> mRNA expression in BM cells.

In paper 1, we reported that constitutive female KI mice are not fertile, probably in relationship with pregnancy complications commonly seen in MPN patients. Therefore, we could not generate homozygous JAK2<sup>V617F</sup> KI mice. Not shown in the paper 2, we could not generate adult homozygous conditional non-induced SCLCreERT / JAK2<sup>V617F</sup> KI. However, we genotyped day 11.5 homozygous SCLCreERT / JAK2<sup>V617F</sup> KI embryo derived from crossing between SCLCreERT / JAK2<sup>V617F</sup> KI mice. These embryos had pale and small livers containing a 90% deficit in erythroid progenitor cells. No JAK2 protein could be detected in livers. Therefore, we concluded that our Flex construct generated a hypomorph phenotype leading to a JAK2 KO embryonic lethal phenotype. In order to generate adult homozygous JAK2<sup>V617F</sup> KI, we could cross male vavCre / JAK2<sup>V617F</sup> KI mice (germinal expression) with non-induced female SCLCreERT / JAK2<sup>V617F</sup> KI (fertile) and treat the homozygous progenies with tamoxifen. However, we did not develop such a complicated approach.

In constitutive KI mice, 3 months after birth, KI mice displayed high hematocrits and elevated RBC numbers with a low mean corpuscular volume indicating the presence of a microcytic erythrocytosis. Analysis of plasma iron revealed low plasma iron levels that could explain the microcytosis. Microscopic examination of blood smears showed RBC abnormalities like poikilocytosis, anisocytosis and polychromatophilia. Leukocyte counts were markedly increased in KI animals. Circulating erythroblasts and myeloblasts were also observed in blood

smears. Platelet counts and volume were increased along with the presence of giant platelets indicating the presence of a dysmegakaryopoiesis. Histological examination of BM and spleen showed hypocellularity in BM with an increase in granulocytic components and a decrease in erythroid components. KI mice displayed a severe age-dependent splenomegaly. Spleen histology revealed trilineage hyperplasia with disappearance of the normal splenic architecture and megakaryocytic abnormalities. Grade III fibrosis was evidenced in both BM and spleen around 30 weeks after birth. Cumulative number of erythroid and myeloid precursors and progenitors were increased in BM and spleen of KI mice. Hematopoiesis was also observed in liver of KI animals.

Thus, our JAK2<sup>V617F</sup> KI murine model faithfully recapitulated a human PV-like disease transforming into secondary myelofibrosis.

We analyzed blood parameters in conditional VavCre/JAK2<sup>V617F</sup> KI mice every month for over 10 months. Mice were born with normal hematocrit and hemoglobin levels but with high WBC and platelet counts. RBC numbers significantly increased two weeks after birth, but did not affect hematocrit due to the important microcytosis. Increased hematocrit and hemoglobin were evidenced at 1 month of age with the plateau at 5 months and a decrease afterward. Mice that were 6-month-old and over displayed a decrease in platelet numbers, while hematocrit and hemoglobin levels dropped below WT littermate mouse values. KI mice developed splenomegaly in an age-dependent manner due to erythrocytosis and myeloid hyperplasia. Mice aged over 6 months displayed marked deposits of reticulin fibers and megakaryocyte hyperplasia in spleen and BM, confirming the late onset of MF. Thus, our VavCre/JAK2<sup>V617F</sup> mice initially developed an ET-like disease rapidly evolving into a PV-like disease finally progressing into secondary myelofibrosis. Therefore, this model reproduces the natural clinical evolution of human MPN: ET → PV → MF. Interestingly, not shown in the papers, induction by tamoxifen of adult SCLCreERT/JAK2<sup>V617F</sup> KI mice resulted into rapid erythrocytosis and moderate leukocytosis but platelet counts only increased after 2-3 months to slightly elevated levels. Finally, the disease also evolved into MF. However no ET-like phenotype was observed.

## Discussion

Breeding between WT and constitutive KI mice yielded to heterozygous KI progeny at approximately the expected Mendelian frequency showing that constitutive JAK2<sup>V617F</sup> expression is viable. However, there is no report of a patient with a germinal JAK2<sup>V617F</sup> mutation, but other less potent JAK2 gain-of-function mutations such as JAK2<sup>V617I</sup>, suggesting that it is not viable in human.

A PV-like phenotype and the absence of an ET-like disease in heterozygous JAK2<sup>V617F</sup> KI mice were not expected from human studies. All other KI models using a mouse JAK2<sup>V617F</sup> gene have demonstrated a similar observation and the human JAK2<sup>V617F</sup> TG model of Tiedt *et al.* also showed that an ET-like phenotype was only obtained with a WT/VF ratio of 0.5 that is below the heterozygous ratio of 1. The only KI model showing an ET-like phenotype is the one reported by Li *et al.* using a human gene. The absence of ET-phenotype maybe due to several reasons:

- Heterozygous patients developing PV exist but, compared to heterozygous ET patients, display a majority of mutated progenitor cells. If it is the reason for PV development in “heterozygous” patients, it is not surprising that 100% mutated progenitors in KI mice give rise to a PV phenotype.
- Genetic differences between human and mice and between mouse strains should be taken into consideration. JAK2<sup>V617F</sup> expression in Balb/c mice resulted in a phenotype more pronounced than in C57/Bl6 mice.<sup>180,181</sup>

However JAK2<sup>V617F</sup> does not directly signal and requires cytokine receptors. Thus the stoichiometry between the number of receptors and JAK2 molecules maybe an important determinant in disease phenotype and may greatly differ among species and mouse strains. In addition the JAK2<sup>V617F</sup> signaling complex, including positive and negative regulator interactions, maybe more active, long lasting and more competitive toward the JAK2<sup>WT</sup> signaling complex in some mouse strains (Balb/c) than others (C57/Bl6) and in mice than in human.

Overall, our KI model has provided new insights that endogenous heterozygous JAK2<sup>V617F</sup> expression is sufficient to initiate PV that evolves into secondary myelofibrosis. However, further investigations are needed to decipher how one mutation develops a whole spectrum of MPNs in patients, if one HSC is sufficient to drive the disease, and if other molecular events are required for the development and evolution of MPNs.

## **B) Effect of JAK2<sup>V617F</sup> KI on early hematopoietic cells / clonal dominance**

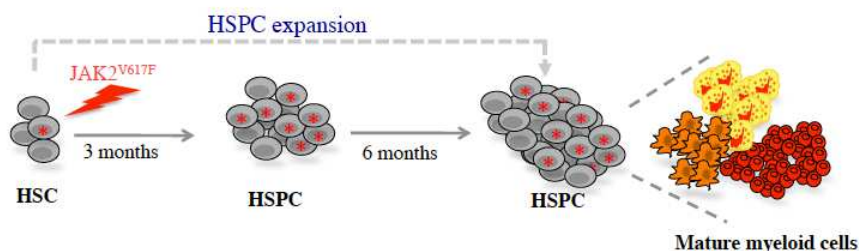
### **Context**

In the past few years, novel mutations in the JAK-STAT pathway (MPL, CBL, LNK), epigenetic regulators (TET2, DNMT3a, ASXL1, EZH2) and RNA splicing machinery (SF3B1, SRSF2, SF3A1, U2AF1) have been evidenced at low frequencies in MPN patients<sup>397</sup>. Some mutations, such as in the TET2 gene, may antedate the acquisition of JAK2<sup>V617F</sup>, suggesting that they may initiate MPN<sup>398</sup>. Mutational status analysis of HSC and progenitor compartments from MPN patients demonstrates that JAK2<sup>V617F</sup> induces a proliferative advantage to the late stages of hematopoiesis<sup>189,203</sup>. On the contrary, DNMT3a<sup>311</sup> and TET2<sup>292</sup> mutations were shown to play an important role in the amplification and self-renewal of HSC in murine models. JAK2<sup>V617F</sup> has demonstrated differential effects on HSC expansion and functional activity in various KI murine models<sup>156,173,196</sup>. After all these observations the role of JAK2<sup>V617F</sup> as an initiator and/or driver of MPN has become a matter of controversy<sup>197</sup>.

### **Results**

#### **1) Effects of JAK2<sup>V617F</sup> on HSC compartment size**

First, we investigated the HSCP compartment from these mice. Hematopoietic progenitor colony forming assays at 3 months of age revealed an expanded pool of erythroid progenitors in spleen and BM of KI animals including around 50% EEC. Expansion of CFU-GM was observed in the spleen of these animals. Flow cytometric analysis revealed an increased pool of Lin<sup>-</sup> cells in 1-month-old mouse BM and spleen without changes in the number of SLAM (LSK/CD48/CD150<sup>+</sup>) and LSK cells compared to their WT littermates. However, these immature cell pools were markedly increased in 3- and 6-month-old KI mice. Detailed analysis within the LK compartment of KI mice including CMP, GMP and MEP progenitors showed progressive increase of CMP and GMP pool in BM and spleen of 3- and 6- month-old KI animals while MEP compartment was not further increased in 6-month-old mice. These results demonstrate that JAK2<sup>V617F</sup> expands late and early HSC compartments in KI mice and that expansion seems to increase with age.



**Figure 27. Effect s of JAK2<sup>V617F</sup> on HSPC compartment**

## 2) JAK2<sup>V617F</sup> HSC competitiveness

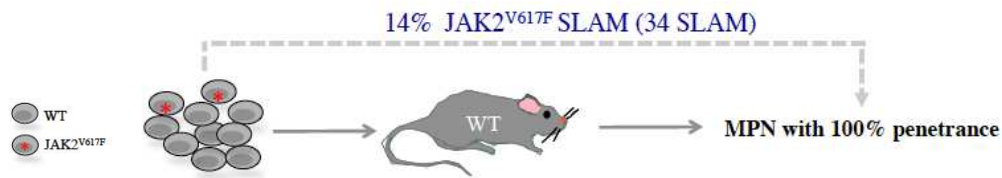
To propagate long-term disease in human JAK2<sup>V617F</sup> is assumed to provide a competitive advantage to HSC compared to JAK2<sup>WT</sup>. We investigated this notion by performing a competitive BMT in WT lethally irradiated mice with 30% KI and 70% WT cells. After 34 weeks, blood myeloid chimerism reached 100% in mice engrafted with 30% KI cells, demonstrating a proliferative advantage of JAK2<sup>V617F</sup>-positive mature myeloid cells over WT cells. Study of Lin<sup>-</sup>, LSK and SLAM compartments revealed a striking increase of KI cells in BM and spleen of these animals. We also noticed a two-fold increase of KI cells in lymphoid pool ( $\approx 50\%$ ) in peripheral blood. This increase maybe the consequence of the 2-fold increased HSC compartment of the 3-month-old KI donors compared with the WT competitive donors (KI/WT HSC compartment ratio  $\approx 50\%$ ). Overall, these results suggest that JAK2<sup>V617F</sup> provides a strong competitive advantage to late and early stages of hematopoiesis in mice.



**Figure 28. Effect of JAK2<sup>V617F</sup> on HSC competitiveness**

## 3) JAK2<sup>V617F</sup>-positive HSC minimum graft size

To determine the minimum graft size necessary to initiate the disease, we performed competitive graft assays with limiting dilutions using whole BM cells from 1-2 months old KI mice. Flow cytometric analysis before transplantation allowed us to calculate the absolute number of SLAM cells grafted in each group. Results demonstrate that at least 50 SLAM (or 30% KI BM cells) always generate a long-term disease with erythrocytosis, thrombocytosis, leukocytosis and splenomegaly. Interestingly, mice grafted with 10% BM cells equivalent to 17 SLAM generated the disease in only 50% of animals. HSC compartment analysis of these chimeric groups by the end of 30<sup>th</sup> week post-BMT demonstrated a significant out growth of KI SLAM, LSK and progenitors cells over their WT counterparts in BM. These results demonstrated that 34 JAK2<sup>V617F</sup> SLAM (or 14% SLAM graft) is necessary to initiate MPN with 100% penetrance in our mouse model, with selective proliferative advantage in HSC and myeloid lineage.



**Figure 29. JAK2<sup>V617F</sup>-positive SLAM minimum graft size**

**4) Effect of JAK2<sup>V617F</sup> on HSC proliferative and apoptotic status:** Finally to investigate the mechanism leading to KI HSC amplification and proliferative advantage, we measured the percentages of BM LSK or SLAM in G0 (quiescent), G1 and S/G2/M phases and showed that KI SLAM are more proliferative than WT SLAM. We also measured the percentage of apoptotic cells (Annexin V+) in BM and spleen of Lin-, LK, LSK, and SLAM cells and showed that BM and spleen LSK and SLAM cells are less apoptotic in KI mice than in WT mice. The results clearly showed that early KI cells are more proliferative and less apoptotic than early WT cells. These properties may explain their amplification and proliferative advantage.

### Discussion

With growing evidences of the presence of other mutations in MPN, the role of JAK2<sup>V617F</sup> MPN has been questioned and demanded an extensive re-evaluation of the effect of JAK2<sup>V617F</sup> in HSC activity.

First, our murine model demonstrated that expression of JAK2<sup>V617F</sup> expanded the HSCP compartment in an age-dependent manner. There were more HSCP cells in 6-month-old compared to 3-month-old mice, whereas 1-month-old KI mice displayed no amplification of HSCP cells compared to WT littermates. Expansion of the HSCP compartment was also observed in another JAK2<sup>V617F</sup> KI model<sup>156</sup>, and can be explained by the presence of type I (MPL, GCSFR) and type II (interferon- $\gamma$ ) receptors that are present on HSC surface and were shown to play crucial roles in HSC self-renewal and proliferation<sup>399-401</sup>. Since JAK2 is an essential component of these receptors signaling, its constitutive activation may alter signaling and deregulate HSC activity. Moreover, the high cycling and low apoptotic status of JAK2<sup>V617F</sup>-positive cells could explain their competitive advantage and the progressive increase in compartment size with age. The acquisition of additional events that may confer a proliferative advantage to HSC and support disease progression is a possibility. CGH array from mice of different ages will shed some light on this hypothesis. There might be a bias in the SLAM/LSK phenotypic analysis due to the upregulation of Sca-1 (a target of interferon- $\gamma$  signaling) on myeloid progenitor cells that could eventually lead to contamination of the “analyzed SLAM/LSK compartment” with highly proliferative or low quiescence cells. We addressed this concern by reanalyzing the SLAM cells regardless of Sca-1 and obtained identical results.

Next, competitive BMT with a mixture of KI and WT cells demonstrated that JAK2<sup>V617F</sup> gives a proliferative capacity to HSC in this setting, which could explain the emergence and life-long persistence of MPN-cells in patients. This competitive advantage was also demonstrated in Mullally’s KI model. In contrast, Li *et al.* have demonstrated deleterious effects of JAK2<sup>V617F</sup> on the HSC compartment that could be related to the use of the hJAK2<sup>V617F</sup> which could behave differently than mJAK2<sup>V617F</sup> in the murine settings. Indeed, mJAK2<sup>V617F</sup> was shown to induce an enhanced erythrocytosis, as compared to its human counterpart, in a RV BMT mouse model<sup>200</sup>.

We have identified the minimal JAK2<sup>V617F</sup>-positive SLAM pool to be at least 34 cells required to rapidly initiate LT-MPN, likely without the cooperation of an additional event. Interestingly, at the limiting dilution of 17 SLAM cells only 50% of the animals developed the disease. This incomplete penetrance can be explained by technical limitation of injecting such a small number of SLAM cells, non-mutually exclusive with the fact that only 1 out of 2.1 SLAM harbors some LT-hematopoietic capacity<sup>5</sup>, a fact that we were not able to reproduce with WT or KI SLAM cells using CD150- cells as carrier and CD45 1 or 2 haplotypes. In our preliminary experiments, 30 SLAM cells were still necessary to detect > 5% donor cells in 50% recipients, a number similar to the number of SLAM necessary to generate MPN in mice.

Overall, our JAK2<sup>V617F</sup> KI mouse model emphasizes the fact that JAK2<sup>V617F</sup> is sufficient to initiate the disease with a very limited number of LT-HSC without the cooperation of additional event. Moreover, JAK2<sup>V617F</sup> expression induces the expansion of the HSCP compartment in an age-dependent manner based on an increased competitive advantage.

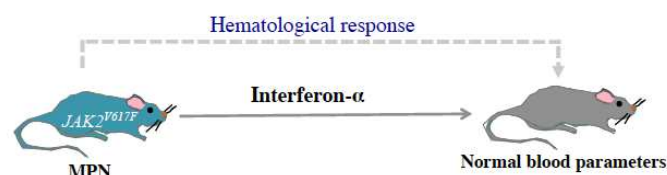
### C) Effect of IFN $\alpha$ therapy

#### Context

MPN-initiating JAK2<sup>V617F</sup> cells reside in the LT-HSC compartment<sup>49,196</sup> and eradication of these cells seems to be required to definitively cure the disease. Interferon- $\alpha$  (IFN $\alpha$ ) treatment in MPN patients has been used since the last 2 decades with hematological and molecular responses. Recently, its has been shown to achieve a complete and sustained molecular responses by eradicating JAK2<sup>V617F</sup>-positive clones in ET and PV patients<sup>327,402</sup> knowing that the sensitivity of the molecular technique is around 1%. However, its mechanism of action is largely unknown. We have used our chimeric KI mouse model to study the effects of interferon  $\alpha$ .

#### Results

We tested the effect of interferon- $\alpha$  (IFN $\alpha$ ) treatment on MPN development in duplicate using cohorts of mice with two different JAK2<sup>V617F</sup> allele burden; in fact corresponding at different levels of chimerism. We found that IFN $\alpha$  corrected the PV phenotype by suppressing leukocytosis, and decreasing hematocrit and the high platelet count without the development of leukopenia or anemia but by inducing a thrombocytopenia.

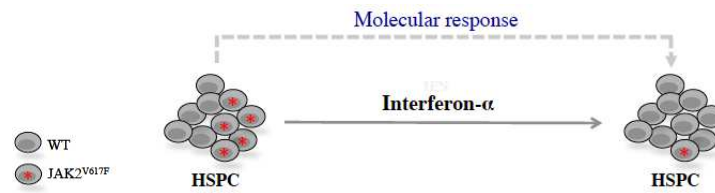


**Figure 30. Hematological response in JAK2<sup>V617F</sup> KI mouse model upon IFN $\alpha$  exposure**

More interestingly, long-term IFN $\alpha$  treatment diminished or greatly reduced blood KI granulocyte chimerism in both low- and high-allele burden groups (low or high KI chimerism) by the end of 13 or 11 weeks of treatment, respectively. IFN $\alpha$  treatment selectively suppressed KI Lin<sup>-</sup>, LK, LSK and SLAM cells in BM of these animals. Overall, these results suggest that IFN $\alpha$  not only targeted maturing hematopoietic JAK2<sup>V617F</sup> cells but also early hematopoietic compartment suggesting that it may eradicate the disease. . Secondary transplantation from IFN $\alpha$



treated mice confirms this hypothesis because no disease was observed in secondary mice demonstrating that IFN $\alpha$  eradicated the JAK2<sup>V617F</sup> LT-HSC disease initiating cells, the equivalent of a cancer stem cell.



**Figure 31. Molecular response in JAK2<sup>V617F</sup> KI mouse model upon IFN $\alpha$  exposure**

Thus, IFN $\alpha$  treatment has the potential to treat MPN by targeting early JAK2<sup>V617F</sup>-positive HSC in contrast to what has been observed for JAK2 inhibitors.

### Discussion

Using 30% of JAK2<sup>V617F</sup> KI in a competitive BMT, mimicking PV patients with clonal hematopoiesis along with residual polyclonal hematopoiesis, we have demonstrated that IFN $\alpha$  treats MPN by selectively targeting JAK2<sup>V617F</sup>-positive cells at the HSC level. These results reflect the recent molecular remissions obtained in ET and PV trials<sup>327,402</sup> and explain that the decrease in JAK2<sup>V617F</sup> cells in human requires a long treatment because it requires a HSC exhaustion. Intriguingly, we did not observe an enhanced cell cycling in JAK2<sup>V617F</sup>-positive cells upon exposure to IFN $\alpha$ , as an underlying mechanism proposed by A. Mullally's team<sup>403</sup>. Because we have confirmed our results regardless of Sca-1 expression, we cannot rule out the possibility that the LSK compartment in the study of Mullally *et al.* was contaminated by Sca-1-expressing myeloid progenitor cells in high cell cycling state. Moreover, these differences maybe due to experiment settings; we did a short-term treatment of 3 days while they chose a treatment of 4 weeks before investigating cell cycle status.

This work demonstrates that our mouse model is a valuable preclinical model able to predict the success of novel therapies. It provides the opportunity to study the mechanism of action of IFN $\alpha$  and evaluate the potential of other therapeutic agents for the treatment of MPN.

**Article 1**

**Myeloproliferative neoplasm induced by constitutive expression of JAK2<sup>V617F</sup> in Knock-in mice**

## Brief report

Myeloproliferative neoplasm induced by constitutive expression of JAK2<sup>V617F</sup> in knock-in mice

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The *Jak2*<sup>V617F</sup> mutation is found in most classical BCR/ABL-negative myeloproliferative neoplasms (MPNs). Usually, heterozygosity of the mutation is associated with essential thrombocythemia (ET) and homozygosity with polycythemia vera (PV). Retrovirally transduced or transgenic animal models have shown that the mutation is sufficient for MPN development but that the level of expression is

crucial for MPN phenotypes. Therefore we investigated the effect of an endogenous heterozygous expression of *Jak2*<sup>V617F</sup> in knock-in (KI) mice. These animals displayed constitutive JAK2 activation and autonomous erythroid progenitor cell growth. Mice suffered from marked polycythemia, granulocytosis and thrombocytosis. Spleens and marrows displayed myeloid trilineage hyperplasia. Most ani-

mals survived to develop advanced fibrosis in these organs at around 9 months of age. In conclusion, constitutive heterozygous expression of JAK2<sup>V617F</sup> in mice is not embryo-lethal but results in severe PV-like disease with secondary myelofibrosis and not in ET-like disease as expected from patient study. (*Blood*. 2010; 116(5):783-787)

## Introduction

The acquired *Jak2*<sup>V617F</sup> mutation is present in 95% of polycythemia vera (PV) patients and approximately 50% of patients suffering from essential thrombocythemia (ET) or primary myelofibrosis (PMF).<sup>1-3</sup> Interestingly, heterozygosity of the *Jak2*<sup>V617F</sup> mutation is usually associated with ET and homozygosity, resulting from mitotic recombination, with PV.<sup>4</sup> Animal models using JAK2<sup>V617F</sup> retrovirally (RV)–transduced bone marrow (BM) cells<sup>5-8</sup> or transgenesis (TG)<sup>9,10</sup> have shown that JAK2<sup>V617F</sup> expression in hematopoietic stem cells (HSC) is sufficient to induce myeloproliferative neoplasms (MPNs) in mice. As suggested by patient studies, animals develop either an ET- or a PV-like disease depending on the JAK2<sup>V617F</sup> expression levels.<sup>6,9</sup> Therefore, considering the crucial role of JAK2<sup>V617F</sup> expression level in disease development, we generated a knock-in (KI) model allowing for a physiologic expression of JAK2<sup>V617F</sup> from the endogenous *Jak2* promoter to further our understanding of MPN physiopathology and JAK2<sup>V617F</sup> mechanism of action. We report here the phenotype of these mice.

embryonic stem (ES) cell genomic DNA to introduce the GTC > TTC point mutation (617V > F) and subcloned in a vector with a floxed neomycin resistance (NeoR) cassette resulting in a step1 plasmid. A 5' homologous arm was amplified by PCR and subcloned in step1 plasmid to generate a step2 plasmid. Finally, a 3' homologous arm was subcloned in step2 plasmid to generate the final targeting construct. Targeted 129S2/SvPas ES clones were confirmed by PCR and Southern blot and injected into C57BL/6J blastocysts to generate chimeric mice. Chimeras (L2) were crossed with flippase (FLP) TG C57BL/6 mice to excise the FRT site-flanked NeoR cassette on F1 progenies (L-). Finally, F1 animals (L-) were crossed with 129Sv mice to generate F2 animals.

## Mouse analysis

Blood parameters were determined using an automated MS9 counter (Schloessing Melet). For histology, femur and spleen sections were stained with hematoxylin-eosin-safran and reticulin fibers were revealed by silver staining according to the Gordon Sweet method. Progenitor cell assays were carried out in duplicate in methylcellulose (StemCell Technologies) supplemented or not with cytokines and erythropoietin (EPO).<sup>6</sup>

## Methods

Establishment of the *Jak2*<sup>V617F</sup> KI mouse line

*Jak2*<sup>V617F</sup> KI animals were generated at the Mouse Clinical Institute, Illkirch, France (<http://www-mci.u-strasbg.fr>). All animal experiments were carried out at the Animal Facilities of the Institut Gustave Roussy under the conditions established by the European Community (Directive 86/609/CCE) and according to French law. The targeting vector (Figure 1A) was constructed as follows. A fragment encompassing *Jak2* exon 13 was amplified by polymerase chain reaction (PCR) on 129S2/SvPas mouse

## Results and discussion

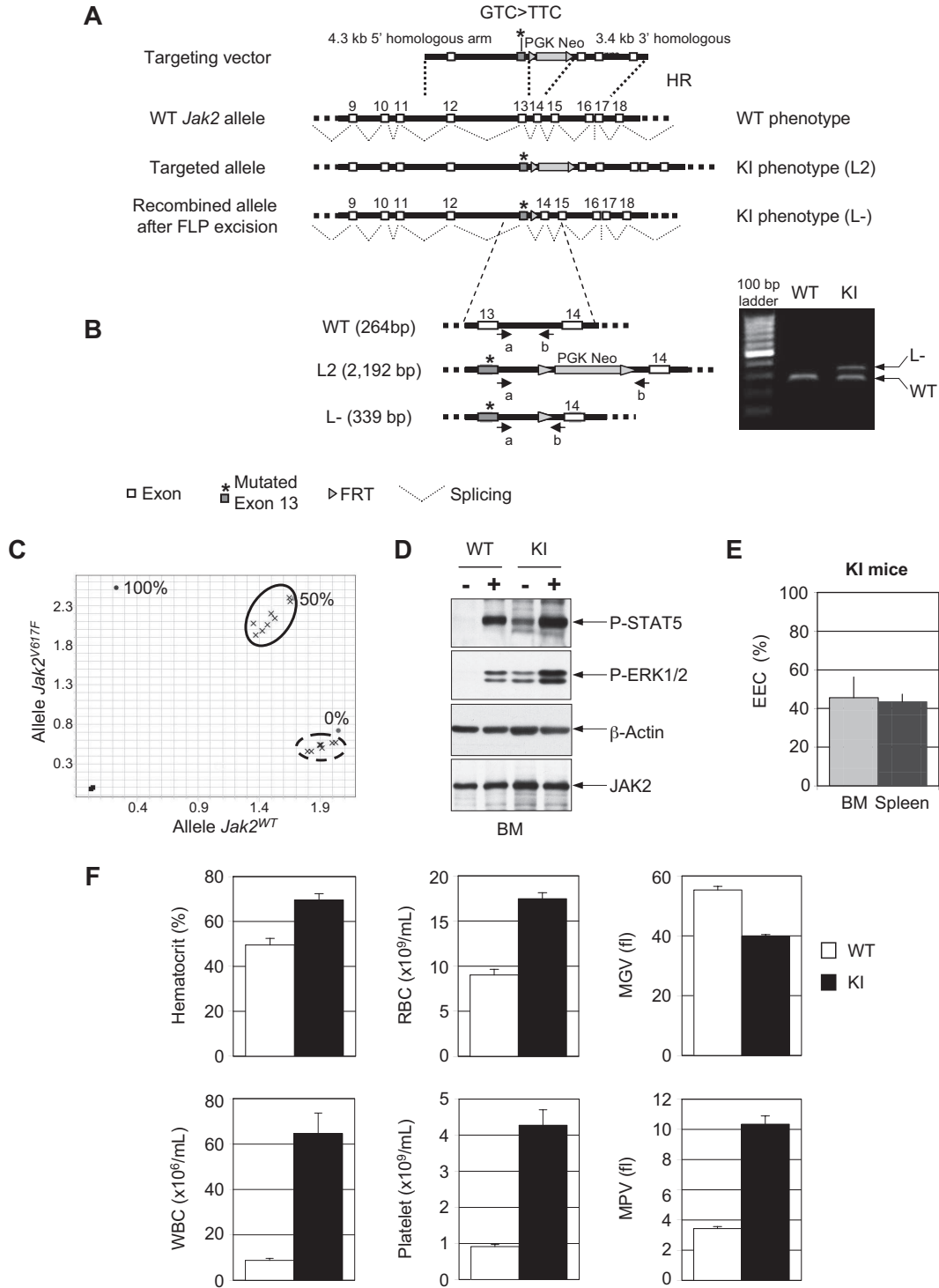
The heterozygous status of all KI mice was confirmed by tail DNA PCR genotyping (Figure 1B). Quantitative allele-specific RT-PCR (Figure 1C) showed that heterozygosity resulted in equal amounts of mutated and wild-type (WT) *Jak2* mRNAs, suggesting normal regulation of the *Jak2*<sup>V617F</sup> KI allele. Western blot analysis showed that JAK2 protein levels in KI and WT mice were comparable

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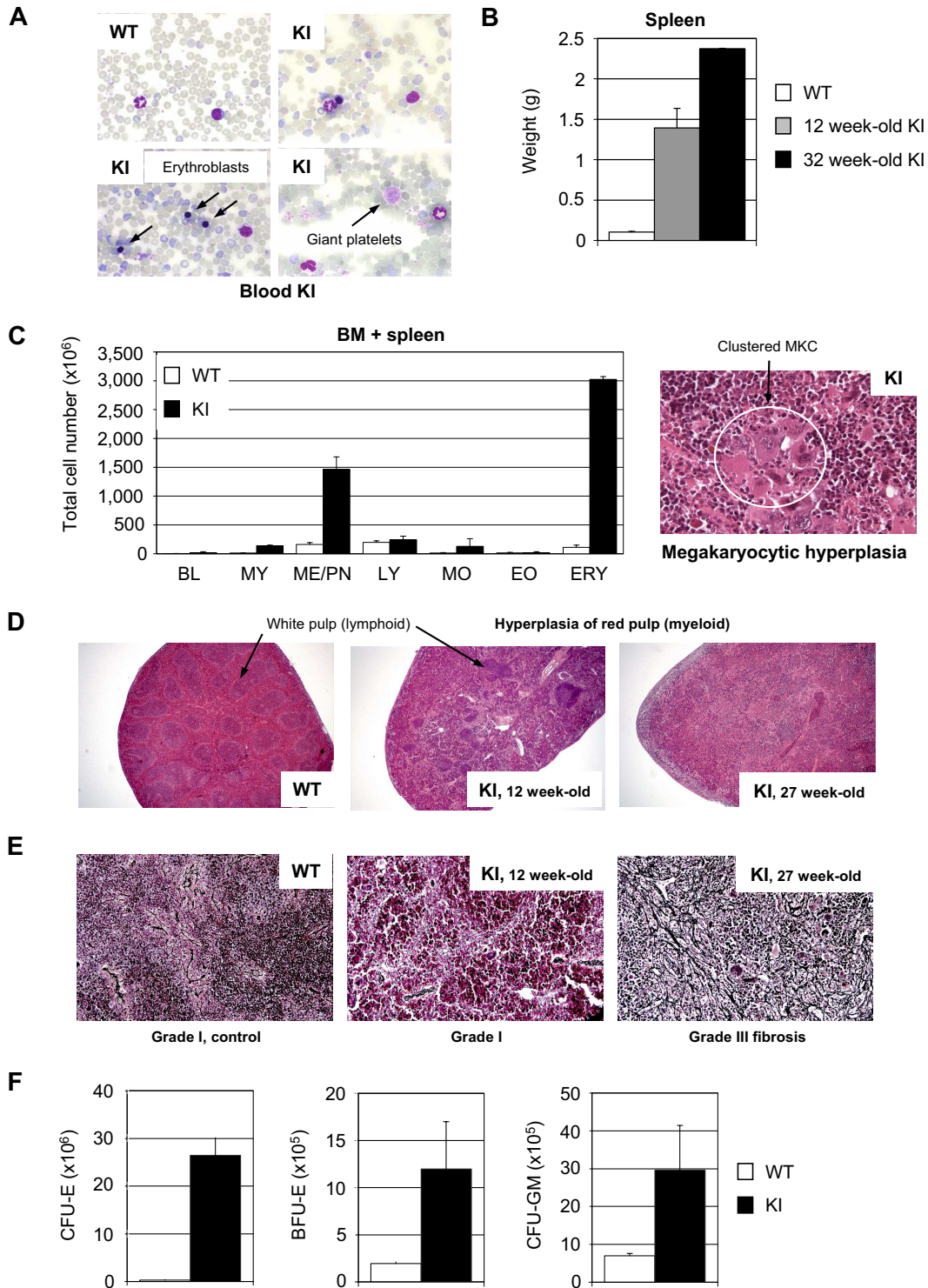
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**Figure 1. Characterization of the *Jak2*<sup>V617F</sup> KI mouse model.** (A) Schematic representation of the targeting vector. Homologous recombination into the *Jak2* WT allele of mouse embryonic stem (ES) cells resulted into the L2 knock-in (KI) genotype. A correctly targeted ES clone was injected into blastocyst stage embryos to generate L2 chimeric mice. Chimeras were bred with flippase (FLP) transgenic mice to generate L-. (B) PCR analysis of genomic tail biopsy DNA using primers a (5'-CCTGCTCAGAATCCTTCT-CATTAGGG-3') and b (5'-CTCCAGGGTTACACGAGTCACC-3') detects successful recombination events in the L- KI mice (right panel). (C) Wild-type (WT) and mutated *Jak2* Taqman allele specific amplification from bone marrow (BM) and spleen RNA samples of 2 KI (within the circle in plain line, studied in duplicate 12 and 20 weeks after birth) and WT mice (in duplicate, within the circle in dashed line) was performed. Standard curve (0%, 50%, and 100%) was carried out from mixtures of plasmids containing *Jak2*<sup>V617F</sup> or *Jak2*<sup>WT</sup> cDNAs. (D) Constitutive phosphorylation of ERK1/2 (Thr 202/Tyr 204) and STAT5 (Tyr 694) and total JAK2 protein in KI mice identified by Western blot analysis (antibodies from Ozyme). Cells isolated from WT and KI mice were starved for 40 hours and stimulated (+) or not (-) with interleukin-3 and granulocyte-macrophage colony-stimulating factor for 15 minutes. β-actin served as loading control. (Sigma). (E) Autonomous growth in KI mice represented as the percentage of CFU-Es derived from BM or spleen KI mice forming endogenous erythroid colonies (EECs) in the absence of added erythropoietin (mean value ± SD, n = 4). No EEC was detected from control mice. (F) Blood cell parameters (mean value ± SE) from KI (n = 11) and WT (n = 8) mice studied at 12 (± 2) weeks of age. RBC indicates red blood cell; MGCV, mean globular volume; WBC, white blood cell; and MPV, mean platelet volume.



**Figure 2. Blood, BM, and spleen features from *Jak2<sup>V617F</sup>* KI mice.** (A) Blood smears from a 31-week-old *Jak2<sup>V617F</sup>* KI mouse revealed RBC polychromatophilia, anisocytosis and poikilocytosis, large platelets, and erythroblastosis (May-Grünwald-Giemsa staining). Images were obtained (63 $\times$  lens) using a Zeiss Axiophot microscope with a Zeiss Axiocam Mrc camera and AxiVision Rel.4.3 acquisition software. (B) Spleen weight in KI mice proportionally increased with age (mean value  $\pm$  SD, n = 3). (C) Myeloid trilineage hyperplasia in KI mice. The left panel shows no blockade in the differentiation processes and increases in the cumulative numbers (BM plus spleen) of granulocytic and erythroid precursor cells in KI compared with WT mice (mean value  $\pm$  SD, n = 2, 27 and 31 weeks old). Calculation is based on the assumption that 1 femur represented 6% of the total marrow and from the number of cells isolated from the spleen. Cell types were identified from May-Grünwald-Giemsa stained cell cytopspins. BL indicates blast; MY, myeloblast/myelocyte; ME/PN, metamyelocyte/polymorph; LY, lymphocyte; MO, monocyte; EO, eosinophil; and ERY, erythroblast. The right panel shows clustered megakaryocytes (MKs) with multilobulated nucleus from a hematoxylin/eosin/safran (HES)-stained KI mouse spleen (400 $\times$  magnification). Images were obtained using a DM2000 Leica microscope and a DFC300FX Leica camera with Leica Application Suite v.2.5.OR1 acquisition software. (D-E) Histology of the spleen. (D) Hematoxylin-eosin coloration of a 12-week-old KI mouse revealed hyperplasia of the red pulp with the partially preserved white pulp that was clearly visible in a WT mouse (arrows). In contrast, white pulp of a 27-week-old KI mouse was completely blended. (25 $\times$  magnification). (E) Silver stain coloration indicated high-grade fibrosis in a 36-week-old mouse. Fibrosis was absent in 12-week-old KI and 27-week-old WT mice (200 $\times$  magnification). (F) Cumulative numbers of progenitor cells in BM and spleen from KI and WT mice scored after 2 (CFU-E) or 7 (BFU-E and CFU-GM) days of culture (mean value  $\pm$  SE, n = 4, 21  $\pm$  5 weeks of age). WT littermates were usually used as controls.

(Figure 1D) but, in the absence of antibody recognizing specifically the mutated or WT proteins, we could not verify that both forms were in equal amounts in KI mice. STAT5 and ERK1/2 downstream signaling molecules were phosphorylated in the absence of added growth factors in KI BM (Figure 1D) and spleen (data not shown). Finally, around 45% of colony forming unit–erythroid (CFU-E) from BM or spleen of KI mice gave rise to endogenous erythroid colonies (EECs; Figure 1E). These results show that JAK2<sup>V617F</sup> was constitutively activated in KI mice, resulting in EPO hypersensitive or independent growth.

Breeding between WT and KI mice yielded heterozygous KI progeny at approximately the expected mendelian frequency (10 of 42 pups). Of 1F1 and 10F2 KI studied mice, 5 died unexpectedly (15 ± 3 weeks), 5 were killed (23 ± 10 weeks) and one 12-week-old mouse is still alive. Parameters from peripheral blood of KI mice were profoundly modified (Figure 1F). The hematocrit increased, the number of red blood cells (RBCs) doubled but their volume decreased (Figure 1F). Iron deficiency (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article) probably contributed to microcytosis. RBC polychromatophilia, anisocytosis and poikilocytosis were observed (Figure 2A). WBC counts increased 7.3-fold ( $P < .05$ ) due to a rise in total numbers of granulocytes (12.5-fold), monocytes (3.5-fold), and lymphocytes (3.3-fold). Furthermore, circulating granulocytic precursors (myeloblasts, metamyelocytes; 0.5%-5%) and erythroblasts (0.5%-8.5%) were seen, especially in old mice (Figure 2A). Platelet counts and volume (MPV) increased 4.7-fold and 3-fold, respectively with the presence of giant platelets, indicative of dysmegakaryopoiesis (Figure 2A).

The number of nucleated cells collected from femurs ( $16 \pm 2 \times 10^6$ /femur) was lower than in controls ( $21 \pm 2 \times 10^6$ /femur;  $P < .05$ ,  $n = 5$ , week 21 ± 6). An increase in neutrophil precursors (1.5-fold) and a drop in both erythroblasts (2-fold) and lymphocytes (3-fold) were observed. There was no shift in the proportion of immature precursor cells showing that maturation was unaffected. Spleen weights from KI mice increased over the time of survey (Figure 2B). Splenomegaly was due to an increase in erythroid (88-fold) and myeloid (82-fold) precursors without any noticeable blockage of maturation. Cumulative numbers in BM plus spleen showed an increase in erythroid and myeloid precursors but no change in the total lymphocyte population (Figure 2C). Differential count data in blood, BM and spleen were confirmed by fluorescence-activated cell sorting analysis with lineage markers (data not shown). Histologic examination of the spleens also revealed clusters of megakaryocytes (MKs) with a multilobulated nucleus and granulocyte emperipoiesis demonstrating megakaryocytic hyperplasia (Figure 2C right panel). Spreading of the red pulp with progressive extinction of the white pulp with age emphasized myeloid trilineage hyperplasia (Figure 2D). Grade 3 fibrosis with low collagen deposition was evidenced in spleen (Figure 2E) and BM of aged mice (around week 30). Extramedullary hematopoiesis was observed in the liver with the presence of MKs, granulocytes and erythroblasts (data not shown). Finally, cumulative numbers of CFU-E, burst-forming unit–erythroid (BFU-E), and colony-forming unit–granulocyte-macrophage (CFU-GM) progenitor cells in BM and spleen of KI mice were increased 87-, 6-, and 4-fold, respectively, compared with control (Figure 2F). The progenitor pool amplification was mostly due to splenic hematopoiesis.

This study shows for the first time that heterozygous expression of JAK2<sup>V617F</sup> in KI mice induces a severe PV-like disorder leading to myelofibrosis. The disease is similar to some phenotypes previously described in RV-transduced<sup>5,6</sup> and especially low ex-

presser TG<sup>9,10</sup> models. In contrast, JAK2<sup>V617F</sup> heterozygosity in patients is usually associated with the development of ET.<sup>4</sup> The absence of ET-like disease in the KI mice may be due to several reasons. First, the embryonic development of this inherited disease, compared with the adult acquired human disease, may have changed the disease phenotype. However, heterozygous KI induced in adult life (our preliminary results and Akada et al<sup>11</sup>) also lead to PV-like phenotypes. Second, heterozygous patients developing PV exist but, compared with heterozygous ET patients, display a majority of mutated progenitor cells.<sup>12</sup> If it is the reason for PV development in heterozygous patients, it is not surprising that 100% mutated progenitors in KI mice give rise to a PV phenotype. Third, genetic differences between human and mice and between mouse strains<sup>5</sup> should be taken into consideration. Among these differences, the JAK2<sup>V617F</sup> signaling complex, including receptors, positive and negative regulator interactions, may be more active, long-lasting, and more competitive toward the JAK2<sup>WT</sup> signaling complex in mice than in humans.

To date, no JAK2<sup>V617F</sup> inherited human disorder has been described although JAK2<sup>V617F</sup> acquired familial forms of MPNs exist.<sup>13</sup> The severity of the disease suggests that an inherited disorder may be embryo-lethal in human. In conclusion, we have described the effects of a constitutive heterozygous JAK2<sup>V617F</sup> mutation in mice. Work remains to understand the absence of an ET-like phenotype and to study potential nonhematopoietic phenotypes.

#### Addendum

During the review of this paper Akada et al<sup>11</sup> demonstrated that heterozygous and homozygous JAK2<sup>V617F</sup> KI induced after birth developed a PV-like disease that is transplantable in recipient mice. Hematologic parameters of our constitutive KI mouse resemble those of the homozygous inducible KI mice, both models being analyzed at similar ages in a C57Bl6/129Sv background.

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#### Authorship

Contribution: C.M. and C.L. designed the study, performed research, and analyzed data; A.M. performed the histopathology; S.H. performed research; S.J. and M.-C.B. generated the KI mice; W.V. designed the study; and J.-L.V. designed the study, performed research, analyzed data, and wrote the paper.

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

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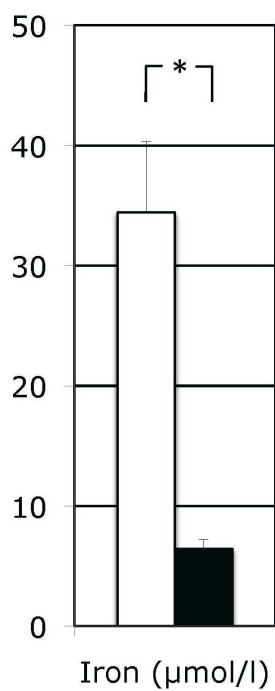
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# Myeloproliferative neoplasm induced by constitutive expression of JAK2<sup>V617F</sup> in knock-in mice

## Supplemental materials for: Marty et al

### Files in this Data Supplement:

- [Figure S1. Iron deficiency in KI mice \(JPG, 61.3 KB\)](#) - Iron levels were analyzed in the plasma of WT (white column, n=4, VGM=51 ± 3) and KI (dark column, n=3, VGM=41 ± 6) mice. Mean values ± SD are indicated. A low level was found in KI mice compared to WT mice (p = 0.0005).





## **Article 2**

**JAK2<sup>V617F</sup> expression in mice amplifies early hematopoietic stem cells and gives them a competitive advantage that is hampered by IFN $\alpha$**

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# Regular Article

## MYELOID NEOPLASIA

### JAK2<sup>V617F</sup> expression in mice amplifies early hematopoietic cells and gives them a competitive advantage that is hampered by IFN $\alpha$

Salma Hasan, Catherine Lacout, Caroline Marty, Marie Cuingnet, Eric Solary, William Vainchenker, and Jean-Luc Villeval

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#### Key Points

- JAK2<sup>V617F</sup> amplifies in mouse early hematopoietic cells, giving them a proliferative advantage through high cell cycling and low apoptosis.
- IFN $\alpha$  prevented myeloproliferative neoplasm development by specifically inhibiting JAK2<sup>V617F</sup> cells at an early differentiation stage.

The acquired gain-of-function V617F mutation in the Janus Kinase 2 (JAK2<sup>V617F</sup>) is the main mutation involved in BCR/ABL-negative myeloproliferative neoplasms (MPNs), but its effect on hematopoietic stem cells as a driver of disease emergence has been questioned. Therefore, we reinvestigated the role of endogenous expression of JAK2<sup>V617F</sup> on early steps of hematopoiesis as well as the effect of interferon- $\alpha$  (IFN $\alpha$ ), which may target the JAK2<sup>V617F</sup> clone in humans by using knock-in mice with conditional expression of JAK2<sup>V617F</sup> in hematopoietic cells. These mice develop a MPN mimicking polycythemia vera with large amplification of myeloid mature and precursor cells, displaying erythroid endogenous growth and progressing to myelofibrosis. Interestingly, early hematopoietic compartments [Lin<sup>-</sup>, LSK, and SLAM (LSK/CD48<sup>-</sup>/CD150<sup>+</sup>)] increased with the age. Competitive repopulation assays demonstrated disease appearance and progressive overgrowth of myeloid, Lin<sup>-</sup>, LSK, and SLAM cells, but not lymphocytes, from a low number of engrafted JAK2<sup>V617F</sup> SLAM cells. Finally, IFN $\alpha$  treatment prevented disease development by specifically inhibiting JAK2<sup>V617F</sup> cells at an early stage of differentiation

and eradicating disease-initiating cells. This study shows that JAK2<sup>V617F</sup> in mice amplifies not only late but also early hematopoietic cells, giving them a proliferative advantage through high cell cycling and low apoptosis that may sustain MPN emergence but is lost upon IFN $\alpha$  treatment. (*Blood*. 2013;122(8):1464-1477)

#### Introduction

The acquired gain-of-function V617F mutation in the Janus Kinase 2 (JAK2<sup>V617F</sup>) is the major mutation involved in BCR/ABL-negative classical myeloproliferative neoplasms (MPNs).<sup>1-4</sup> It is present in 95% of polycythemia vera (PV) patients and ~50% to 60% of patients suffering from essential thrombocythemia (ET) or primary myelofibrosis. Retrovirally induced<sup>5-7</sup> and transgenic (TG) animals<sup>8</sup> have demonstrated that the sole JAK2<sup>V617F</sup> mutation is able to generate these diseases and that the level of expression of JAK2<sup>V617F</sup> is crucial in determining MPN diversity.<sup>8</sup> Several knock-in (KI) mouse models using the murine gene have been described and all demonstrate that endogenous heterozygous expression of mJAK2<sup>V617F</sup> leads to a PV-like disease, usually followed by secondary myelofibrosis,<sup>9-11</sup> whereas homozygosity increases the severity of the PV-like phenotype.<sup>9</sup> In contrast, a mild ET-like disease was reported in a KI model using hJAK2<sup>V617F</sup>.<sup>12</sup>

Besides JAK2<sup>V617F</sup>, several other acquired mutations have been identified in BCR/ABL-negative MPN, although with a frequency often <10%. These mutations target several pathways/complexes: JAK/STAT signaling directly responsible for MPN phenotype, DNA methylation/demethylation, and PRC2 and RNA splicing.<sup>13</sup> Functional analyses of several of these additional mutations have questioned the precise contribution of JAK2<sup>V617F</sup> to MPN disease

initiation, as deletion of *Tet2*<sup>14,15</sup> or *Dnmt3a*<sup>16</sup> in mice cause hematopoietic stem cell (HSC) amplification and increased self-renewal. On the contrary, the contribution of JAK2<sup>V617F</sup> to HSC selective amplification has become a matter of controversy.<sup>17</sup> Accordingly, analysis of human PV patients has suggested that JAK2<sup>V617F</sup> amplified terminal stages of hematopoietic cell differentiation without amplifying the early stages, as opposed to *Tet2* mutations.<sup>18,19</sup> Therefore, the ability of JAK2<sup>V617F</sup>-positive HSCs to compete with normal HSCs is largely questioned, and actual data suggest that other mutations, occurring before or after JAK2<sup>V617F</sup>, are instead responsible for clonal emergence of JAK2<sup>V617F</sup>-positive MPN.

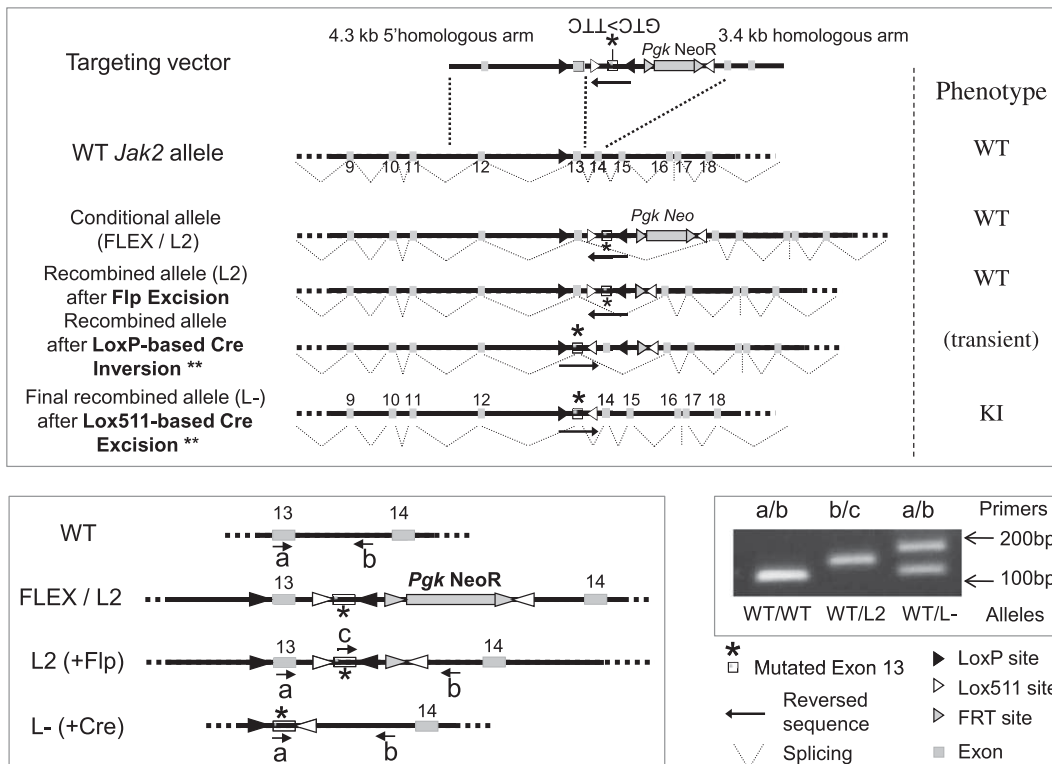
To reevaluate the role of JAK2<sup>V617F</sup> in HSC amplification, we developed a new conditional JAK2<sup>V617F</sup> KI model and analyzed early stages of differentiation. We found that endogenous expression of JAK2<sup>V617F</sup> greatly amplified multipotent progenitor cells and gave a proliferative advantage in a competitive setting. We also showed that interferon- $\alpha$  (IFN $\alpha$ ), a drug promoting cycling of dormant cells<sup>20,21</sup> and clinically effective in PV patients,<sup>22</sup> prevented disease and JAK2<sup>V617F</sup>-positive disease-initiating cell development. Overall, these results show that JAK2<sup>V617F</sup> provides a strong competitive advantage to HSCs that may account by itself for MPN emergence and that is lost upon IFN $\alpha$  treatment.

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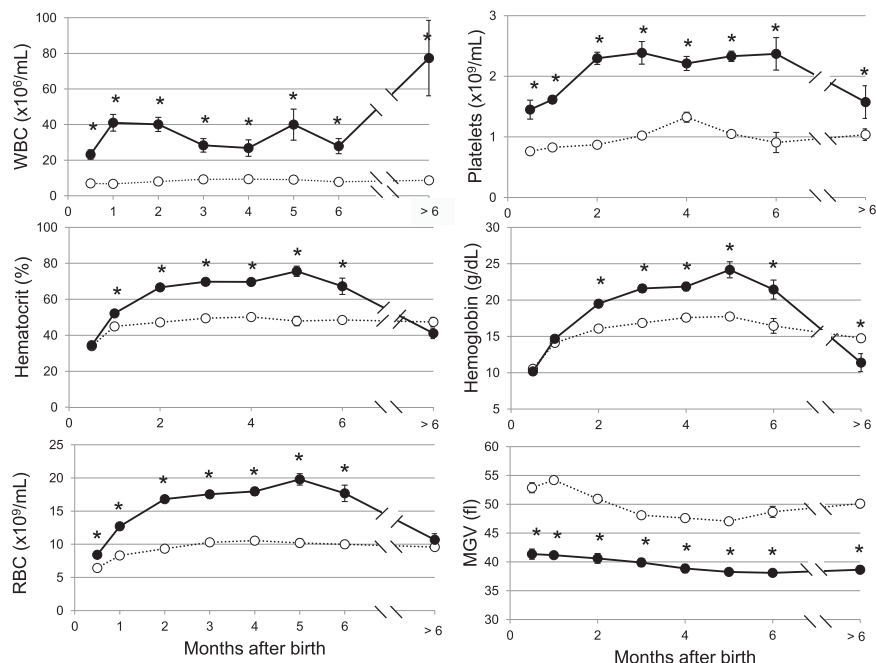
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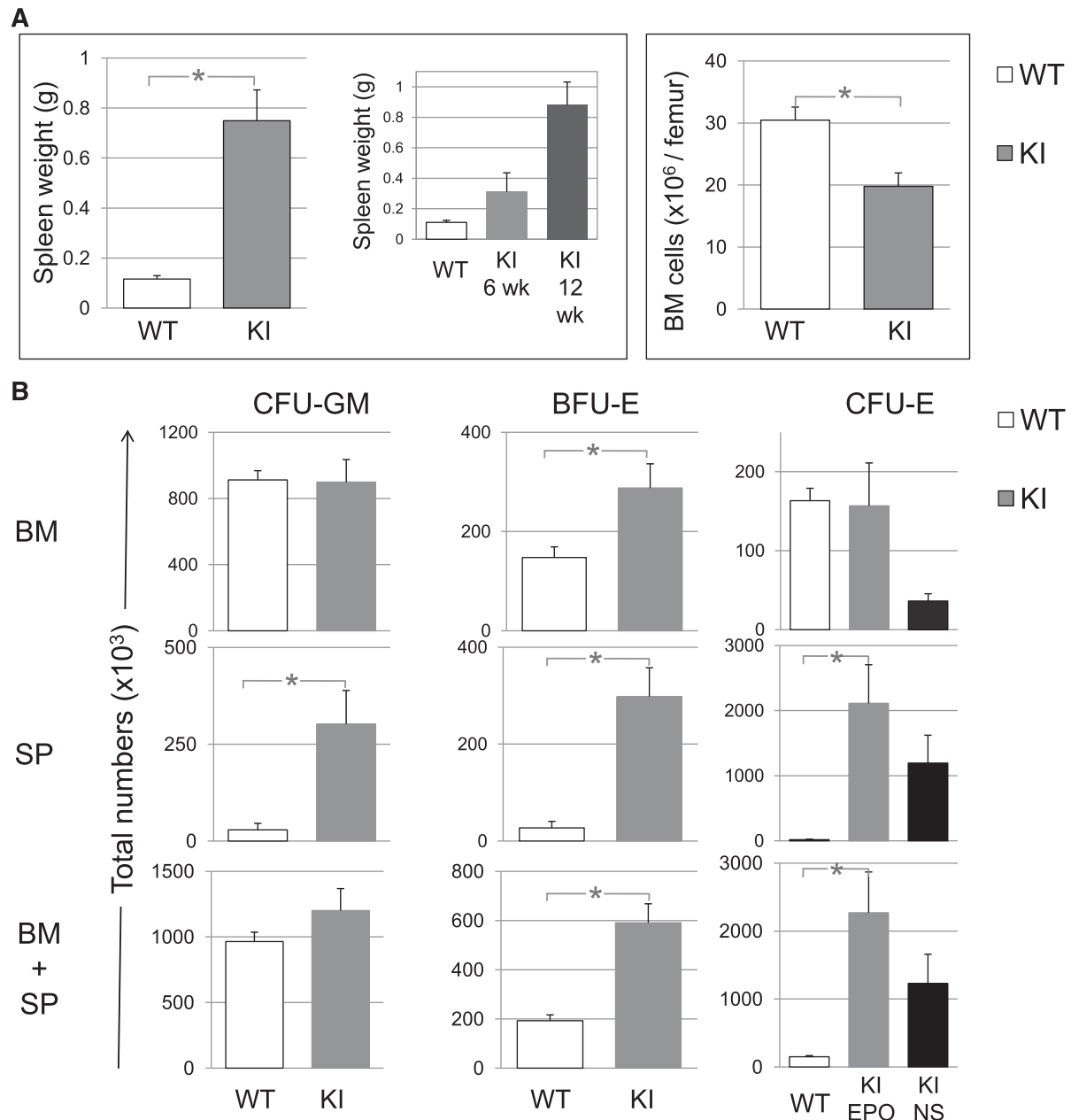
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**Figure 1. Schematic representation of the targeting vector and resulting modified allele to generate the *JAK2*<sup>V617F</sup> KI mouse model.** Homologous recombination into the *Jak2* WT allele of mouse embryonic stem (ES) cells resulted into the FLEX (F) or L2 conditional allele. A correctly targeted ES clone was injected into blastocyst stage embryos to generate L2 chimeric mice (WT phenotype). Chimeras were bred with flippase (FLP recombinase) TG mice to remove the FRT-flanked selection *PgkNeoR* cassette from the progeny. To generate the L- KI offspring, expressing the mutated exon 13 and the resulting JAK2<sup>V617F</sup> protein, K1<sup>flex(F)/+</sup> animals were crossed with TG animals expressing the Cre recombinase. Cre recombination induced the LoxP site-directed inversion of a KI construct, resulting in the Lox511-directed excision of the WT exon13 and transcription of the mutated exon13 into the G1849U-mutated mRNA that results into the JAK2<sup>V617F</sup> protein translation (KI phenotype). The WT, L2 (FLEX), and L- alleles could be discriminated using PCR analysis with different primers (a, b, c) whose location is represented in the figure. A typical result from tail-DNA genotyping, showing correct heterozygous recombination (L- construct), is shown with the a (TGTCCTACTAAAGCCAGGTGATGG)/b (GCTCCAGGTTACACGAGTC) couple of primers resulted into the 105-bp, 186-bp, and 884-bp (the latter not visible with our PCR conditions) PCR-amplified fragments within the WT, L-, and L2 allele, respectively. The c (GTCTGTCCAAAGAGTCTGTAAGTAC)/b couple of primers amplified a fragment (144 bp) only within the L2 allele. \*\* indicates the Lox511-based inversion can also precede the LoxP-based excision, resulting in an identical KI phenotype.



**Figure 2. Blood cell parameters in *JAK2*<sup>V617F</sup> KI mice.** Blood parameters from KI (filled circles, n = 5-41) and WT littermate (open circles, n = 5-24) mice were studied from 2 weeks of age to over 6 months of age. Values were grouped to the closest month. Hematocrit values at 2-3 months were 70 ± 2% n = 13, controls 49 ± 1% n = 13. Platelet numbers reach at 2 months 2.3 ± 0.1 × 10<sup>9</sup>/mL, n = 31, controls 0.84 ± 0.04 × 10<sup>9</sup>/mL, n = 20. WBCs remained high for 6 months (36 ± 2 × 10<sup>9</sup>/mL, n = 117, controls 8.0 ± 0.3 × 10<sup>9</sup>/mL, n = 89). After 6 months, WBCs reached 77 ± 21 × 10<sup>9</sup>/mL n = 5; controls: 8.7 ± 0.7 × 10<sup>9</sup>/mL, n = 8; but platelet decreased to subnormal levels (1.6 ± 0.21 × 10<sup>9</sup>/mL, n = 5; controls 1.03 ± 0.09 × 10<sup>9</sup>/mL, n = 8) along with hemoglobin levels (11.4 ± 1.2 g/L, n = 5, control values 14.7 ± 0.5 g/L, n = 8). Results are mean value ± SEM, \*P ≤ .05 with the 2-tailed unpaired Student *t* test. MCV, mean corpuscular volume; MGV, mean globular volume; MPV, mean platelet volume.



**Figure 3. Tissue cellularity, progenitor and early cell content in KI (gray bars), and WT littermate (white bars) mice.** (A) Spleen weight (also showing spleens collected in 6- and 12-week-old KI mice) and BM cell content (6-12 weeks) per femur in KI ( $n = 11$ ) and WT littermate ( $n = 9$ ) mice. (B) Total numbers ( $\times 10^3$ ) in BM (top), spleen (SP, middle) and cumulative numbers ( $\times 10^3$ ) in BM plus spleen (BM+SP) of CFU-GM, BFU-E and CFU-E from KI ( $n = 9$ ) and WT littermate ( $n = 7$ ) mice. CFU-E numbers from KI mice growing without added Epo (endogenous colonies) are represented in the black bars (NS for nonstimulated). No endogenous CFU-E was found in organs from WT littermate mice. The calculation is detailed in "Methods." (C) Percentages of Lin-, LSK, and SLAM cells in BM and spleen from KI and WT littermates measured by FACS at 1, 3, and 6 months of age,  $n = 3$  mice for 1- and 6-month-old mice and  $n = 8-12$  mice for 3-month-old mice. (D) Total numbers ( $\times 10^6$ ) of Lin-, LSK, and SLAM (LSK, CD48<sup>-</sup>, CD150<sup>+</sup>) cells in BM plus spleen from KI and WT littermates measured at 1, 3, and 6 months of age. Calculation is detailed in "Methods" from percentage of Lin-, LSK, and SLAM cells represented in C.  $n = 3$  for SLAM, LSK, and Lin- in 6-month-old mice or 8-9 mice for 3-month-old mice. Results are mean value  $\pm$  SEM. \* $P \leq .05$  with the 2-tailed unpaired Student  $t$  test.

## Methods

### Generation of the conditional JAK2<sup>V617F</sup> KI mice

Animal experiments were approved by the Institut Gustave Roussy review board, protocol no. 2012-061. The method to generate and genotype the conditional flexed JAK2 (JAK2<sup>FLEX/+</sup> or L2<sup>+/+</sup>) KI mice, backcrossed  $>15$  times with C57Bl/6 mice, is described in supplemental Data and Figure 1 (available on the *Blood* Web site). To express the mutation, KI mice were

crossed with *CmvCre* TG mice to validate the KI construct and with *VavCre* TG mice<sup>23,24</sup> for the rest of the study. We termed these heterozygous recombined JAK2<sup>V617F/WT</sup> mice (JAK2<sup>VF/+</sup>), with a wild-type (WT) and a mutated *Jak2* allele, as JAK2<sup>V617F</sup> KI mice.

### Treatment and analysis of mice

For bone marrow (BM) transplantation,  $3 \times 10^6$  C57/Bl6/6J BM cells were engrafted into lethally irradiated (9.5 Gy) congenic recipient mice. For competitive BM transplantation, lethally irradiated Ly5.1 WT recipient

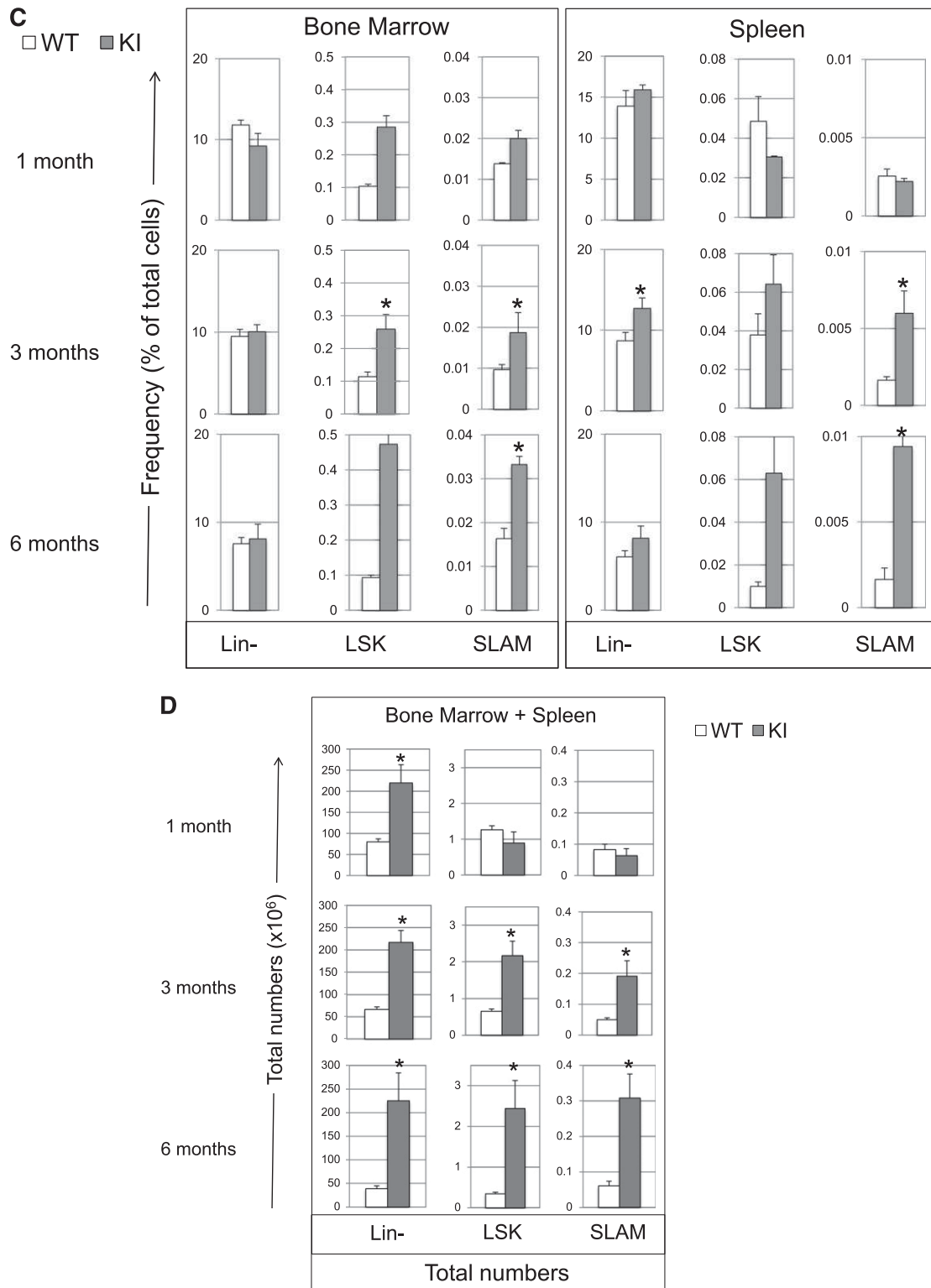


Figure 3. (Continued).

mice were injected with BM cells from Ly5.1+2 WT donor mice mixed with BM cells from Ly5.2 *Vav*Cre/JAK2<sup>V617F/+</sup> mice. Murine IFN $\alpha$  (Miltenyi Biotech; 10<sup>7</sup> IU/mL) was subcutaneously injected every day at a dose of 3  $\times$  10<sup>4</sup> IU in 0.2 mL phosphate-buffered saline/mouse (1.5  $\times$  10<sup>6</sup> IU/kg). Control vehicle mice were injected with phosphate-buffered saline.

Hemoglobin, mean corpuscular volume, hematocrit, red blood cell (RBC), platelet, and white blood cell (WBC) counts were determined

using an automated counter (MS9; Schloessing Melet, France) on blood collected from the retro-orbital plexus in citrated tubes. BM cells were removed by flushing both femurs. Spleens were weighed and single cell suspensions were prepared. For histopathology analysis, spleens were fixed in formaldehyde and sections (4.5  $\mu$ m) were stained with hematoxylin/eosin, periodic acid Schiff, and Giemsa for cytology analysis. Reticulin fibers were revealed by silver staining according to the Gordon Sweet method.

Flow cytometry of blood, BM, and spleen was used as described in supplemental Data.

### Progenitor cell study

Progenitor cell assays were carried out in 1 mL methylcellulose “MethoCult 32/34” (Stemcell Technologies) without stimulus or maximally stimulated by 10 ng/mL IL3, IL6, TPO, 100 ng/mL SCF, and 2 IU/mL EPO. Cultures in duplicate or quadruplicate were scored after 2 days for colony forming unit-erythroid (CFU-E) assays and 8 days for burst forming unit-erythroid (BFU-E) and colony forming unit granulocyte-macrophage (CFU-GM) assays. Total progenitor cell number was calculated assuming that one femur represents 6% of the total marrow and from the total number of cells isolated from the spleen.

### Cell cycle and apoptosis analysis

BM or spleen cells were stained for LSK and SLAM isolation. For apoptosis studies, cells were stained with Annexin V-PB antibody (Biollegend). For cell cycle status, cells were fixed, permeabilized, and then labeled with Ki-67-FITC (Becton Dickinson) and Hoechst 33342 (Invitrogen). Flow cytometry was performed using an LSR II analyzer (Becton Dickinson).

### Statistical analysis

Results are presented as mean  $\pm$  SEM and data were analyzed with the 2-tailed Student *t* test.

## Results

### Constitutive expression of JAK2<sup>V617F</sup> results in PV-like phenotype

We generated conditional JAK2<sup>V617F</sup> KI mice using the FLEX switch strategy<sup>25</sup> (Figure 1; supplemental Data). We crossed the JAK2<sup>FLEX/+</sup> KI mice with TG mice expressing the Cre recombinase under the control of the *Vav* promoter.<sup>26,27</sup> *Vav*Cre TG mice targets *in vivo* Cre recombination in HSCs<sup>27</sup> and some endothelial cells.<sup>24</sup> Allele specific reverse-transcription polymerase chain reaction in BM revealed an equal amount of JAK2<sup>WT</sup> and JAK2<sup>V617F</sup> mRNA, demonstrating that all BM cells were recombined with a normal transcriptional activity of the mutated locus (supplemental Figure 1). These conditional *Vav*Cre/JAK2<sup>V617F/+</sup> animals were born with severe thrombocytosis and leukocytosis (Figure 2). Surprisingly, the hemoglobin and hematocrit levels remained normal during the first month of life. RBC number was slightly in excess in 2-week-old mice, but severe microcytosis precluded any effect on hematocrit (Figure 2). At 2 to 3 months of age, hematocrit values were elevated and reached a plateau for 5 months. Reticulocyte counts were elevated ( $17 \pm 2\%$ , controls  $2.8 \pm 0.4\%$ ,  $n = 13$ ). Platelet numbers rapidly increased and reached a plateau for 6 months. In aged mice, WBCs reached high values, but platelet counts along with hemoglobin levels decreased to subnormal levels, as observed during myelofibrosis development in other models (Figure 2).<sup>5,28</sup>

Hematopoietic tissues were abnormal. BM cellularity was reduced (60% of control) (Figure 3A) with an increase in the percentage of granulocytes (from  $41 \pm 2\%$  in WT littermates to  $70 \pm 3\%$ ;  $P = .02$ ) and a decrease in both mature erythroblasts (from  $30 \pm 4\%$  in WT to  $8 \pm 1\%$ ;  $P = .03$ ), as previously reported in JAK2<sup>V617F</sup> mouse models,<sup>5,10</sup> and B cells (from  $20 \pm 2\%$  in WT to  $10 \pm 3\%$ ). Splenomegaly was mild at 6 weeks (3-fold) but severe at 12 weeks (8-fold) (Figure 3A) and was due to an increase in erythroblasts (from  $7 \pm 5\%$  in WT to  $14 \pm 2\%$ ) and myeloid cells (from  $2 \pm 1\%$  in WT to  $18 \pm 4\%$ ), with a decreased

percentage of B cells (from  $51 \pm 3\%$  in WT to  $27 \pm 4\%$ ). In aged mice (>6 months), we observed high-grade fibrosis in BM and spleen and extra-medullary hematopoiesis in liver (supplemental Figure 2).

These data show that heterozygous endogenous JAK2<sup>V617F</sup> expression in hematopoietic cells leads to hyperplasia of mature and maturing erythroid, granulocytic, and megakaryocytic cells in blood and hematopoietic tissues that models human PV with secondary myelofibrosis.

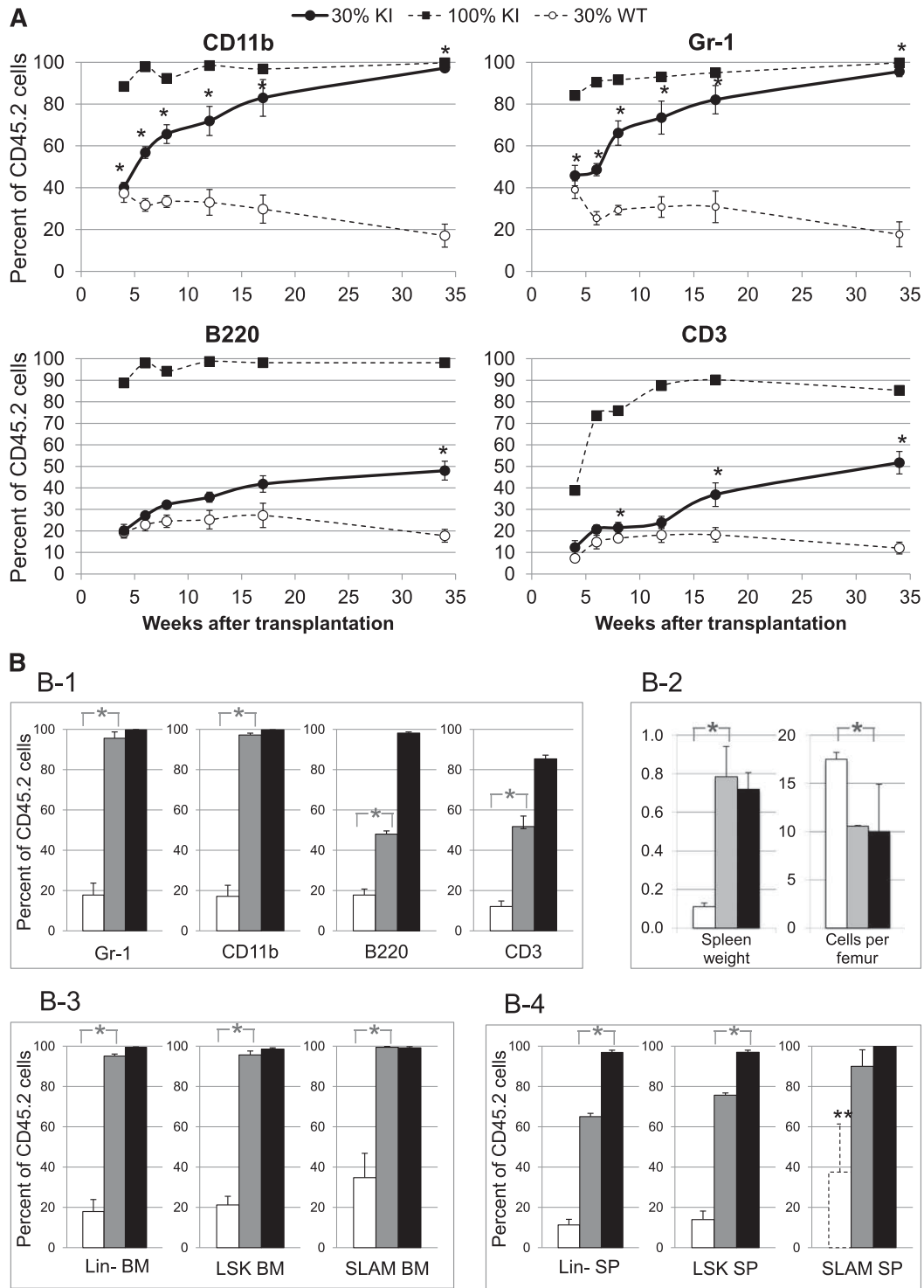
### Endogenous JAK2<sup>V617F</sup> expression increases early stages of differentiation

We next studied the effect of JAK2<sup>V617F</sup> on early stages of differentiation. We first analyzed progenitor cell numbers from *Vav*Cre/JAK2<sup>V617F/+</sup> animals using clonogenic assays. In the marrow, the frequencies of all progenitor cells except CFU-E increased (supplemental Figure 3). Taking into account the decrease in BM cellularity (Figure 3A), the total numbers of myeloid progenitors (CFU-GM) and late erythroid progenitors (CFU-E) did not differ from littermates, but early erythroid progenitor cells (BFU-E) were 2-fold increased (Figure 3B). In the spleen, frequencies of all progenitor cells were increased (supplemental Figure 3) and taking into account splenomegaly, the myeloid progenitor cell compartment was largely amplified, particularly CFU-E (124-fold) (Figure 3B), with 56% of them being Epo-independent. Finally, cumulative numbers of CFU-GM, BFU-E, and CFU-E in marrow plus spleen were increased by 1.2-, 3-, and 15-fold, respectively (Figure 3B).

We then analyzed by flow cytometry Lin<sup>-</sup>, LSK, and LSK/CD48-/CD150+ cells, so-called SLAM cells, which are highly enriched (1 of 2.2)<sup>29</sup> in long-term reconstituting activity (LT-HSC) (supplemental Figure 4). In 3-month-old mice, JAK2<sup>V617F</sup> KI animals demonstrated an increase in marrow no change in the frequency of Lin<sup>-</sup> cells but an increase in LSK ( $\times 2.3$ ) and SLAM ( $\times 2$ ) cells (Figure 3C). In spleen, the percentages of Lin<sup>-</sup>, LSK, and SLAM cells increased 1.5-, 1.7-, and 3.7-fold, respectively. We noticed a progressive increase with age. A normal frequency was observed in 1-month-old mice, but the percentages of BM and spleen LSK and SLAM cells were increased in 6-month-old mice more than in 3-month-old mice. Overall, we observed a 3-, 4-, and 5-fold increase in the total numbers (BM plus spleen) of Lin<sup>-</sup> cells in 1-, 3-, and 6-month-old mice, respectively. In contrast, there was no increase in the total numbers of LSK and SLAM cells in 1-month-old mice but a 3- or 7-fold and a 4- or 6-fold increase in the total numbers of LSK and SLAM cells in 3- or 6-month-old mice, respectively (Figure 3D). One possible pitfall could be the upregulation of Sca-I by JAK2<sup>V617F</sup>, but SLAM cells, analyzed regardless of Sca-I, were similarly increased. Related to c-Kit+/Lin<sup>-</sup> progenitor cells (LK), the total numbers of MEP, GMP, and CMP augmented in 3- and 6-month-old mice, mainly due to the spleen content with marked and early increase in MEP (supplemental Figures 4 and 5). Overall, the differences between KI and WT mice in the pools of LSK, GMP, CMP, and particularly SLAM cells increased with the age, whereas these differences in MEP and Lin<sup>-</sup> pools seemed precociously established. These results show that endogenous JAK2<sup>V617F</sup> progressively amplifies the earliest stages of differentiation with the age while late stages are already amplified in young mice.

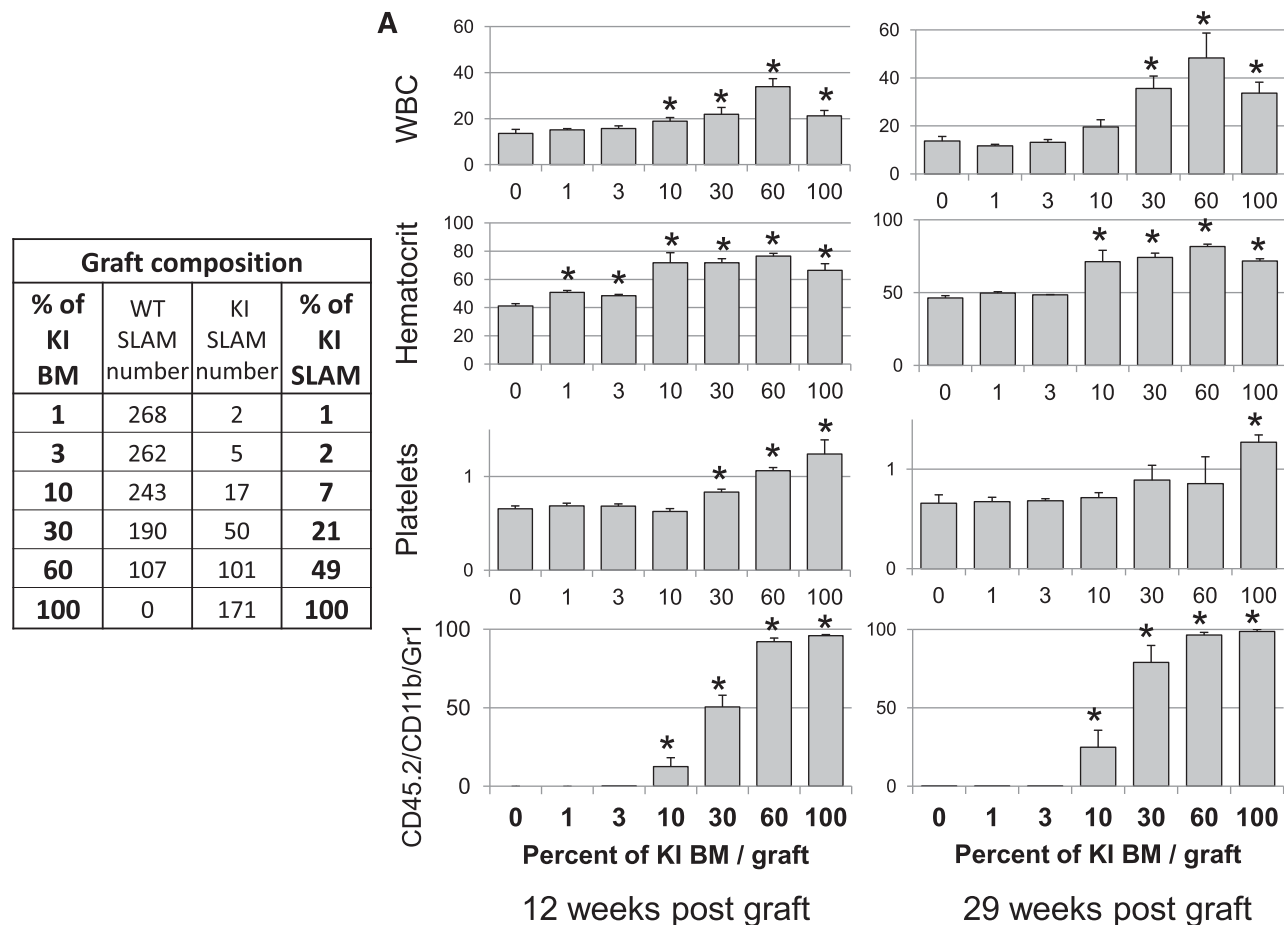
### JAK2<sup>V617F</sup> provides a competitive advantage to hematopoietic cells at early stages of differentiation

We next investigated if JAK2<sup>V617F</sup> expression confers a proliferative advantage to LT-HSC using competitive grafts in CD45.1 WT



**Figure 4. Competitive grafts in recipient mice transplanted with 3-month-old donor mice showing proliferative advantage of KI cells over WT cells.** (A) Percentage of CD45.2<sup>+</sup> blood myeloid (CD11b<sup>+</sup> or Gr-1<sup>+</sup>) and lymphoid (B220<sup>+</sup> or CD3<sup>+</sup>) cells from KI or WT origin in recipient mice showing progressive overgrowth of KI cells in competitive grafts. CD45.1 recipient mice were transplanted with either 100% KI cells (CD45.2) (■, dashed line, n = 5) or a mixture of 30% KI cells (CD45.2) plus 70% WT CD45.1+2 cells (●, solid line, n = 5). As a control, CD45.1 recipient mice were transplanted with a mixture of 30% WT CD45.2 cells and 70% WT CD45.1+2 cells (○, dashed line, n = 5). (B) Percentages of CD45.2<sup>+</sup> cells from KI or WT origin in blood and tissues analyzed 34 weeks after competitive grafts showing progressive overgrowth of KI cells in late and early stages of differentiation. CD45.1 recipient mice were transplanted with either 100% KI cells (CD45.2) (black columns) or a mixture of 30% KI cells (CD45.2) plus 70% CD45.1+2 WT cells (gray columns). As a control, CD45.1 recipient mice were transplanted with a mixture of 30% WT CD45.2 cells plus 70% WT CD45.1+2 cells (white columns). (B-1) CD45.2, Gr-1, CD11b, B220, and CD3-positive cells were determined in blood. (B-2) Spleen weight (g) and marrow cellularity (×10<sup>6</sup> cells/femur) in recipient mice. (B-3-4) CD45.2<sup>+</sup>, Lin-, LSK, and SLAM (LSK, CD48<sup>-</sup>, CD150<sup>+</sup>) cells were determined in BM (3) or spleen (4). \*\* indicates that the numbers of SLAM cells detected in the WT spleen samples were too low (10 ± 5 cells) to provide a reliable value. Data are mean values ± SEM, n = 3. \*P ≤ .05 with the 2-tailed unpaired Student *t* test.





**Figure 5. Competitive grafts with different ratio of KI/WT BM cells showing disease propagation from a low number of KI SLAM cells.** (A) Left: composition in WT/KI SLAM (LSK, CD48<sup>+</sup>, CD150<sup>+</sup>) cells, determined by FACS analysis, in the  $3 \times 10^6$  cells grafts mixing different percentages of KI and WT BM cells from 1-month-old donor mice. Right: WBC ( $10^9$ /mL), hematocrit (%), and platelet ( $10^9$ /mL) levels and frequency of blood KI myeloid cells (CD45.2/CD11b/Gr-1) measured 12 and 29 weeks after transplantation of BM samples containing 0%, 1%, 3%, 10%, 30%, 60%, and 100% KI BM cells ( $n = 5$ /graft mixture). Results show that a disease may develop (3/5 recipients) from a graft containing 10% KI BM cells or 17 KI SLAM cells. (B) BM cellularity, spleen weight, and Lin<sup>-</sup>, LSK, and SLAM cells from KI origin found 29 weeks after transplantation in mice transplanted with 0%, 10%, 30%, and 100% KI BM cells. Results show low BM cellularity, splenomegaly, and amplification of early cells from the initial 7% and 21% KI SLAM cell input present in the 10% and 30% KI BM graft. (C) Percent of CD3, B220, and CD11b/Gr-1 cells from KI origin in the blood of mice transplanted with 10%, 30%, 60%, and 100% KI BM cells. Results show that myeloid cells but not lymphoid cells were amplified after transplantation from the initial 7%, 21%, and 49% KI SLAM cell input present in the 10%, 30%, and 60% KI BM graft. Data are mean values  $\pm$  SEM,  $n = 5$ /graft mixture, except for mice grafted with 10% KI cells in graphs B and C, where only 3 mice developed the disease, the 2 other mice having no KI cells detectable 6 months after transplantation. \* $P \leq .05$  with the 2-tailed unpaired Student *t* test compared with recipients grafted with 0% KI cells.

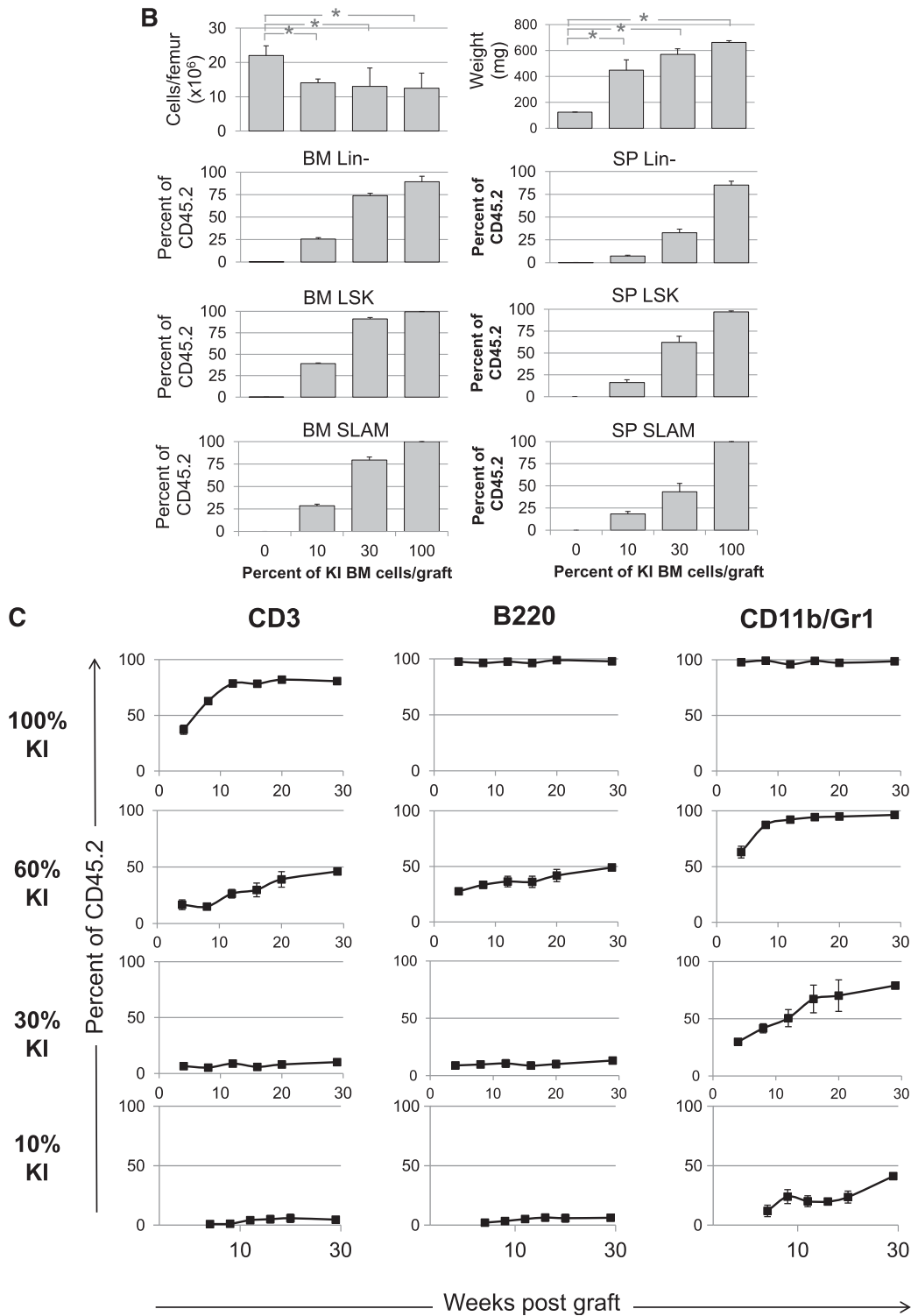
lethally irradiated recipient animals transplanted with 30% CD45.2 KI and 70% CD45.1+2 WT BM cells collected from 3-month-old donor mice. As a WT control, we used 30% CD45.2 WT and 70% CD45.1+2 WT grafts and as a control for full disease engraftment, we used 100% CD45.2 KI grafts.

The disease was transmitted to all recipients grafted with 30% KI graft (supplemental Figure 6). Blood KI myeloid cells (CD45.2/CD11b/Gr-1) increased from  $\sim 40\%$  to  $\sim 90\%$  (similarly to mice transplanted with 100% KI cells) from 4 to 34 weeks posttransplantation (Figure 4A). In contrast, the percentages of blood KI lymphoid cells (CD45.2/B220/CD3) only reached  $\sim 50\%$  at 34 weeks posttransplant (Figure 4A-B). Mice were killed 34 weeks posttransplant to analyze chimerism in Lin<sup>-</sup>, LSK, and SLAM cells. Strikingly, the percentages of BM Lin<sup>-</sup>, LSK, and SLAM cells from KI origin in mice grafted with 30% KI cells reached similarly high percentages ( $95 \pm 1\%$ ,  $96 \pm 2\%$ , and  $99 \pm 1\%$ , respectively) as those ( $99 \pm 1\%$ ,  $98 \pm 2\%$ , and  $99 \pm 1\%$ , respectively) found in mice transplanted with 100% KI BM cells (Figure 4B). The percentages of spleen KI Lin<sup>-</sup>, LSK, and SLAM cells ( $65 \pm 2\%$ ,  $76 \pm 1\%$ , and

$90 \pm 8\%$ , respectively) were slightly lower (Figure 4B). The extent of splenomegaly and the reduction in marrow cellularity were similar in mice transplanted with 30% and 100% KI cells, showing that disease severity was comparable in both groups (Figure 4B).

These results show that endogenous JAK2<sup>V617F</sup> expression confers a selective advantage to LT-HSC and myeloid cell progenies.

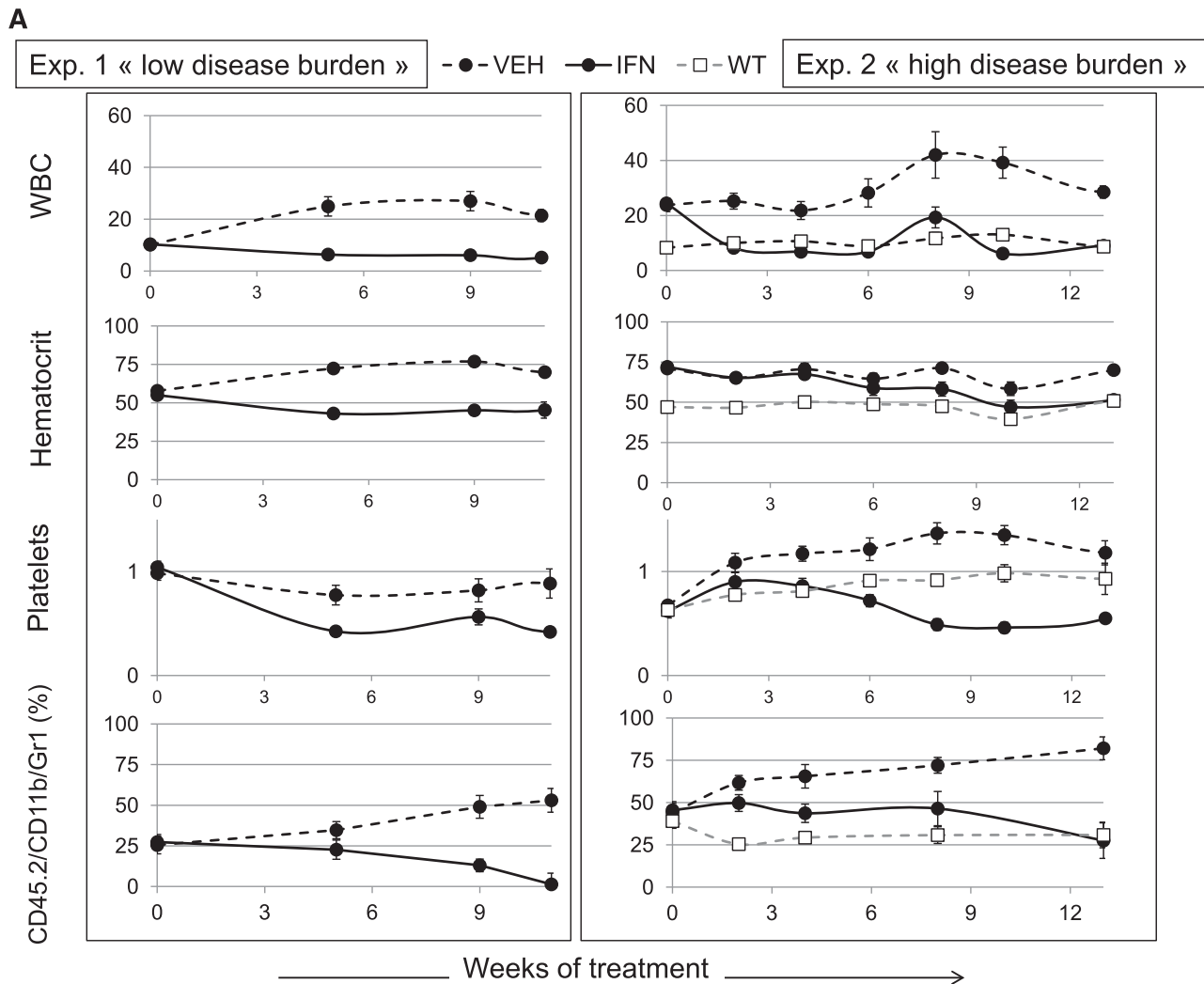
Our 30% KI graft initiated from 3-month-old mice should correspond to a 46% KI graft at the stem cell level due to the previously shown 2-fold BM SLAM amplification. Therefore, to know the minimum graft necessary for disease emergence, we developed competitive grafts from young donor mice, including different KI/WT BM cell percentages corresponding to similar KI/WT SLAM cell percentages and known KI SLAM numbers (Figure 5A). Results showed that 30% KI BM containing  $\geq 50$  KI SLAM cells always generated a long-term disease (29 weeks posttransplant) with polycythemia, leukocytosis, thrombocytosis, splenomegaly, and low BM cellularity, although long-term thrombocytosis was not always observed (Figure 5A-B). In contrast, only 3 of 5 recipients grafted with 10% KI BM or 17 KI SLAM cells



developed the disease. Experiments including 10 and 15 mice confirmed that 10% KI BM grafts generated only long-term disease (15 months) in ~50% recipients (5/10 and 7/15). These results suggest that in competition assays, ~30 JAK2<sup>V617F</sup> SLAM cells are sufficient to generate MPN with complete penetrance. Interestingly, the percentages of blood lymphocytes from KI origin 30 weeks posttransplant were similar or lower to the percentages of KI SLAM cells initially transplanted, suggesting no or very

delayed KI lymphoid cell amplification (Figure 5C). In contrast, as previously shown, the percentages of KI blood myeloid cells, BM/spleen Lin-, LSK, and SLAM cells were higher than the initial KI SLAM cell input, showing overgrowth of these cells in recipients (Figure 5B-C).

These results show that endogenous JAK2<sup>V617F</sup> expression generates MPN in mice from a low number of HSCs and confers no selective advantage or bias to lymphoid cells.



**Figure 6. Effect of IFN $\alpha$  treatment on hematological parameters and malignant JAK2<sup>V617F</sup>-positive cell proliferation.** KI recipient animals (●) were transplanted with 30% CD45.2 KI and 70% CD45.1+2 WT BM cells from 1 (exp. 1, left) or 3 (exp. 2, right) month-old donor mice and treated for 11 (exp. 1) or 13 (exp. 2) weeks with IFN $\alpha$  (continuous line) or vehicle (dashed line) starting 7 weeks after transplantation. Control WT recipient animals (□) represent untreated mice grafted with 30% CD45.2 WT and 70% CD45.1+2 WT cells. (A) Variations in WBC ( $10^6$ /mL), hematocrit (%), platelet ( $10^9$ /mL), and the percentage of KI-derived myeloid blood cells (Gr-1/CD11b/CD45.2) analyzed during 11 or 13 weeks of treatment of primary recipient animals. Data are mean values  $\pm$  SEM of 10 or 5 IFN $\alpha$ -treated, 10 or 5 vehicle-treated (exp.1 or 2, respectively) and 5 nontreated (WT) mice. (B) Variations after 11 weeks of treatment (from exp. 1) in the percentages of cells from KI origin in BM, spleen, and blood. Are represented the early cell pools in BM (Lin-, MEP, CMP, GMP, LK, LSK, and SLAM cells determined as supplemental figure 4) (upper left) and CD11b/Gr1, B220, CD3 in BM (upper right), blood (lower left) and spleen (lower right). Data are mean values  $\pm$  SEM,  $n = 3$ . (C) Secondary transplantation from exp. 2: variations in the hematological parameters and the percentages of KI-derived cells, analyzed by allele-specific PCR, of animals transplanted with BM from donors treated during 13 weeks with IFN $\alpha$  (continuous line) and vehicle (dashed line). Data are mean values  $\pm$  SEM of 2 groups of 5 mice, each transplanted with BM from 2 different donors. Results from the secondary transplantation of exp. 1 are figured in supplemental Figure 11.

### Effect of long-term treatment by IFN $\alpha$

Previous results in KI mice have shown that a JAK2 inhibitor could not eradicate disease-initiating cells.<sup>11</sup> Therefore, we investigated if another drug, IFN $\alpha$ , could hamper this early cell reservoir for MPN propagation. IFN $\alpha$  seemed a good candidate, because it induces HSC exhaustion<sup>20,21</sup> and molecular response in patients suffering from PV through still unclear mechanisms.<sup>22,30</sup>

IFN $\alpha$  treatment was initiated in 2 groups of CD45.1 WT recipient mice 7 weeks after the transplantation of 30% KI BM cells collected from 1- or 3-month-old donor mice in order to generate low or high disease burden, as previously shown. In both groups of mice, IFN $\alpha$  treatment induced a rapid suppression of leukocytosis without leukopenia (Figure 6A). All cell types dropped, but mainly B cells and the percentage of T cells, particularly CD3+LY6C+, and myeloid cells increased (supplemental Figure 7).

Platelet levels also dropped quickly after IFN $\alpha$  treatment but reached a plateau lower than control (Figure 6A). Hematocrit, hemoglobin, and RBC counts normalized early or late after treatment according to low or high disease severity (Figure 6A). Microcytosis was corrected after polycythemia (supplemental Figure 8), suggesting correction of iron deficiency.<sup>10</sup> The percentage of myeloid cells from KI origin (CD45.2/CD11b/Gr-1) in vehicle-treated mice increased with time, highlighting disease progression. IFN $\alpha$  cancelled out this progression, and myeloid chimerism at the end of treatment was reduced to  $\sim 0\%$  or  $27 \pm 10\%$  vs  $53 \pm 3\%$  or  $85 \pm 6\%$  in vehicle-treated mice in the first or second group of mice, respectively (Figure 6A). Chimerism was also strongly reduced in BM lymphoid, myeloid progenitor, and SLAM cells but not in blood and spleen lymphocytes (Figure 6B; supplemental Figure 9). Splenomegaly was slightly reduced in the severe disease group ( $323 \pm 4$  mg vs

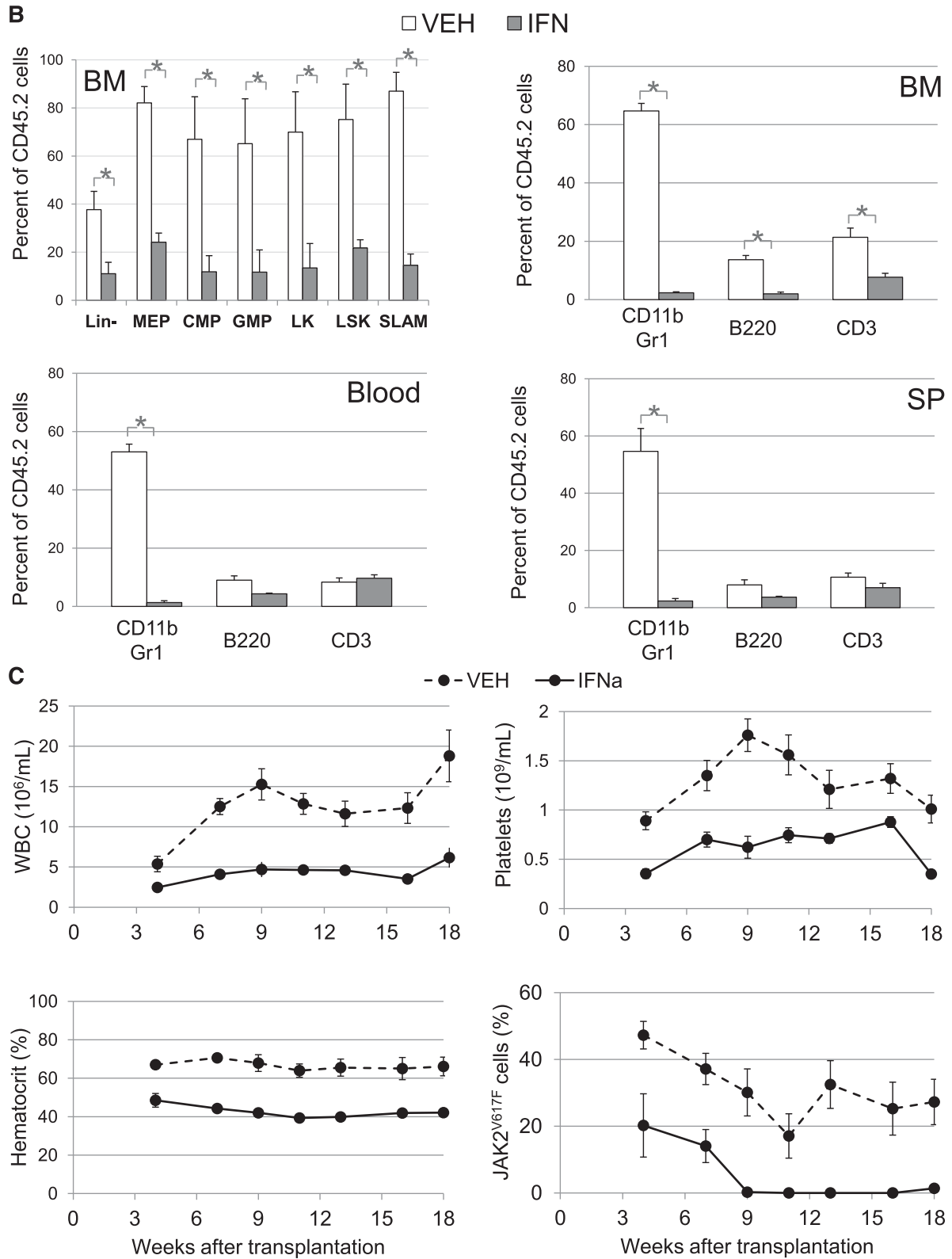


Figure 6. (Continued).

485 ± 20 mg). In the low severity group, splenomegaly was strongly reduced (176 ± 3 mg vs 580 ± 27 mg; *P* = .001) with normalization of its architecture. BM cellularity was increased. Myeloid and MKC hyperplasia were suppressed in BM and spleen (supplemental Figure 10).

These results show that IFN $\alpha$  hampers JAK2<sup>V617F</sup> cell proliferation with normalization of most blood hematological values, except thrombocytopenia, according to disease severity.

To check if IFN $\alpha$  targeted the disease-initiating stem cells, we carried out secondary transplantation from 3 × 10<sup>6</sup> BM cells collected

from IFN $\alpha$ - or vehicle-treated mice. As expected, mice transplanted with the vehicle-treated BM cells developed MPN with high hematocrit and WBC values (Figure 6C; supplemental Figure 11). In contrast, mice transplanted with the IFN $\alpha$ -treated BM cells developed normal hematocrit, platelet, and WBC values. We finally checked whether JAK2<sup>V617F</sup>-positive cells were still present in transplanted mice. From the IFN $\alpha$ -treated mice displaying the low severity disease, no cell from KI origin (CD45.2) was detected in the secondary recipients in contrast to vehicle-treated mice (supplemental Figure 11). Because we used CD45.2 recipients for IFN $\alpha$ -treated mice displaying the severe disease, we developed an allele-specific PCR assay to determine JAK2<sup>V617F</sup> allele burden in WBC. JAK2<sup>V617F</sup> was detected in WBC up to 7 weeks posttransplant and thereafter became undetectable (Figure 6C). In contrast, the level of JAK2<sup>V617F</sup>-WBC remained stable in mice transplanted with vehicle-treated BM cells from week 7 to 20 of survey.

These results show that long-term treatment by IFN $\alpha$  is able to alleviate JAK2<sup>V617F</sup>-derived MPN by suppressing long-term disease-initiating cells.

IFN $\alpha$  is known to induce antiproliferative and proapoptotic activities. To know if the effect of IFN $\alpha$  on JAK2<sup>V617F</sup> disease-initiating cells was mediated through cell cycle or apoptosis, we measured these parameters in LSK/SLAM cells from KI and WT littermate mice treated 3 days with IFN $\alpha$  ( $3 \times 10^4$  IU) or vehicle. First, the results showed that LSK/SLAM cells from KI mice were more proliferative and less apoptotic than those from WT mice (Figure 7A-B; supplemental Figures 12 and 13). These properties may explain their amplification and proliferative advantage. Second, IFN $\alpha$  strongly induced the proliferation of WT LSK/SLAM, which, as previously reported,<sup>20</sup> had an effect on KI LSK cell cycle but none on KI SLAM cell cycle. Overall, IFN $\alpha$  treatment induced WT SLAM cells to become as proliferative as KI SLAM cells. We noticed that IFN $\alpha$  increased Sca-1 expression, as reported for IFN $\gamma$ ,<sup>31</sup> and could bias our results, but we obtained similar results without including Sca-1 (that is dispensable) in SLAM cell characterization. Third, LSK and SLAM cells were clearly less apoptotic in KI than in WT mice. However, IFN $\alpha$  markedly induced apoptosis of spleen, but not BM, KI LK, and LSK/SLAM cells, and KI SLAM became as apoptotic as WT SLAM (Figure 7B; supplemental Figure 13).

These results suggest that IFN $\alpha$  eradicates JAK2<sup>V617F</sup> disease-initiating cells in long-term treatment by increasing WT HSC cell cycle and KI HSC apoptosis, suppressing overall the proliferative advantage of KI over WT HSC.

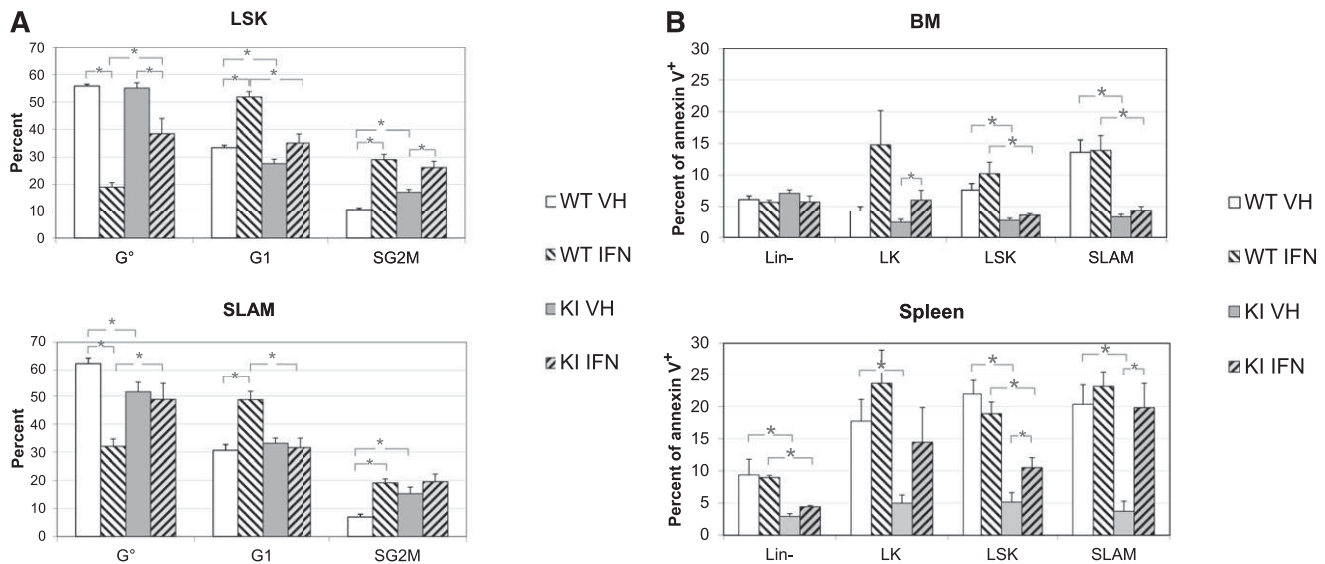
## Discussion

In this study, we show that endogenous expression of JAK2<sup>V617F</sup> in mouse HSCs induces a progressive amplification of early hematopoietic cells and provides to HSCs a strong competitive advantage over normal ones that could be explained by their high proliferative and low apoptotic status. Furthermore, long-term IFN $\alpha$  treatment suppresses disease progression by targeting JAK2<sup>V617F</sup>-expressing disease-initiating cells. Overall, this study suggests that JAK2<sup>V617F</sup> is capable of driving MPN emergence by itself from a low number of HSCs and that IFN $\alpha$  hampers this ability.

We previously described a constitutive JAK2<sup>V617F</sup> KI.<sup>10</sup> In this report, a conditional KI with restricted expression in hematopoietic and some endothelial cells<sup>24</sup> also generated a 2-stage MPN-mimicking

human PV evolving into secondary myelofibrosis but with lower/more delayed splenomegaly/leukocytosis and polycythemia than our constitutive KI. This may be due to the late JAK2<sup>V617F</sup> gene expression controlled by the *Vav* promoter during fetal but not embryo life.<sup>32</sup> All JAK2<sup>V617F</sup> Retroviral,<sup>5-7</sup> TG,<sup>8</sup> and KI<sup>9-12</sup> mouse models have described the occurrence of MPN, emphasizing the responsibility of the mutation in this disorder. However, the phenotype of these models often differs. Studies have shown that the mutated/WT JAK2 ratio is crucial in determining disease diversity through various levels of RBC and platelet counts.<sup>5-8</sup> However, it is not yet well understood why heterozygous expression of mouse JAK2<sup>V617F</sup> in all KI models induces a PV-like disease, one without thrombocytosis,<sup>11</sup> whereas heterozygosity is usually associated with ET in humans. An ET-like disease is observed only in the heterozygous KI using the human gene.<sup>12</sup>

The effect of JAK2<sup>V617F</sup> on the amplification of late mature and maturing myeloid cells is well established in mice<sup>5-11</sup> as in humans.<sup>18,33</sup> In contrast, the effect of JAK2<sup>V617F</sup> on the amplification of early hematopoietic cells remains a matter of controversy.<sup>17</sup> Our study shows that JAK2<sup>V617F</sup> increases in 6-month-old mice the percentage of LSK/SLAM cells by 5-/2-fold and 6-fold in marrow and spleen, respectively. These phenotypically defined cell pools are the most HSC-enriched compartments.<sup>29</sup> Taking into account the BM and spleen cellularity, we measured a 7- or 5-fold expansion in the total numbers of LSK or SLAM cells in these mice, not including the contribution of the extra-medullary hematopoiesis evidenced in these animals. Interestingly, the pool of HSCs is quasi-normal at 1 month and expands only with age, while more mature cells amplify precociously. Our study also demonstrates the ability of JAK2<sup>V617F</sup>-expressing HSCs to compete with normal HSCs in transplantation settings with JAK2<sup>V617F</sup>-expressing blood granulocytes and tissue Lin-, LSK, and SLAM cells rapidly prevailing upon normal ones. Our results are compatible with 2 other KI models reporting an increase in BM LSKCD34-Flk2- cells<sup>34</sup> and a competitive advantage of JAK2<sup>V617F</sup>-expressing HSCs recorded after 1 year of competitive transplantation.<sup>35</sup> In contrast, others reported a functional impairment of HSCs by JAK2<sup>V617F</sup>,<sup>12</sup> which could be related to the use of a human JAK2<sup>V617F</sup> gene. Overall, murine models indicate that JAK2<sup>V617F</sup> provides a clonal advantage to HSCs. Our study further shows that ~30 JAK2<sup>V617F</sup> SLAM cells are sufficient to generate MPN with complete penetrance, which further emphasizes the sole role of JAK2<sup>V617F</sup> in this model and is compatible with a mathematic model suggesting that clonal dominance would need more than a single transformed cell to appear in a mouse lifetime.<sup>36</sup> We also demonstrated that KI SLAM cells are less apoptotic and more proliferative than normal SLAM cells. This result may explain the proliferative advantage and amplification of KI over WT HSCs. The use of JAK2 by cytokines involved in HSC regulation, such as TPO,<sup>37,38</sup> granulocyte colony stimulating factor,<sup>39</sup> or IFN $\gamma$ ,<sup>31</sup> may explain this result. This study shows no or very delayed lymphoid cell amplification by JAK2<sup>V617F</sup>, which emphasizes the mild role of JAK2 in lymphocyte signaling or suggests that amplified SLAM cells are myeloid biased.<sup>40</sup> Intriguingly, amplification of KI SLAM cells in vehicle-treated primary recipients did not result in the same amplification of KI donor cells in secondary hosts. This result suggests that the amplification of SLAM cells by JAK2<sup>V617F</sup> does not correlate with HSC activity. Similar results were reported by Mullally et al,<sup>41</sup> indicating that further experiments are needed to precisely determine the HSC properties of SLAM cells amplified by the mutation, with the caveat that high proliferative status may compromise their long-term self-renewal capacities.



**Figure 7. Effect of IFN $\alpha$  on cell cycle and apoptosis of LSK and SLAM cells from KI and WT mice.** (A) Percentages of LSK (top) or SLAM (bottom) in G0 (Ki67<sup>low</sup>/H33342<sup>2n</sup>, quiescent), G1 (Ki67<sup>high</sup>/H33342<sup>2n</sup>, activated), and S/G2M (Ki67<sup>high</sup>/H33342<sup>4n</sup>, cycling) phases after 3 days of in vivo treatment by vehicle or IFN $\alpha$ . Results show that KI SLAM are more proliferative than WT SLAM but that IFN $\alpha$  induced proliferation of WT LSK and SLAM cells, reducing the differences in cycle between WT and KI SLAM cells. (B) Percentage of apoptotic cells (annexin V) in BM (top) and spleen (bottom) of Lin<sup>-</sup>, LK, LSK, and SLAM cells after 3 days of in vivo treatment by vehicle or IFN $\alpha$ . Results showed that BM and spleen LSK and SLAM cells are less apoptotic in KI mice than in WT mice and that IFN $\alpha$  slightly increased apoptosis, especially of spleen KI cells. \* $P \leq .05$  with the 2-tailed unpaired Student *t* test. Both cycle and apoptosis results are mean values  $\pm$  SEM,  $n = 6$ , of 2 independent experiments each including 3 IFN $\alpha$ -treated and 3 VEH-treated WT and KI mice around 3 months of age.

In ET and PV patients, most studies agreed on the fact that the percentage of JAK2<sup>V617F</sup>-positive cells is lower in progenitor cells than in differentiated stages. In contrast, an amplification of JAK2<sup>V617F</sup>-positive cells was identified in progenitor and HSC of PMF or heterozygous PV patients,<sup>18,33,42</sup> that may present more evolved forms of disease. Therefore, as shown by this study, JAK2<sup>V617F</sup> might give only a subtle advantage to HSCs that can be detected after several months in mice but only after several years in humans. As previously predicted,<sup>18,19</sup> amplification of heterozygous JAK2<sup>V617F</sup> HSCs could also explain why heterozygosity leads to PV in mice but only to ET in humans unless additional molecular events, such as *TET2* mutations, accelerate the upstream amplification of heterozygous cells. This hypothesis is corroborated by the association between an ET-like phenotype and the lack of early amplification in our young KI mice or KI mice using human JAK2<sup>V617F</sup>.<sup>12</sup> In humans, the presence of other mutations affecting *TET2*,<sup>19</sup> *DNMT3a*,<sup>16</sup> or *ASXL1* might not only give a further opportunity to the clone to develop the disease but also modify the phenotype or accelerate disease evolution. However, the possibility remains that human JAK2<sup>V617F</sup> is less functional, at least in a mouse context, than its mouse homolog and that these mutations become mandatory for disease emergence itself.

JAK2 inhibitors are currently tested in MPN patients and ruxolitinib was approved for use in the treatment of myelofibrosis.<sup>43</sup> These drugs have essentially demonstrated palliative virtues with limited effects on the selective reduction of JAK2<sup>V617F</sup>-positive cells in human as in mice.<sup>11,44</sup> In contrast, IFN $\alpha$  treatment has demonstrated hematological and molecular responses, including complete molecular responses in PV patients.<sup>22</sup> Therefore, we tested the efficacy of IFN $\alpha$  in competitive transplantation models that included normal hematopoiesis and 2 different levels of JAK2<sup>V617F</sup> disease burden. IFN $\alpha$  rapidly reduced platelet and WBC counts and corrected erythrocytosis and splenomegaly according to disease severity. This is in agreement with previous studies in normal mice showing that IFN $\alpha$  impaired lymphopoiesis<sup>45</sup> as well as myelopoiesis,<sup>46</sup> inducing anemia, leukopenia, and thrombocytopenia.

Prolonged IFN $\alpha$  exposures (11-13 weeks) were used, because it seems an important factor for complete molecular responses in humans.<sup>22</sup> Development of thrombocytopenia was observed. It has been attributed in humans to inhibition of platelet production by megakaryocytes.<sup>47</sup> Most interestingly, the treatment targeted granulocytes but also SLAM JAK2<sup>V617F</sup> cells that were considerably reduced at the end of treatment. Secondary transplantations using treated marrow showed that disease developed normally from vehicle-treated cells but not from IFN $\alpha$ -treated cells. This result indicated that IFN $\alpha$  treatment suppressed JAK2<sup>V617F</sup> HSC amplification and proliferative advantage. This finding may give the opportunity to understand the mechanism of action of this drug that is largely unknown. Our results show that IFN $\alpha$  promoted cycling of normal stem cells, as previously reported,<sup>20,21</sup> but did not further increase proliferation of JAK2<sup>V617F</sup>-SLAM cells. The reason could be that JAK2<sup>V617F</sup> already maximally stimulates the JAK/STAT signaling pathway that the IFN $\alpha$  receptor uses to promote HSC proliferation. However, cycling induced through the IFN $\alpha$  receptor and through other receptors<sup>31,37-39</sup> using JAK2<sup>V617F</sup> may have distinct/antagonist effects<sup>31</sup> on HSC maintenance (self-renewal or differentiation). It is also conceivable that increased signaling may be deleterious for HSCs, leading to loss of function, apoptosis, and finally exhaustion. Indeed, IFN $\alpha$  also increased apoptosis of SLAM cells in the spleen, and this effect may be major in reducing splenomegaly and suppressing neoplastic proliferation in a location that mostly contributed to its amplification. Among other mechanisms possibly involved, IFN $\alpha$  may promote an antitumor immune response through the stimulation of central memory T cells and activated dendritic cells.<sup>48,49</sup> In humans, IFN $\alpha$  was shown to eradicate JAK2<sup>V617F</sup> cells without affecting *TET2*-mutated cells,<sup>50</sup> suggesting a mechanism of action that is related to JAK2 signaling. Altogether, our results are in part similar to those recently obtained by Mullally et al<sup>41</sup> in KI mice by showing that IFN $\alpha$  targets JAK2<sup>V617F</sup> HSCs, but our results differ in that the mechanism of IFN $\alpha$  is not yet clearly related to an entry of JAK2<sup>V617F</sup> HSCs in cell cycle and their subsequent exhaustion.

In conclusion, our study demonstrates that JAK2<sup>V617F</sup> is self-sufficient for HSCs to drive MPN in mice. Additional mutations may not be necessary to promote clonal emergence of MPN. The fact that IFN $\alpha$  alleviates the disease in mice offers the opportunity to understand the mechanism of action of this drug.

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## Authorship

Contribution: S.H., C.L., M.C., and C.M. performed research and analyzed data; J.-L.V. designed experiments, performed research, analyzed data, and wrote the paper; and W.V. and E.S. designed experiments.

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

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## **Supplemental Methods:**

### **Generation of the conditional JAK2<sup>V617F</sup> Knock-In (KI) mice**

The targeting vector is derived from a genomic DNA fragment spanning exons 12 to 16 of murine *Jak2* gene obtained from 129S2/SvPas mouse genomic DNA (figure 1). It included a 4.8kb 5' homology arm and a 3.7kb 3' homology arm and a *PgkNeoR* cassette flanked by FRT sites. To generate the conditional KI mice we used the FLEX switch strategy.<sup>1</sup> A mutated exon 13 (including the GTC>TTC mutation coding for the JAK2<sup>V617F</sup> mutation) was introduced in inversed direction (untranslated) into intron 13. This mutated exon, upstream the WT exon 13 and downstream the *Pgk-NeoR* cassette, was surrounded with a Lox511 and a LoxP site. A loxP site and a lox511 site were also introduced downstream to the WT exon 13 and upstream to the *Pgk-NeoR* cassette, respectively (figure 1). The linearized homologous recombination vector construct was electroporated into 129S2/SvPas embryonic stem (ES) cells followed by G418 selection. Polymerase chain reaction (PCR) and southern blot verified homologously recombined ES cells were injected into blastocysts and reimplanted into the uterine horns of pseudo-pregnant foster females to allow embryos to develop to term. Chimeras were crossed with C57BL/6N Tac mice carrying the *Flp* recombinase transgene in order to excise the *Pgk-NeoR* cassette. Agouti color hair progenies were genotyped for germinal transmission of the targeted allele using PCR and Southern blotting analyses. This work was carried out at the Institut Clinique de la Souris (ICS, Illkirch, France). Subsequently, these flexed JAK2 (JAK2<sup>F/+</sup> or L2<sup>+/+</sup>) KI mice were transferred, bred and studied in the animal facilities of the IGR. Due to the particular inverted or similar orientations of the LoxP and Lox511 sites in the KI construct (Fig. 1), Cre recombination after crossing the JAK2<sup>F/+</sup> mice with Cre expressing TG mice, results in flipping of the mutated exon, which became translated, and flipping of the WT exon 13, which became reversed and then excised. We termed these JAK2<sup>VF/+</sup> mice, with a WT and a mutated *Jak2* allele, as JAK2<sup>V617F</sup> KI mice.

For tail genotyping, the GTCTGTCCAAAGAGTCTGTAAGTAC and GCTCCAGGGTTACACGAGTC primers generated a 356bp PCR amplification fragment with the KI mouse DNA and the TGTCTTACTAAAGCCCAGGTGATGG and GCTCCAGGGTTACACGAGTC primers amplified a 105bp, 884bp (not observed with our

PCR conditions) or a 186bp PCR fragment with the WT, KI (FLEX/L2) or recombined KI (L-) mouse DNA.

For dosage of WT and mutated JAK2 mRNA, quantitative allele specific PCR from cDNA, generated by reversed transcriptase from tissue mRNA extracted using Trizol reagent (Invitrogen), was conducted using the TTCTCACAAGCATTTGGTTTTGAAT forward primer and the AAAGACAAGCAGCTGGCTTTACTTAC reversed primer for PCR amplification and the GTGTCTGTTTCTGTGGA JAK2<sup>V617F</sup> probe or the GTGTCTGTGTCTGTGGA JAK2<sup>WT</sup> probe to identify amplified fragments from the mutated or WT JAK2 mRNA, respectively. It was performed on a 7500 Real Time PCR System AB (Applied Biosystems, Foster City, USA) and analyzed with associated software.

To check the functionality of the KI construct, JAK2<sup>FLEX/+</sup> KI mice were first crossed with TG mice expressing the Cre recombinase under the control of the ubiquitous CMV promoter.<sup>2</sup> JAK2<sup>VF/+</sup> progenies displayed leukocytosis, polycythemia, thrombocytosis and splenomegaly with endogenous CFU-E demonstrating that Cre recombination of the KI allele led to the correct expression of the mutated JAK2<sup>V617F</sup> protein and associated MPN phenotype.

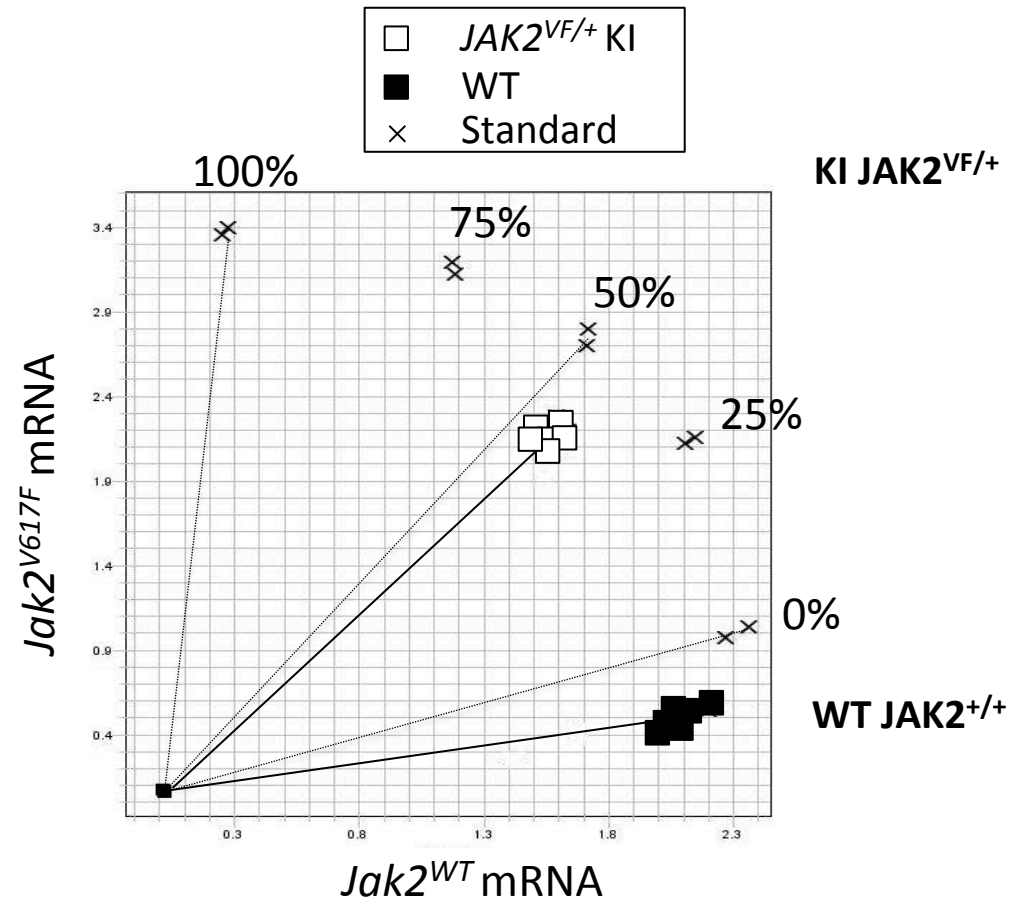
### **Flow cytometry**

Flow cytometry (CANTO II®, DIVA6® analyzer, Becton Dickinson, Mountain View, USA) was used to determine cell composition in blood, BM and spleen after RBC lysis and labelling with anti-CD45.1 R-phycoerythrin (PE), anti-CD45.2 allophycocyanin (APC), anti-Mac1 (CD 11b) fluorescein isothiocyanate (FITC), anti-Gr-1 PerCP-cyanine (Cy) 5.5, anti-B220 APC-Cy7, anti-CD3 Pacific Blue, anti-Ter119 PE-Cy7 antibodies (Pharmingen, San Diego, USA). Appropriated rat IgG2a-conjugated were used as controls. Lineage-negative (Lin-) cells were isolated using APC-conjugated lineage panel (Gr-1, CD11b, B220, CD3 and TER119). Lin<sup>-</sup>, Sca1<sup>+</sup>, c-Kit<sup>+</sup> (LSK) cells were analyzed from Lin- cells labeled with anti-Sca-I/PE-Cy7 and anti-c-Kit/PerCP-Cy5.5 antibodies. So-called “signaling lymphocyte activation molecule» marker fractionated (SLAM) cells were analyzed from LSK cells labeled with anti-CD48/PB and anti-CD150/PE antibodies. MEP, GMP and CMP progenitors were analyzed with anti-CD34/FITC and anti-FcRγII/III/APC-

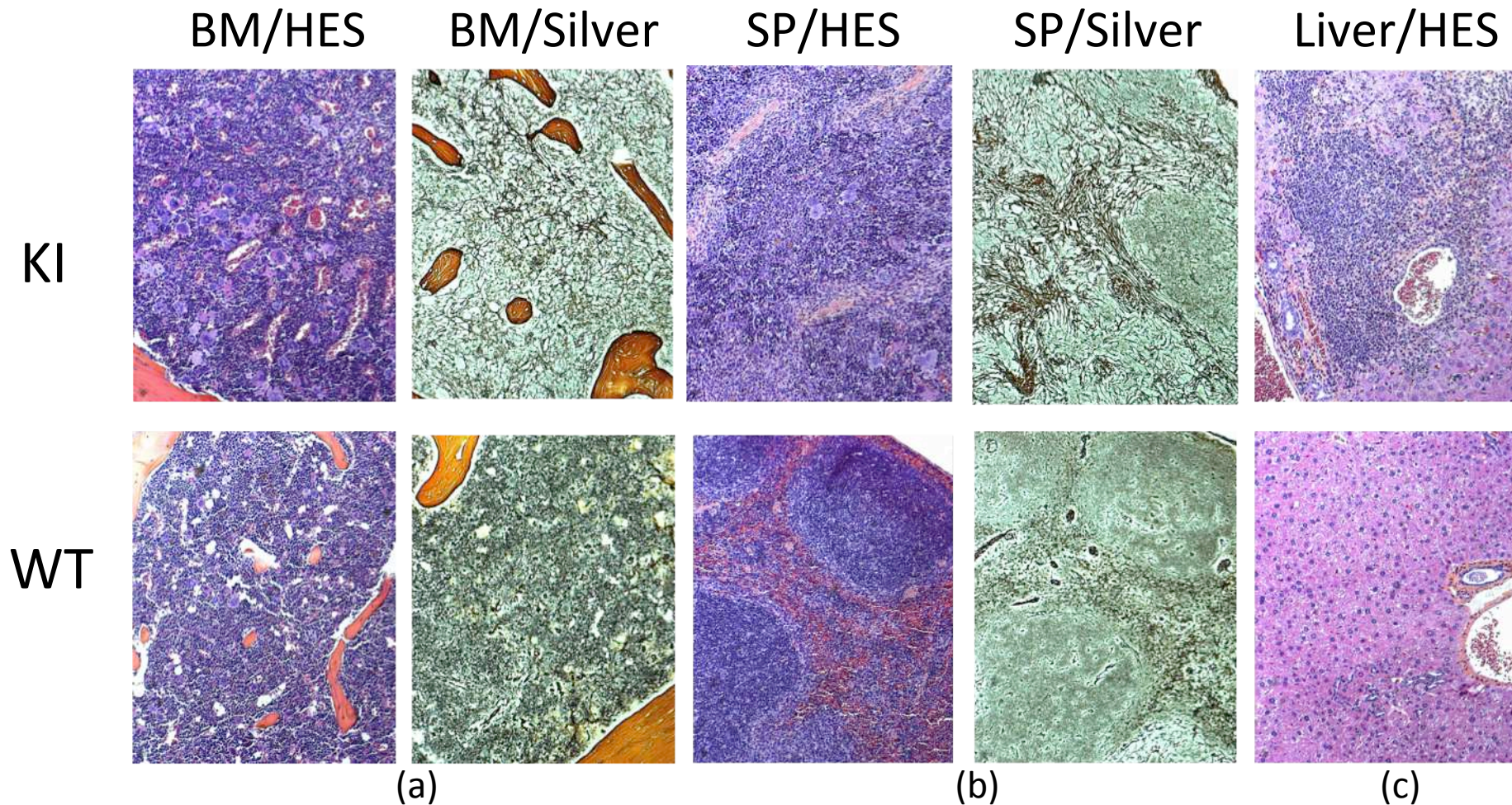
Cy7. Biotinylated anti-CD45.1/Pacific orange streptavidin and anti-CD45.2/AlexaFluor 700 were used to differentiate between WT (CD45.1) and KI JAK2<sup>V617F</sup> (CD45.2) cells.

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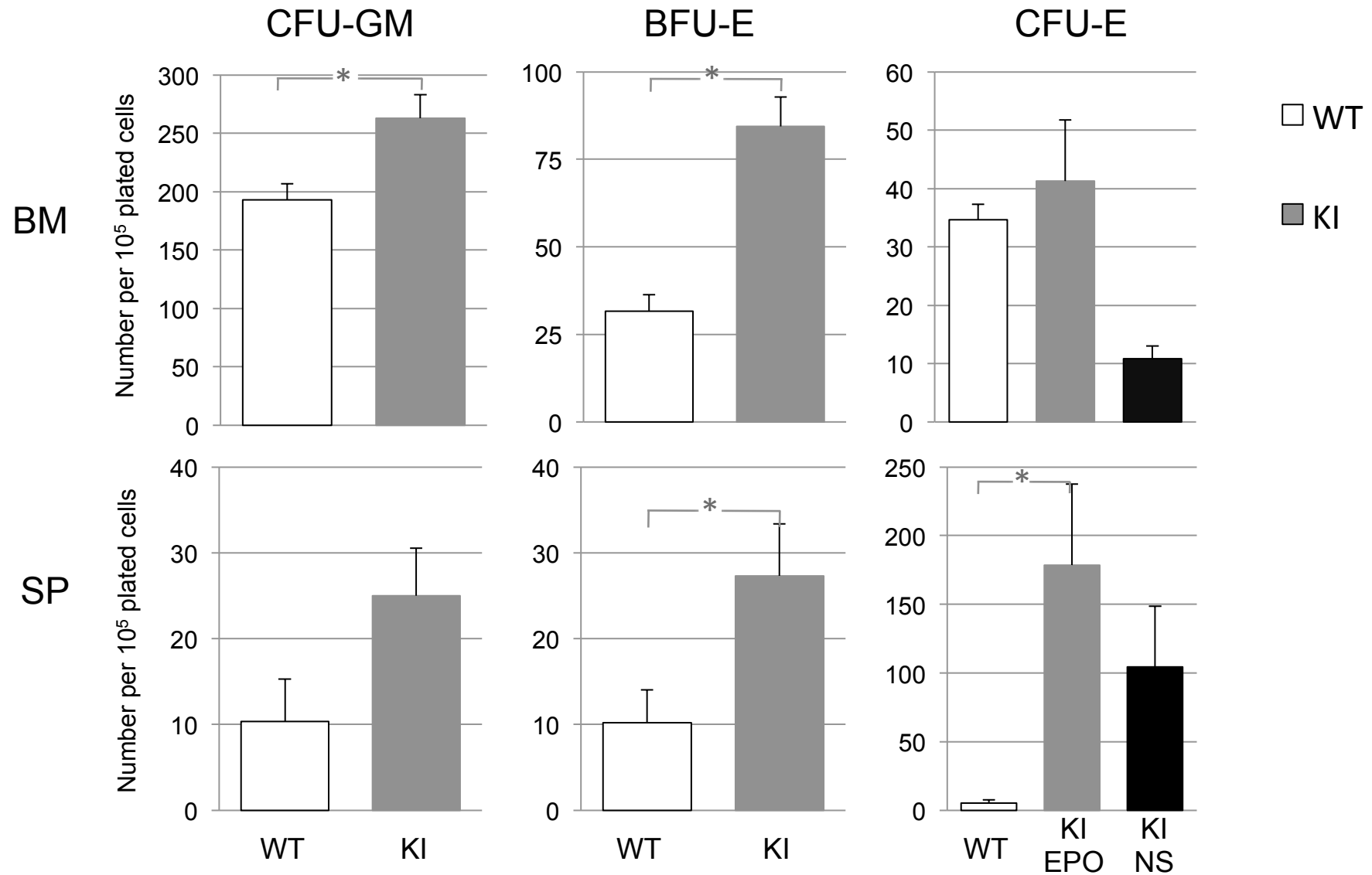
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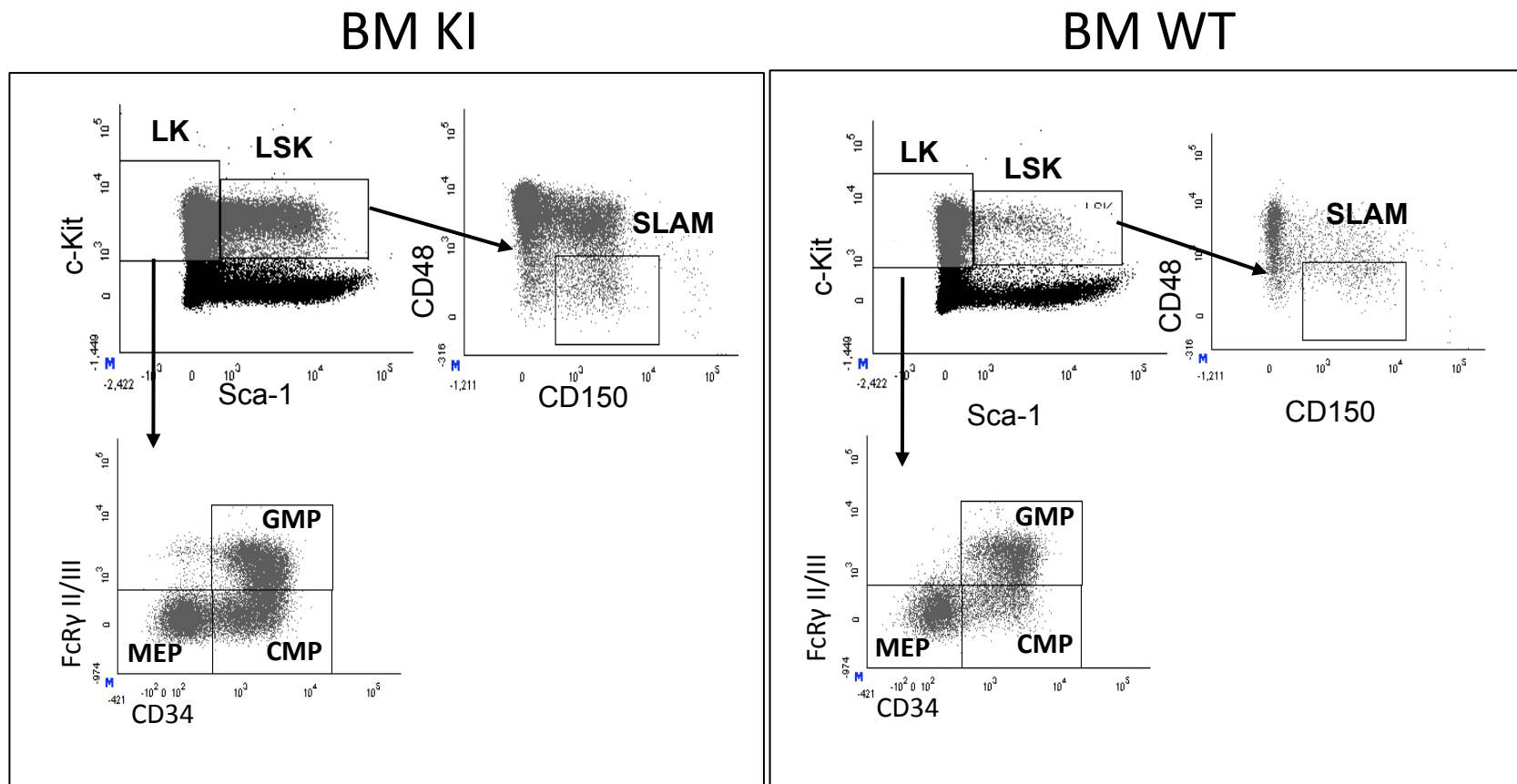
**Heterozygosity in  $JAK2^{V617F/+}$  KI mice.**  $Jak2^{WT}$  and mutated  $Jak2^{V617F}$  Taqman<sup>®</sup> allele specific amplification from BM samples of  $JAK2^{V617F}$  KI (white squares, 3 mice studied in duplicate) and WT (filled squares, 3 mice studied in duplicate) mice was performed around 3 months after birth. Standard curve (0, 25, 50, 75 and 100%) was carried out from mixtures of plasmids containing  $Jak2^{V617F}$  or  $Jak2^{WT}$  cDNAs.



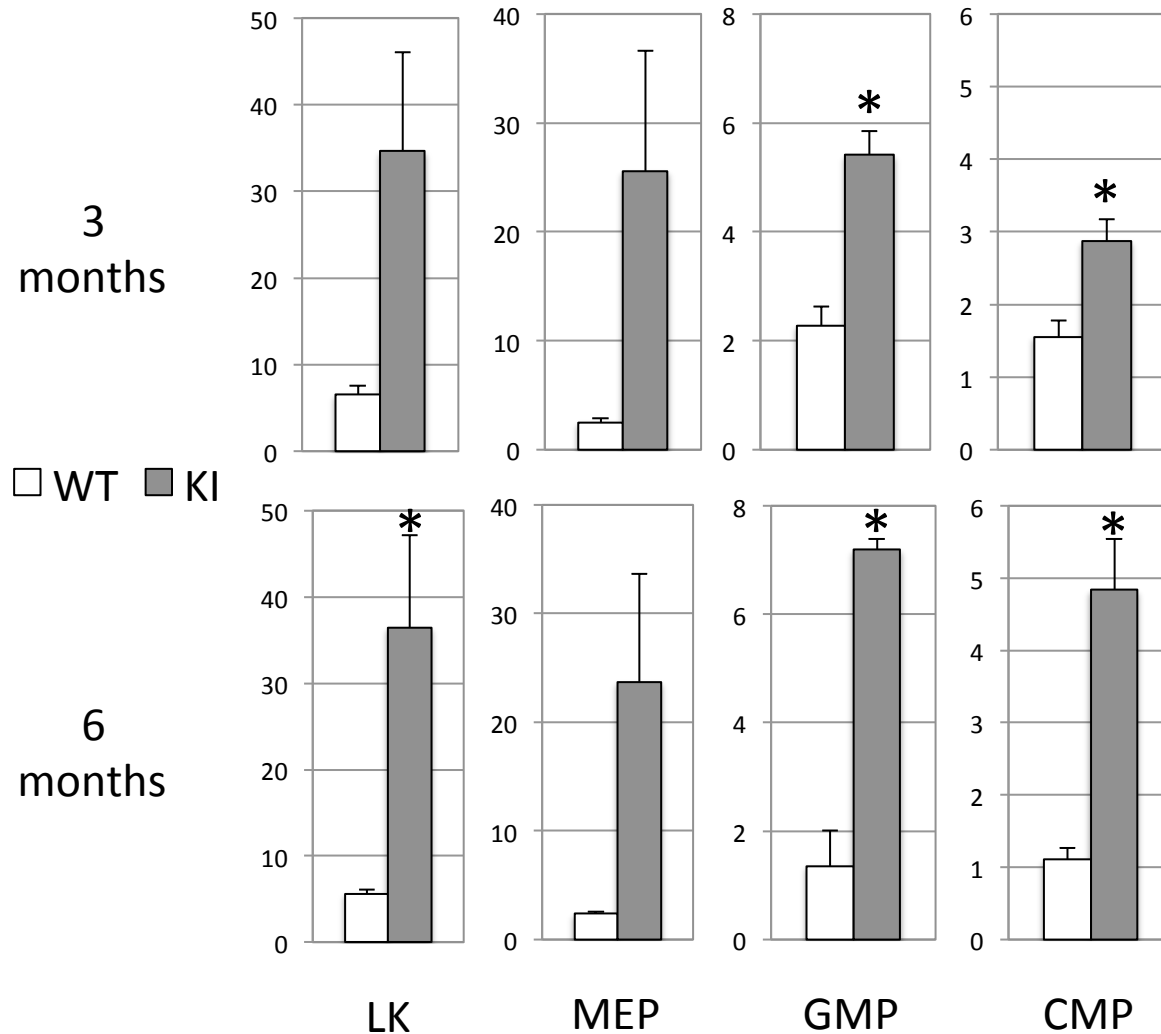
**Histology of bone marrow (BM), spleen (SP) and liver from a 11 month-old KI (top) and WT littermates (bottom).** Hematoxylin-eosin/safran (HES) and silver stains. HES staining showed (a) in the BM inflated blood vessels, clustered megakaryocytes (MKC) and fibrosis may have contributed to reduced BM cellularity ; (b) in the spleen trilineage hyperplasia of the red pulp with numerous clustered MKC and fibrotic tissue; (c) in the liver a large extramedullary hematopoietic foci containing erythroblast, granulocytes and MKC. Silver staining indicated high-grade fibrosis with large deposit of reticulin fibers in the BM and spleen of KI mouse. Images were obtained (10x/0.3 Zeiss lens) using a Zeiss Axiophot microscope with a Zeiss AxioCam Mrc camera and the AxioVision Rel. 4.3 acquisition software (Oberkochen, Germany).



**Frequencies of progenitor cells in tissues from KI (grey column) and WT littermate (white column) mice.** The numbers per 10<sup>5</sup> plated cells of BFU-E, CFU-GM and CFU-E (including autonomous CFU-E in black columns) from KI (n=9) and WT littermate (n=7) mice are shown. BM and spleen cells were plated 5x10<sup>4</sup> or 20-5x10<sup>4</sup> cells/mL. Mean values±SEM are represented. \* indicates p≤0.05 with the two-tailed unpaired student t-test.

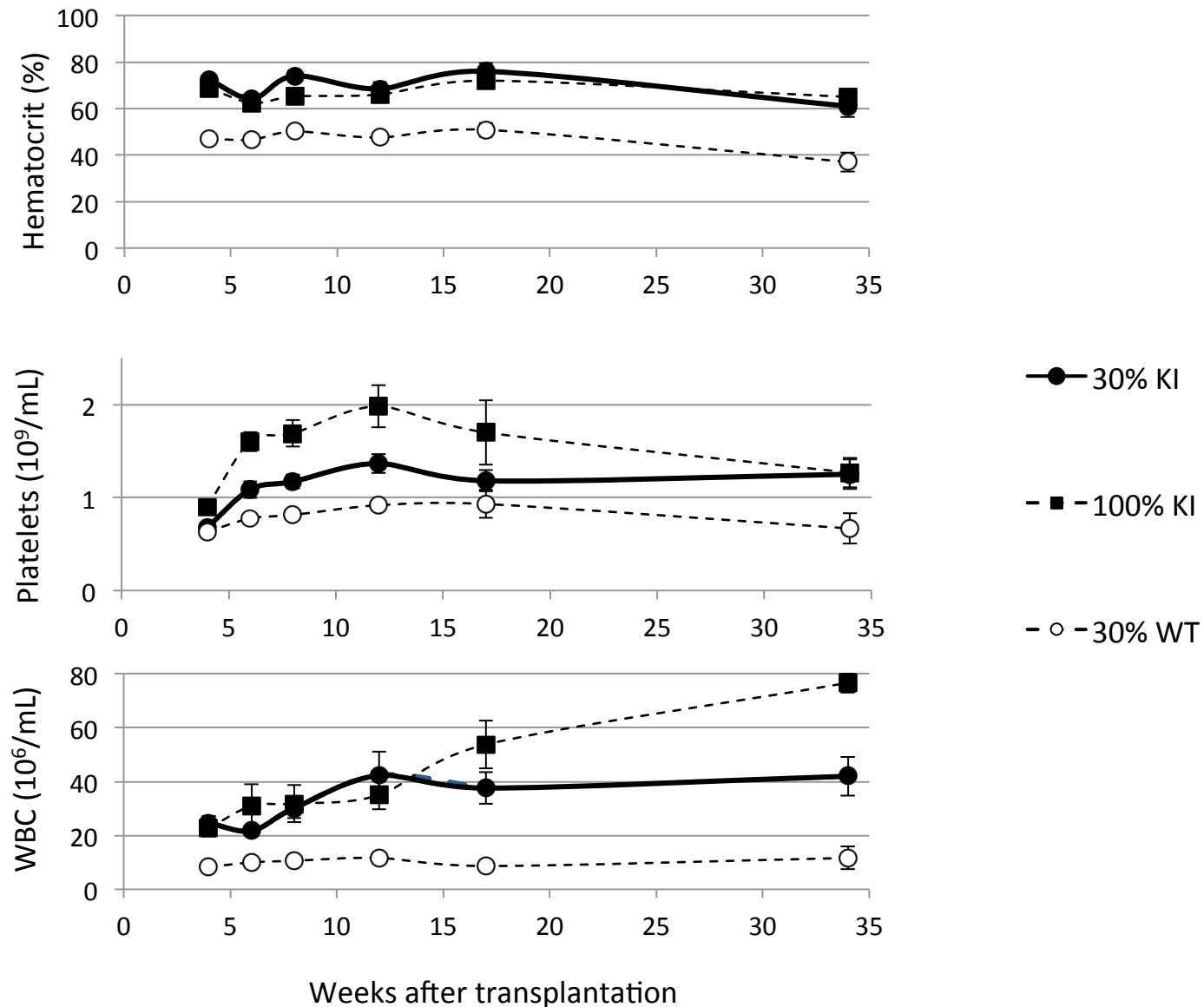


Identification in BM from KI (left) or WT (right) mice of Lin<sup>-</sup> cell subpopulations according to their immunophenotype by FACS analysis: LSK (progenitor cells with multipotent activity, lin<sup>-</sup>, c-kit<sup>+</sup>, Sca-1<sup>+</sup>), SLAM (highly enriched in LT-HSC activity, LSK, CD48<sup>-</sup>, CD150<sup>+</sup>) and subpopulation of LK myeloid progenitor cells (Lin<sup>-</sup>, c-kit<sup>+</sup>) including MEP (megakaryocyte-erythroid progenitors, LK, CD34<sup>-</sup>, FcRyII/III<sup>-</sup>), GMP (granulocyte-macrophage progenitors, LK, CD34<sup>+</sup>, FcRyII/III<sup>high</sup>) and CMP (common myeloid progenitors, LK, CD34<sup>+</sup>, FcRyII/III<sup>low</sup>).

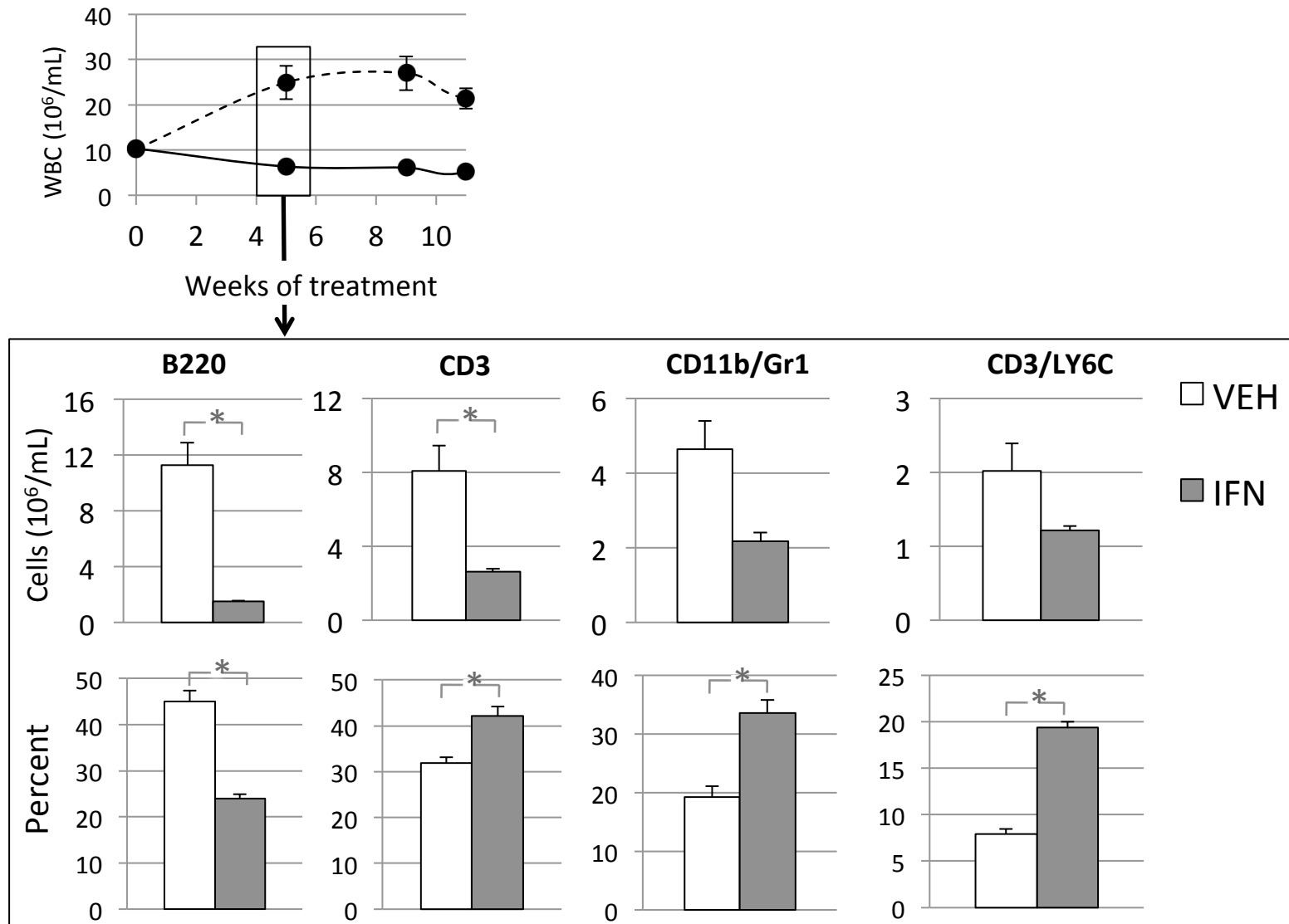


Total numbers ( $\times 10^6$ ) of c-kit<sup>+</sup>/Lin<sup>-</sup> (LK) progenitor cells including MEP, GMP, CMP cells in BM and spleen from KI and WT littermates measured at 3 and 6 months of ages. Calculation is detailed in materials and methods. Cell types were determined as supplementary data, figure S4. Results are mean values  $\pm$  SEM from 3 mice of 3 or 6 months. The non significance of the large increase in KI MEP compared to WT could be due to the low number of studied mice (n=3) as Fig. 3, B shows that KI mice display a significantly increased number of total erythroid progenitor cells.

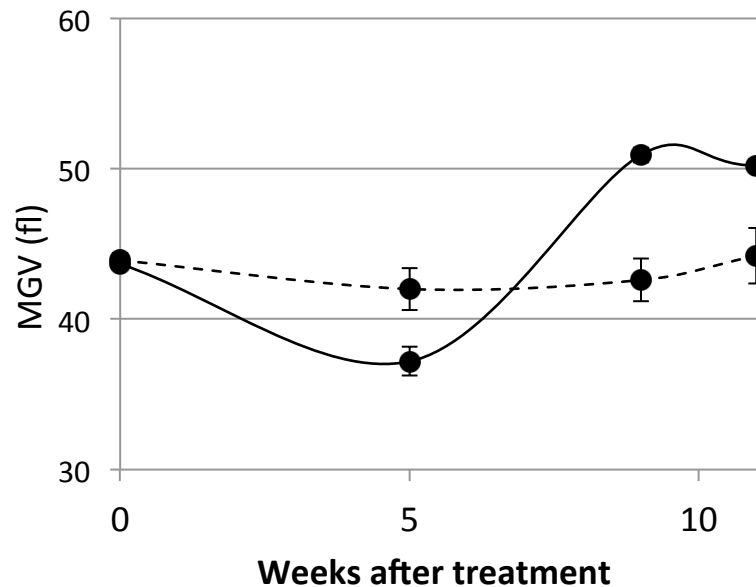
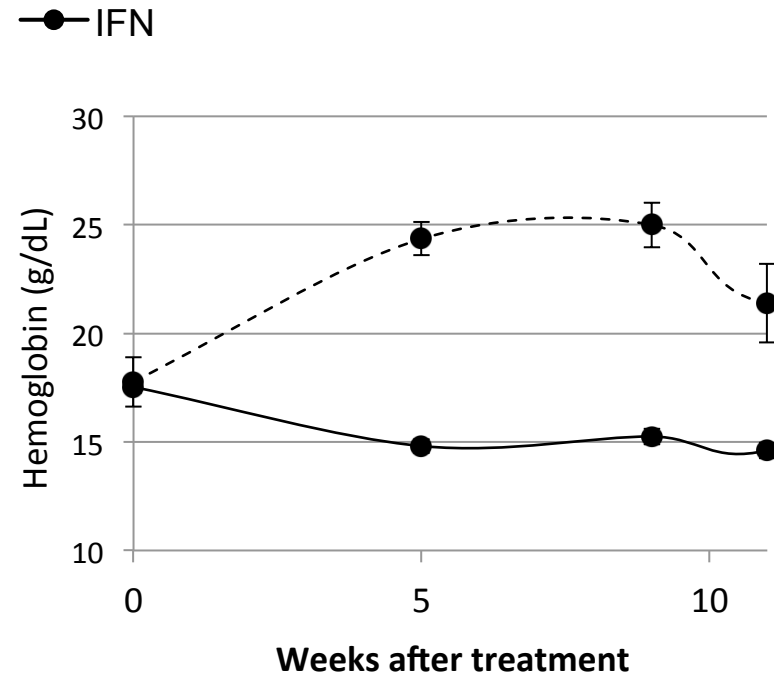
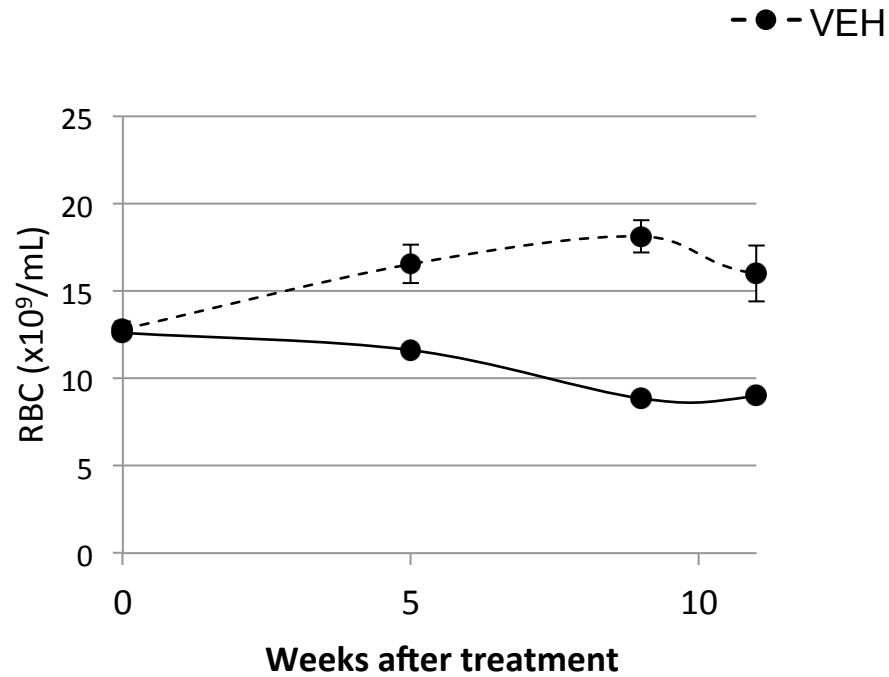




**Blood cell parameters from recipients transplanted with competitive BM grafts from 3 month-old KI mice.** Recipient mice were transplanted with  $3 \times 10^6$  cells originating from either 100% WT cells (○, dashed line, n=5), 100% KI cells (■, dashed line, n=5) or a mixture of 30% KI cells and 70% WT littermate cells (●, solid line, n=5). Low platelet and high WBC counts in 100% compared to 30% KI cells late after BMT, suggests a more rapid evolution towards myelofibrosis in mice grafted with 100% versus 30% KI cells



**Analysis of suppression of leukocytosis induced by IFN $\alpha$ .** Blood from recipient mice treated with vehicle (●, dashed line, n=10) or IFN $\alpha$  (●, solid line, n=10) was analyzed by FACS after 5 weeks of treatment. Results showed that IFN $\alpha$  reduced all cell lineages but mainly B cells (B220), whose percentage decreased. The percentages of T cells (CD3-positive including particularly CD3/Gr1 on the right) and myeloid cells increased. CD3/LY6C were identified with the anti-Gr1 antibody, which only recognizes LY6C on lymphocytes. These data were generated from the first set of treated mice from 1 month-old donors and low severity. \* indicates  $p \leq 0.05$  with the two-tailed unpaired student t-test.



Effect of IFN $\alpha$  on red blood cell (RBC) count, hemoglobin and mean globular volume (MGV). Animals were transplanted with 30% KI BM cells from 1 month-old donor mice and treated for 11 weeks with IFN $\alpha$  (continuous line) or vehicle (dashed line), starting 7 weeks after transplantation (same experiment as Fig. 6, A left). Data are mean values  $\pm$  SEM of 10 IFN $\alpha$ - or vehicle-treated mice.

**Supplemental Figure S8**

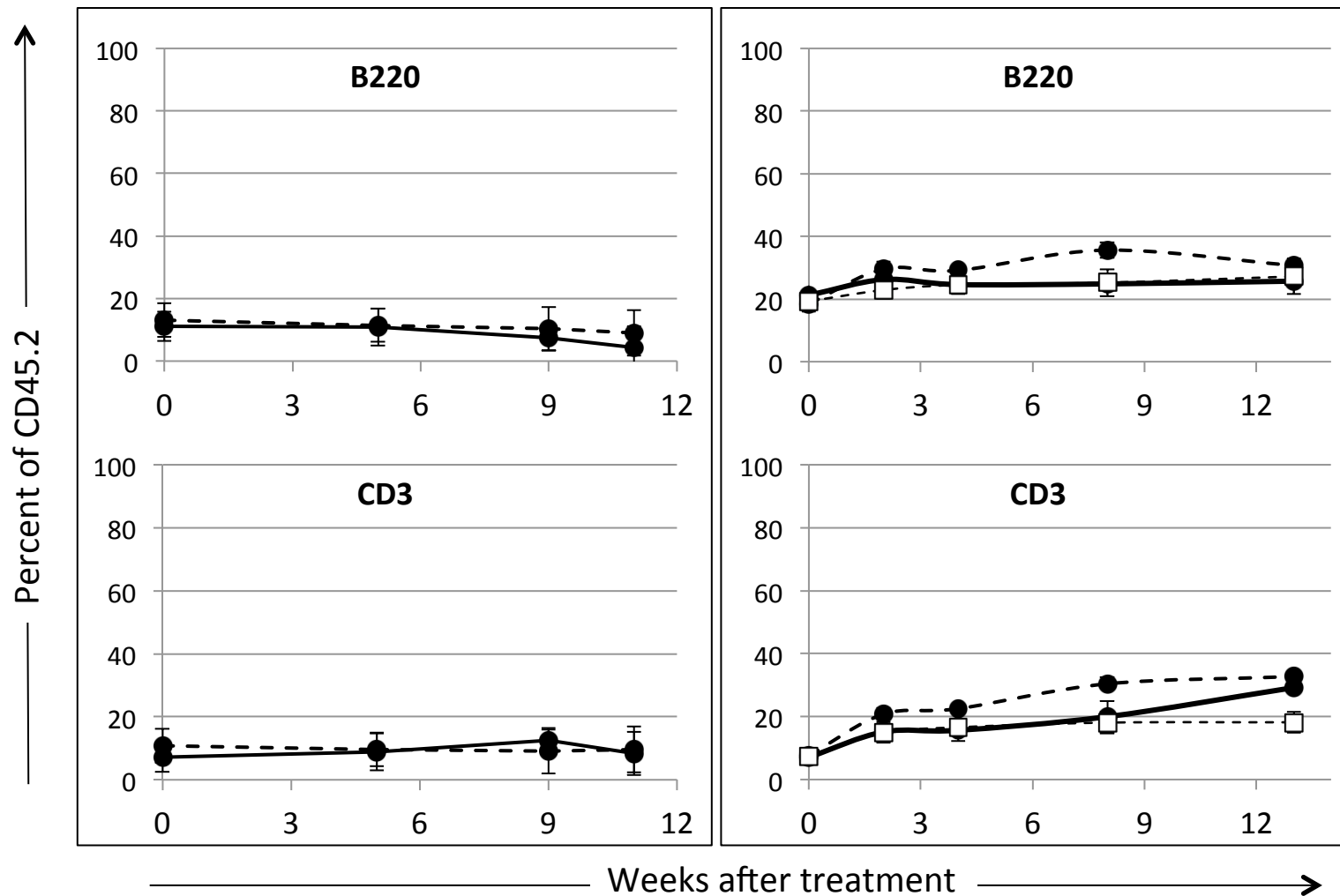
Exp. 1 « low allele burden »

-●- VEH

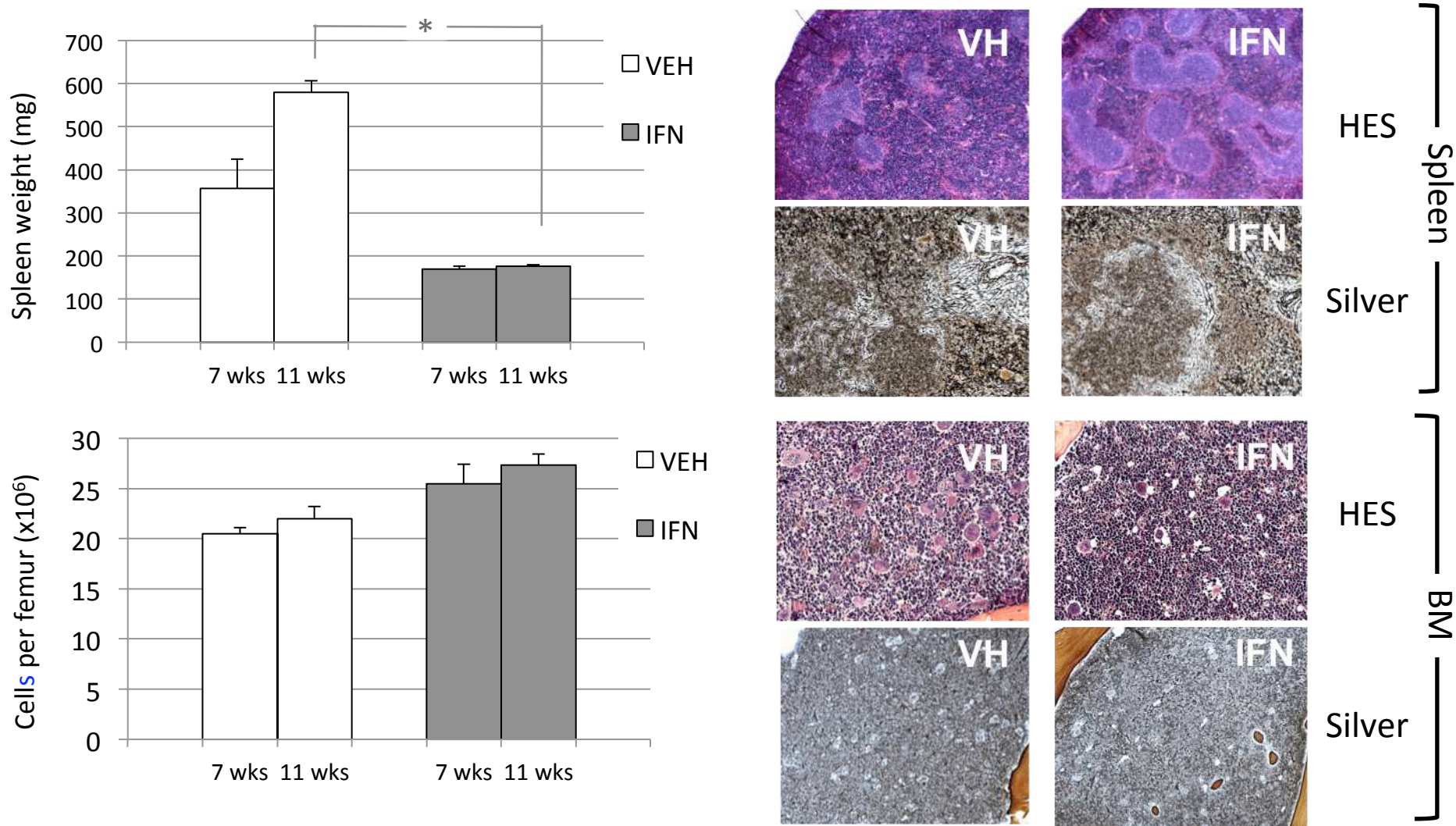
—●— IFN

-□- WT

Exp. 2 « high allele burden »

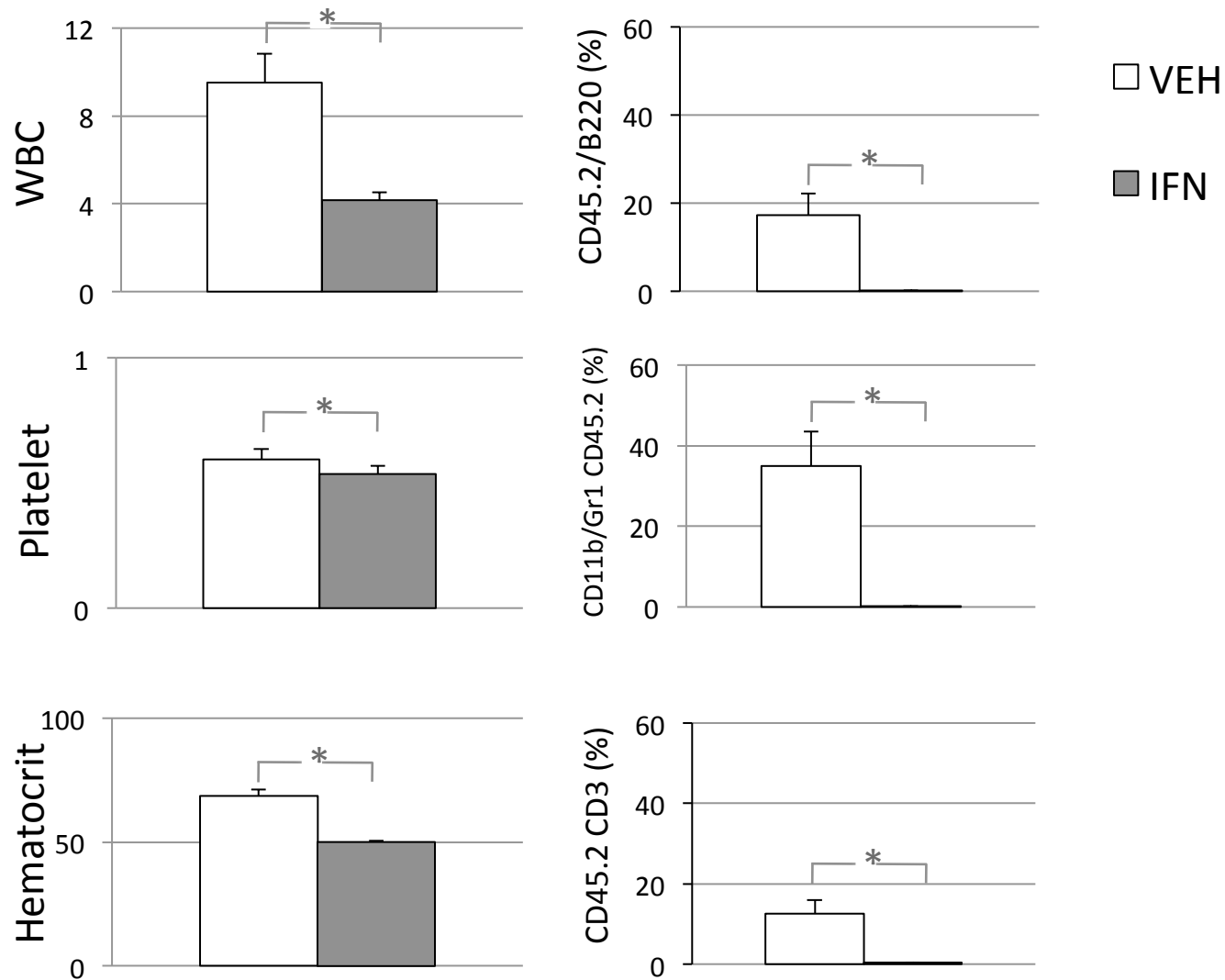


Effect of IFN $\alpha$  treatment on blood B and T cell from KI origin (CD45.2). Results showed two different experiments : first experiment with low allele burden (left) and second experiment with high allele burden (right). In both experiments, no significant effect on lymphoid cell chimerism was observed (result from the same experiments as Fig. 6, A).



**Effect of IFN $\alpha$  on BM and spleen cellularity** from mice treated 7 or 11 weeks with IFN $\alpha$  or vehicle (VEH). HES and silver stains from spleen (top right) and BM (bottom right) treated 11 weeks with VEH or IFN $\alpha$ . Spleen weight (top left) increased in VEH-treated mice during the treatment in contrast to IFN $\alpha$ -treated mice that displayed a quasi normal weight and architecture of the spleen (reduction of red pulp and normal size of the white pulp territory). BM cellularity was more elevated in IFN $\alpha$  treated mice and histology revealed a decrease in the percentage of BM megakaryocytes. Silver staining did not reveal an increase in reticulin fibers at this stage and we could not determine the effect of IFN $\alpha$  on fibrosis. Values are mean  $\pm$  SEM (n=3).

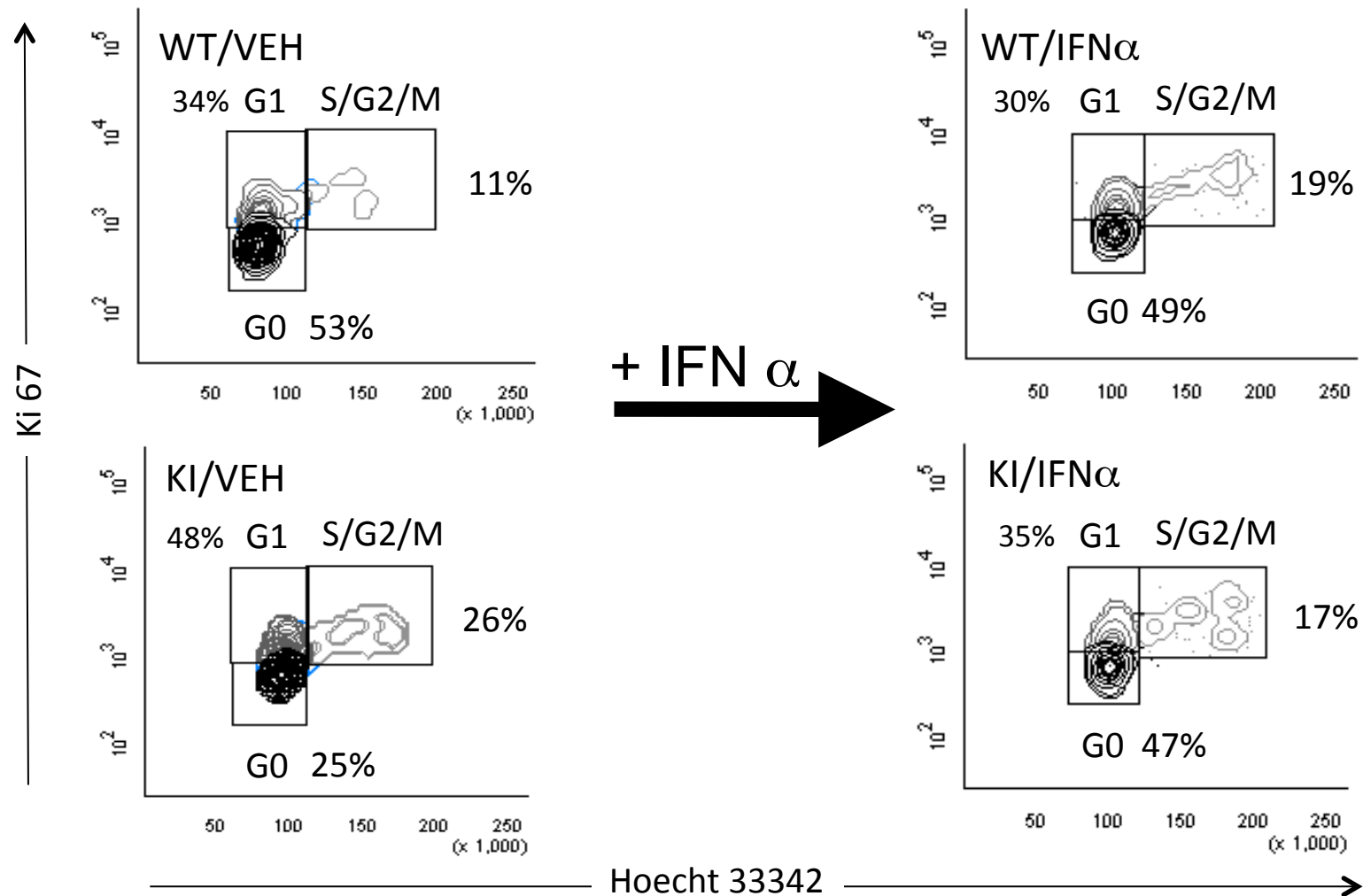
**Supplemental Figure S10**



Variations of hematological parameters and KI-derived B220, Gr-1/CD11b and CD3 cells (CD45.2) in secondary recipients 4 weeks after transplantation of BM cells collected from primary recipients treated for 11 weeks with vehicle or IFN $\alpha$ . Data are mean values $\pm$ SEM of three groups of 5 mice, each transplanted with BM from three different treated donors with low severity disease.

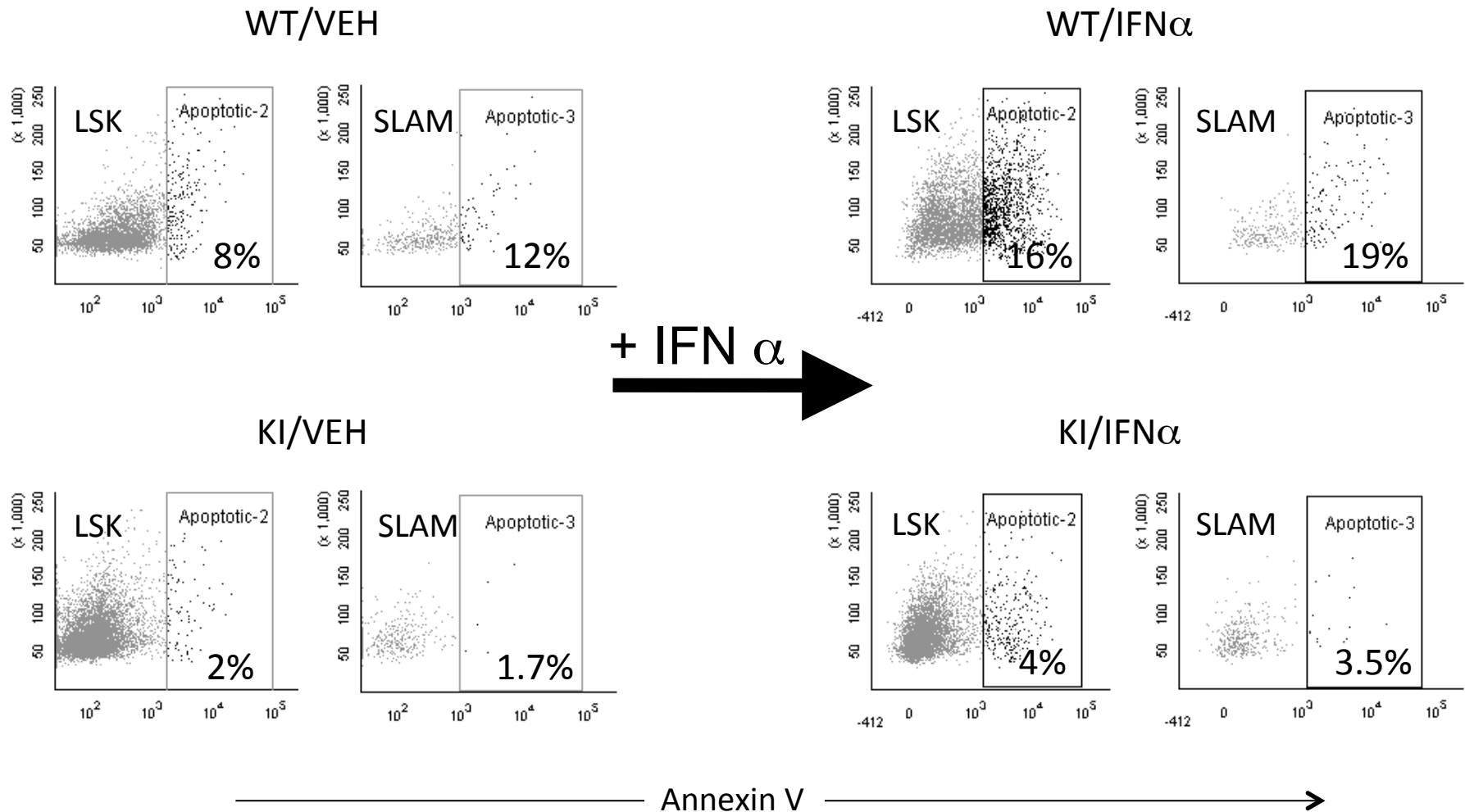
**Supplemental Figure S11**

## BM SLAM



Variations in cell cycle phases of BM SLAM from WT (top) or KI (bottom) mice treated by vehicle (left) or IFN $\alpha$  (right). Analysis showed that KI cells are more proliferative than WT cells and that IFN $\alpha$  induced cell proliferation of WT cells.

## BM LSK/SLAM



Variations of apoptosis of BM LSK or SLAM from WT (top) or KI (bottom) mice treated by vehicle (left) or IFN $\alpha$  (right). Analysis showed that KI cells are less apoptotic than WT cells and that IFN $\alpha$  does not significantly modify this difference in BM.



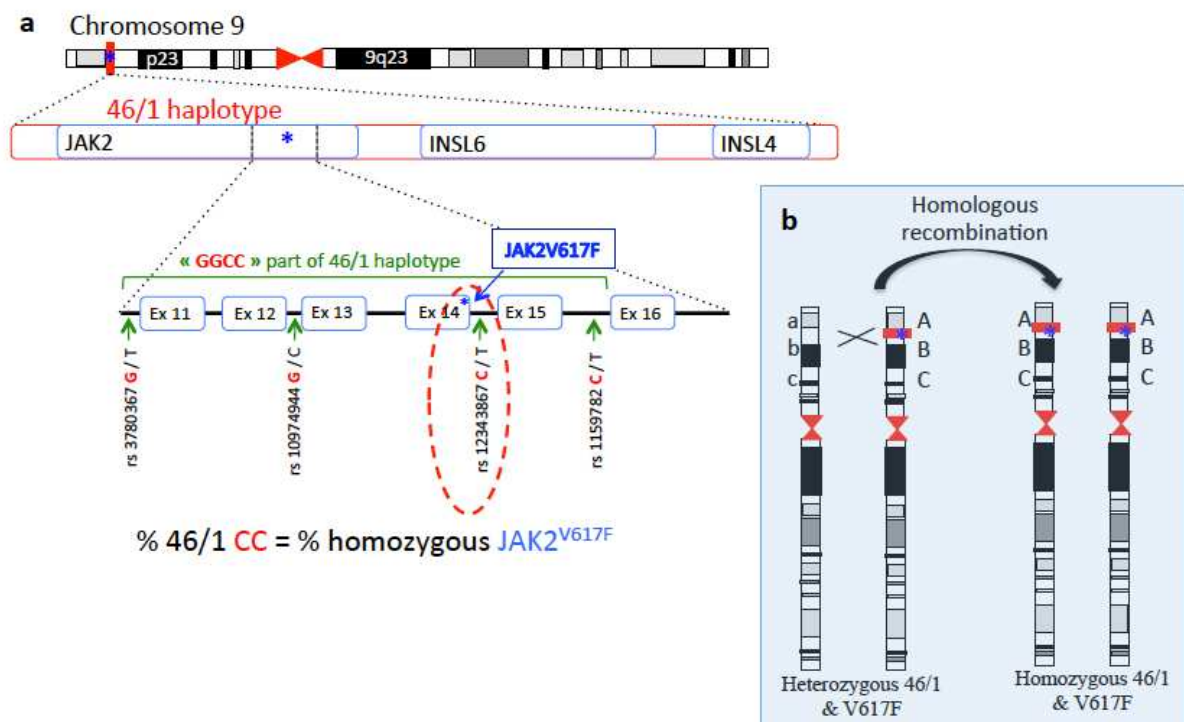
## **Result 2**

### **JAK2<sup>V617F</sup>-positive MPN: Clonal architecture in PV patients**

## Context

The  $JAK2^{V617F}$  mutation results into a guanine to a thymine substitution (G>T) in exon 14 of  $JAK2$  allele located on chromosome 9. Acquisition of chromosome 9p uniparental disomy (UPD 9p) leading to  $JAK2^{V617F}$  homozygosity, is due to mitotic homologous recombination (HR) and is a frequent event in PV and MF but not in ET<sup>121,168</sup>. Appearance of  $JAK2^{V617F}$  homozygous clones have been evidenced during transition of ET towards PV<sup>75</sup> although a recent study shows that homozygotes clones maybe already present in ET, but are not dominant. In fact, the  $JAK2^{V617F}$  allele burden in a cell population is a reflection of WT ( $JAK2^{WT}$ ), heterozygous ( $JAK2^{V617F/WT}$ ) and homozygous ( $JAK2^{V617F/V617F}$ ) clones. Recently,  $JAK2$  haplotype 46/1 has been shown to be strongly associated with the cis-acquisition of  $JAK2^{V617F}$  mutation<sup>206–208</sup>. 46/1 is a 280 Kb long nucleotide sequence consisting of three genes:  $JAK2$ ,  $INSL6$  and  $INSL4$  on chromosome arm 9p. The so-called ‘GGCC’ part of 46/1 is comprised of four single nucleotide polymorphisms (SNPs: rs3780367, rs10974944, rs12343867 and rs1159782) in complete linkage disequilibrium (LD) and includes  $JAK2^{V617F}$ . As UPD 9 involves most of the chromosome 9, a LOH is also present in the SNPs that tag 46/1 and become homozygous when 46/1 and  $JAK2^{V617F}$  are in cis. If they are in trans, the 46/1 haplotype is lost in  $JAK2^{V617F}$  homozygous cells. Thus, the frequency of 46/1 HR reflects  $JAK2^{V617F}$  homozygosity in patients, which are germ line heterozygous for 46/1

**Figure 32.**



**Figure 32. Schematic representation of 46/1 haplotype and homologous mitotic recombination (HR) on chromosome 9** a.  $JAK2^{V617F}$  arises on GGCC part of 46/1 in complete linkage disequilibrium (LD) with a set of four SNPs including rs12343867. b. As Ch. 9p recombines so do its tagging SNPs and the mutation  $JAK2^{V617F}$ .

## Objectives and approach

The only method to obtain the WT, heterozygous and homozygous JAK2<sup>V617F</sup> clonal architecture in PV patients is to genotype individual clones. We hypothesized that by following an informative SNP within 46/1 haplotype, like rs12343867 in our study, we can calculate the percentage of JAK2<sup>V617F</sup> homozygous clones without working on-unicellular levels.

$$\text{HR} = \% \text{ of JAK2}^{\text{V617F/V617F}} \text{ clones} = (\text{rs12343867 allele burden in CD34}^+ - 50\%) \times 2$$

And using global JAK2<sup>V617F</sup> allele burden, the percentages of JAK2<sup>V617F</sup> heterozygous and WT clones can be deduced.

$$\% \text{ of JAK2}^{\text{V617F/WT}} \text{ clones} = (\% \text{ of JAK2}^{\text{V617F}} \text{ allele burden} - \% \text{ of JAK2}^{\text{V617F/V617F}} \text{ cells}) \times 2$$

and

$$\% \text{ of JAK2}^{\text{WT/WT}} \text{ clones} = 100 - (\% \text{ of JAK2}^{\text{V617F/V617F}} + \% \text{ of JAK2}^{\text{V617F/WT}} \text{ cells})$$

The purpose of this study was to validate this model in 9 PV patients heterozygous for 46/1 by comparing the architecture of the clones obtained experimentally from CD34<sup>+</sup> progenitors to that obtained from the formula using global JAK2<sup>V617F</sup> and 46/1 allele burdens in purified CD34<sup>+</sup> cell populations.

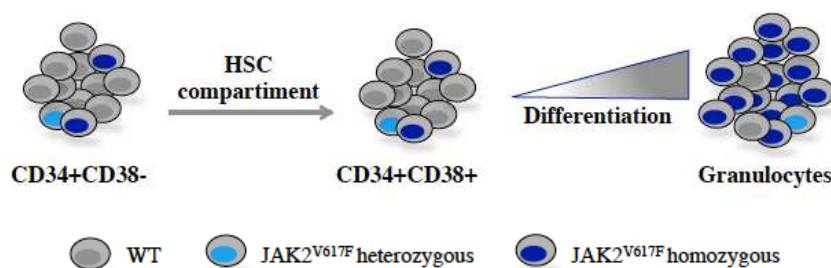
Another aim of this project was to study this technique to evaluate the effects of IFN- $\alpha$  treatment on the JAK2<sup>V617F</sup> clonal architecture of PV patients.

## Results and discussion

If 46/1 recombines frequently independently of JAK2<sup>V617F</sup> the frequency of 46/1 homozygous clones will not reflect the JAK2<sup>V617F/V617F</sup> clonal frequency because the 46/1 haplotype will recombine before the acquisition of JAK2<sup>V617F</sup>. To rule out this possibility we genotyped three JAK2<sup>V617F</sup> negative rs12343867 heterozygous hemochromatosis subjects, no rs12343867 homozygous clones were found in approximately 500 CD34<sup>+</sup>CD38<sup>-</sup> and CD34<sup>+</sup>CD38<sup>+</sup> HSC colonies analyzed. SNP rs12343867 allele burden in purified CD34<sup>+</sup> and CD3<sup>+</sup> cells were found to be 50% confirming that no HR event has occurred. These results suggest that the frequency of 46/1 haplotype recombination in normal subjects is less than 1/500. An analysis of several thousands of clones will be necessary to invalidate this hypothesis. This is important because it has been reported that the 9pLOH may antedate JAK2<sup>V617F</sup> recombination in some patients.

Next, to validate the hypothesis we first determined the rs12343867 and JAK2<sup>V617F</sup> allele burden in purified CD34<sup>+</sup> cells either by allele discrimination Taqman PCR or by NGS in 9 PV patients. Remarking that Taqman PCR tends to be less quantitative for rs12343867 and JAK2<sup>V617F</sup> allele burdens than NGS, we used the NGS results for more precise calculations. Then, we conducted CD34<sup>+</sup>CD38<sup>+</sup> clonal studies for each patient by seeding one cell per well in 96 well plates and genotyped each colony on day 14 of culture. Expected JAK2<sup>V617F</sup> clonal frequencies (derived from calculations) and experimental values (observed in clonal studies) were correlated in the great majority of patients and we could easily reconstitute JAK2<sup>V617F</sup> clonal architecture despite of inter-patients JAK2<sup>V617F</sup> clonal heterogeneity i.e., Patient #1 with only JAK2<sup>V617F/V617F</sup> clones, patients

#5, #7 and #9 with majority of WT clones, Patient #3 with WT and JAK2<sup>V617F/+</sup> clones, patients #6 and #8 with WT and JAK2<sup>V617F/V617F</sup> clones and patient #4 with a mixture of WT, JAK2<sup>V617F/+</sup> and JAK2<sup>V617F/V617F</sup> clones. Interestingly, patients #5 and #7, who were expected to have JAK2<sup>V617F/+</sup> and JAK2<sup>V617F/V617F</sup> clones by our calculation presented much more WT clones as presumed by using single cell analysis through colony assays. As overall the JAK2<sup>V617F</sup> burden was much higher on the global CD34<sup>+</sup> cells than calculated through colony assays, this highly suggests that in fact the culture system induces a marked bias. The most likely hypothesis is that some secondary event present in the JAK2<sup>V617F</sup> impairs its differentiation in short-term clonal assays. In favour of this hypothesis we have found a mutation in an Hox gene in one of these two samples. Moreover, genotyping of CD3<sup>+</sup> cells from each patient showed 0% JAK2<sup>V617F</sup> allele burden, showing that mutation was restricted to myeloid lineage, and 50% rs12343867 allele burden, confirming that all the patients studied were germ line heterozygous for this informative SNP. Of note, the absence of any rs12343867 homozygous JAK2<sup>V617F/+</sup> clone in all patients confirms that UPD 9 is usually a secondary event occurring after the acquisition of JAK2<sup>V617F</sup>. Altogether, our results validate that 46/1 haplotype can be used to construct JAK2<sup>V617F</sup> clonal architecture in HSC of PV patients by a direct measurement in CD34<sup>+</sup> cells. We then exploited rs12343867 to determine the JAK2<sup>V617F</sup> clonal pattern during myeloid differentiation. The same calculations were extrapolated with rs12343867 and JAK2<sup>V617F</sup> allele burden in PNNs of 9 PV patients. Results clearly show that preferentially JAK2<sup>V617F/V617F</sup> clone expands during myeloid differentiation at the expense of WT and JAK2<sup>V617F/+</sup> clones. Moreover, when JAK2<sup>V617F/V617F</sup> clone was absent (patients #3 and #9) JAK2<sup>V617F/+</sup> has no or very little proliferative advantage. In contrast, in patients #5 and #7 majority of PNNs were WT suggesting a clonal disadvantage of JAK2<sup>V617F/V617F</sup> clone during myeloid differentiation. Moreover, we investigated JAK2<sup>V617F</sup> clonal proportions in CD34<sup>+</sup>CD38<sup>-</sup> and CD34<sup>+</sup>CD38<sup>+</sup> HSC compartments. For this purpose we genotype approximately 100 clones per compartment in each patient. Genotype assay revealed no proliferative advantage of JAK2<sup>V617F/V617F</sup> over JAK2<sup>V617F/+</sup>/WT or vice versa within the CD34<sup>+</sup>CD38<sup>-</sup> and CD34<sup>+</sup>CD38<sup>+</sup> compartments in seven patients or between CD34<sup>+</sup>CD38<sup>-</sup>, CD34<sup>+</sup>CD38<sup>+</sup> and CD90<sup>+</sup>CD34<sup>+</sup>CD38<sup>-</sup> cell compartments in one patient. These results are in accordance with previous reports showing that JAK2<sup>V617F/V617F</sup> clones expand during terminal myeloid differentiation but their size remains limited in the HSC compartment<sup>84,170,189</sup>.

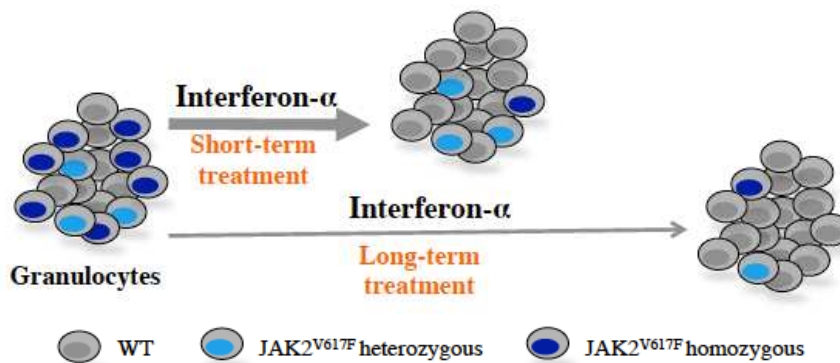


**Figure 33: Proliferative profiling of JAK2<sup>V617F</sup> homozygous clone during hematopoietic differentiation**

However, during evolution of MPN in one patient between 2010 (ET) and 2011 (PV), we observed an amplification of the  $JAK2^{V617F/V617F}$  clone in both the  $CD34^+CD38^-$  and  $CD34^+CD38^+$  HSC compartments from 84-88% to 91-98% suggesting an acquisition of a proliferative advantage to  $JAK2^{V617F/V617F}$  clone over time with the possibility of the occurrence of a secondary genetic hit in the  $JAK2^{V617F}$  clone.

Interferon alpha ( $IFN-\alpha$ ) has been shown to induce molecular response (CMR) in a subset of PV patients (less than 20%), but it is unclear if  $JAK2^{V617F}$  heterozygous and homozygous sub clones behave similarly or differently<sup>327,332</sup>.

We tested the efficacy of  $IFN\alpha$  treatment on  $JAK2^{V617F}$  clonal proportions in 3 non-responding and 4 responding PV patients. First we used NGS to determine rs12343867 and  $JAK2^{V617F}$  allele burden in PNNs of these patients and then, using HR formula, percentages of  $JAK2^{V617F/V617F}$ ,  $JAK2^{V617F/+}$  and WT clones were calculated. Results demonstrated that  $JAK2^{V617F}$  clonal architecture did not change in non-responding patients over 3 years of treatment. However,  $IFN\alpha$  treatment decreased  $JAK2^{V617F}$  allele burden by preferentially and rapidly eradicating  $JAK2^{V617F/V617F}$  clones as compared to  $JAK2^{V617F/+}$  clones, suggesting a link between the levels of JAK2 signaling and  $IFN\alpha$  response. We could overall extend these results to 15 patients. Furthermore, by sequencing *ASXL1*, *TET2*, *IDH1*, *IDH2*, *RUNX1*, *SF3B1*, *U2AF1*, *ZRSR2*, *SUZ12* and *EZH2* in 13 of these patients we only found mutations in *TET2* in 3 patients and mutations in both *ASXL1* and *SUZ12* in one patient. No correlation was observed between the presence of these mutations and the responsiveness to  $IFN\alpha$ , more particularly the patient with *ASXL1* and *SUZ12* was responsive to therapy.



**Figure 34: Effect of interferon alpha exposure on  $JAK2^{V617F}$  sub clones**

Overall, derivation of  $JAK2^{V617F}$  clonal architecture from  $JAK2^{V617F}$  and an informative SNP allele burden provides an excellent model to follow the evolution of disease without working at unicellular levels. Furthermore, preferentially  $JAK2^{V617F/V617F}$  clones amplify during myeloid differentiation but not at immature stages of HSC between the  $CD34^+CD38^-$  and  $CD34^+CD38^+$ . In contrast, the  $JAK2^{V617F/V617F}$  clone is capable to invade the HSC/progenitor compartments during the progression of the disease and  $IFN\alpha$  treatment more specifically target the  $JAK2^{V617F/V617F}$  clone in responding patients apparently irrespective of the presence of other mutations.

### **Article 3**

**Use of the 46/1 haplotype to model JAK2<sup>V617F</sup> clonal architecture in PV patients: clonal evolution and impact of IFN $\alpha$  treatment**

## LETTER TO THE EDITOR

Use of the 46/1 haplotype to model  $JAK2^{V617F}$  clonal architecture in PV patients: clonal evolution and impact of IFN $\alpha$  treatment

Leukemia advance online publication, 8 November 2013;  
doi:10.1038/leu.2013.303

V617F mutation of the protein kinase JAK2 is the most prevalent genetic abnormality in the three myeloproliferative neoplasms (MPNs), namely polycythemia vera (PV, 95%), essential thrombocythemia (ET, 60%) and myelofibrosis (MF, 60%).<sup>1–4</sup> This mutation, which usually affects only one of the  $JAK2$  gene alleles in ET, frequently becomes homozygous in PV and MF. This homozygosity is related to chromosome 9p uniparental disomy (UPD 9p) due to mitotic homologous recombination. It was recently shown that the 46/1 haplotype, defined by four single-nucleotide polymorphisms (SNPs: rs3780367, rs10974944, rs12343867 and rs1159782), was strongly associated with the *cis* acquisition of  $JAK2^{V617F}$  mutation with complete linkage disequilibrium.<sup>5–7</sup> As UPD 9p involves most of the chromosome 9,  $JAK2^{V617F}$  and the SNPs that tag 46/1 are converted into homozygosity simultaneously, and the 46/1 SNP allele burden reflects the percentage of  $JAK2^{V617F/V617F}$  cells in 46/1 heterozygous PV patients. The  $JAK2^{V617F}$  allele burden depends on the fraction of cells with a wild type, heterozygous and homozygous genotype. These fractions are usually determined by single-cell analyses. Here, we report that the combined measurement of 46/1 SNPs and  $JAK2^{V617F}$  allele burdens in hematopoietic progenitors and differentiated myeloid compartments allows dissecting the clonal architecture in each cell compartment. This approach can be used to explore the impact of treatments, as demonstrated in patients exposed to alpha-interferon (IFN $\alpha$ ).

MODELING  $JAK2^{V617F}$  CLONAL ARCHITECTURE IN PV PATIENTS

We first explored three patients with hemochromatosis, wild-type  $JAK2$  and rs12343867 heterozygosity. Genotyping of 500 single-cell colonies established from sorted CD34<sup>+</sup>CD38<sup>-</sup> and CD34<sup>+</sup>CD38<sup>+</sup> progenitors did not identify any rs12343867 homozygous colony (Supplementary Figure S1), indicating that recombination of the 46/1 haplotype was not a common event.

We selected 9 PV patients heterozygous for rs12343867 allele and  $JAK2^{V617F}$  negative in CD3<sup>+</sup> cells using Taqman allelic discrimination PCR. In all the patients, the rs12343867 and  $JAK2^{V617F}$  allele burdens were measured in sorted CD34<sup>+</sup> cells by both Taqman allelic discrimination PCR and next-generation sequencing (NGS), allowing calculation of the percentage of  $JAK2^{V617F}$  cells. The percentage of cells with a homozygous  $JAK2$  mutation ( $JAK2^{V617F/V617F}$ ) was calculated from the formula: (rs12343867 allele burden – 50%)  $\times$  2, the percentage of cells with a heterozygous  $JAK2$  mutation ( $JAK2^{V617F/WT}$ ) by the formula: (% of  $JAK2^{V617F}$  allele burden – % of  $JAK2^{V617F/V617F}$  cells)  $\times$  2, and the percentage of  $JAK2^{WT/WT}$  cells by the formula: 100 – (% of  $JAK2^{V617F/V617F}$  + % of  $JAK2^{V617F/WT}$  cells) described in the Supplementary Methods. In parallel, colonies obtained by CD34<sup>+</sup>CD38<sup>+</sup> culture were genotyped individually using allelic discrimination PCR. In seven out of nine patients, we observed a good correlation between the expected (calculated from PCR and from NGS data) and the observed (in single-cell-derived colonies)

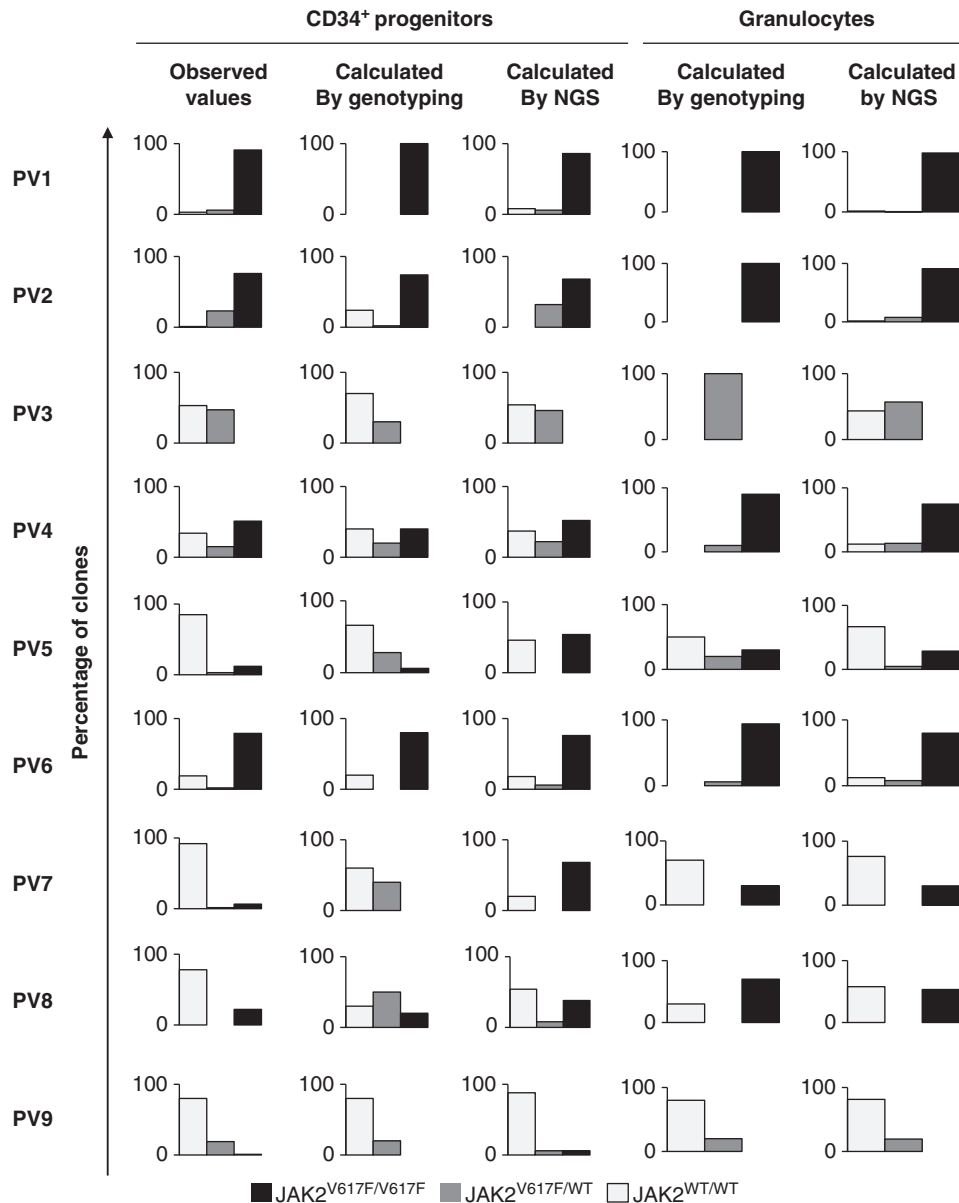
values of homozygous  $JAK2^{V617F/V617F}$  and heterozygous  $JAK2^{V617F/WT}$  cell fractions. Of note, one patient (#1) had only  $JAK2^{V617F/V617F}$  cells and another (#9) had a majority of WT cells. Patient #3 displayed a mixture of WT and heterozygous cells, patients #6 and #8 had a mixture of WT and homozygous cells, and patient #4 of WT, heterozygous and homozygous cells (Figure 1). Interestingly, NGS gave quite similar values than those with allele-specific PCR. Surprisingly, analysis of single-cell-derived colonies of patients #5 and #7 progenitors showed more wild-type cells than expected from our PCR- and NGS-based calculations, that is, the  $JAK2^{V617F}$  burden obtained by genotyping CD34<sup>+</sup>-derived colonies was much lower than measured in the CD34<sup>+</sup> cell population. This result was not related to an artifact because experiments could be reproduced at least three times for each patient. One possibility is that a secondary genetic event affects self-renewal capacities of *in vitro* growth of heterozygous and homozygous  $JAK2^{V617F}$  progenitors and prevents them from growing in short-term culture. This strongly suggests that *in vitro* cell growth induces a bias in clonal architecture analysis. Nevertheless, our results validate the use of the 46/1 haplotype to construct the  $JAK2^{V617F}$  clonal architecture in CD34<sup>+</sup> cells of PV patients.

 $JAK2^{V617F}$  CLONAL AMPLIFICATION PATTERN IN HEMATOPOIETIC STEM CELLS AND MYELOID COMPARTMENTS

Some evidence suggests that, in MPN patients, the  $JAK2^{V617F}$  clone expands during myeloid differentiation.<sup>8–10</sup> In mouse models,  $Jak2^{V617F}$  also stimulates the proliferation of hematopoietic stem cells (HSCs) or early progenitors.<sup>11,12</sup> Calculations obtained from allele-specific PCR and NGS were used with the rs12343867 and  $JAK2^{V617F}$  allele burdens of granulocytes of nine PV patients. Results clearly showed that  $JAK2^{V617F/V617F}$  clone preferentially expands during myeloid differentiation at the expense of WT and  $JAK2^{V617F/WT}$  clones (Figure 1). When  $JAK2^{V617F/V617F}$  clone was absent (patients #3 and #9),  $JAK2^{V617F/WT}$  had no or very little proliferative advantage (Figure 1). Moreover, we investigated  $JAK2^{V617F}$  clonal proportions in the HSC compartments in six patients. Colonies were genotyped from CD34<sup>+</sup>CD38<sup>+</sup>, CD34<sup>+</sup>CD38<sup>-</sup> and CD90<sup>+</sup>CD34<sup>+</sup>CD38<sup>-</sup> cells and revealed no proliferative advantage of  $JAK2^{V617F/V617F}$  or  $JAK2^{V617F/WT}$  (Supplementary Figures S2 and S3). These results are in accordance with previous reports showing that  $JAK2^{V617F/V617F}$  clones expand with myeloid differentiation, but their size remains limited in the HSC compartment.<sup>8–10</sup>

EFFECT OF IFN TREATMENT ON  $JAK2^{V617F}$  CLONAL STRUCTURE

We used our model to assess the therapeutic potential of pegylated IFN $\alpha$  in MPN treatment. We tested 4 molecular nonresponding and 11 responding PV patients according to the criteria of Barosi *et al.*<sup>13</sup> using NGS or allele-specific PCR to determine rs12343867 and  $JAK2^{V617F}$  allele burdens in granulocytes. We observed that the homozygous cells were in all cases targeted by IFN $\alpha$  (P1, P2, P3, P6, P12, P14, P15), whereas heterozygous cells were nonresponders in 53% of patients



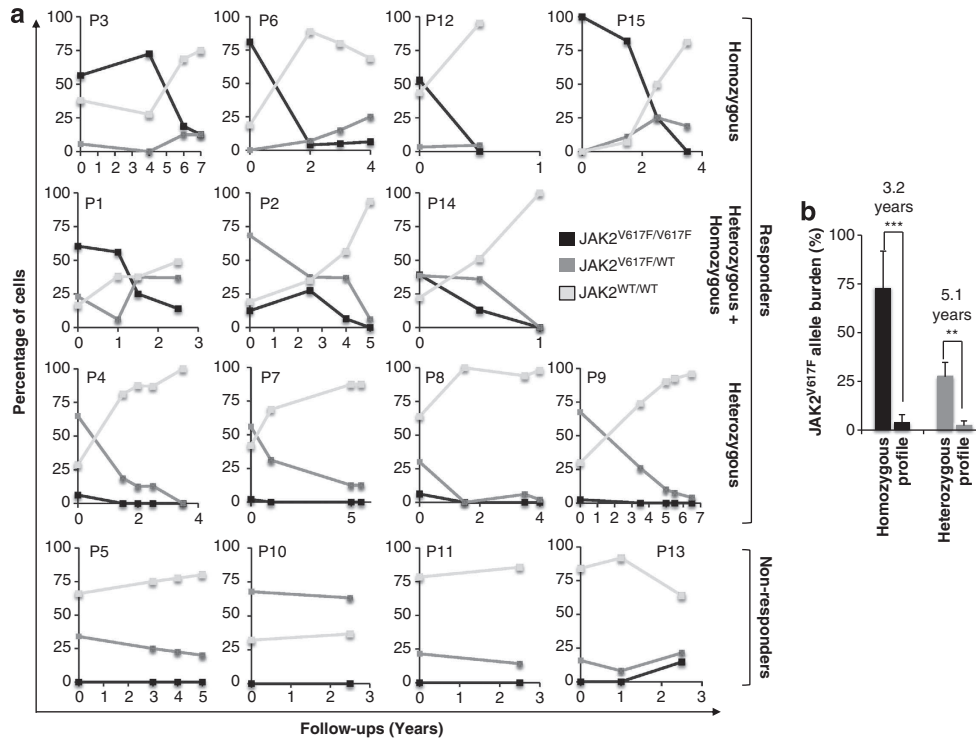
**Figure 1.** Modeling  $JAK2^{V617F}$  clonal architecture in PV patients.  $CD34^+$  cells from nine PV patients were cloned and cultured for 14 days in serum-free medium in the presence of cytokines. DNA was extracted from each colony (an average of 100 colonies were genotyped per patient) and was subjected either to  $JAK2$  or rs12343867 allele discrimination (observed values). Alternatively, quantification of  $JAK2$  mutation and rs12343867 variant was obtained by allelic discrimination 'genotyping' or NGS both in global  $CD34^+$  and granulocyte cells from 9 PV patients. The expected and the observed values of WT,  $JAK2^{V617F/WT}$  and  $JAK2^{V617F/V617F}$  clones are represented in the figure. In none of the nine patients studied, the 9pLOH precedes acquisition of  $JAK2^{V617F}$  mutation.

(P5, P10, P11, P13, P1, P3, P6, P15). Of note, the four nonresponding patients displayed initially only a heterozygous clone (Figure 2a). The percentage of allele burdens decreased more dramatically and faster in homozygous patients than in heterozygous responding patients (73% to 4% in 3.25 years versus 27% to 2% in 5.1 years in average; Figure 2b). Moreover, in patients with mixed cells ( $JAK2^{V617F/V617F}$ ,  $JAK2^{V617F/WT}$ ,  $JAK2^{WT/WT}$ ),  $IFN\alpha$  preferentially targeted homozygous cells during the follow-up (P1, P2, P14). The preferential targeting of homozygous cells by  $IFN\alpha$  is also exhibited by appearance of heterozygous cells during the treatment of patients at the time of eradication of homozygous cells (P1, P3, P6, P15). In addition, the  $JAK2^{V617F}$  burden increased at 48% after 2 years of  $IFN\alpha$  interruption in patient #6 and this relapse was mainly related to the  $JAK2^{V617F/V617F}$  clone. Finally, the screening for

mutations in  $DNMT3A$ ,  $TET2$ ,  $ASXL1$ ,  $SUZ12$ ,  $EZH2$ ,  $ZRSR2$ ,  $SF3B1$ ,  $RUNX1$ ,  $IDH1/2$ ,  $U2AF1$  genes revealed additional mutations in few patients (Supplementary Figure S4), which did not clearly affect the response to  $IFN\alpha$  treatment contrary to what was suggested.<sup>14</sup>

These results demonstrate that this simple modeling can be useful to follow the efficacy and specificity of treatment on  $JAK2^{V617F}$  clones in MPNs, without absolutely requiring investigation at the unicellular level. In addition, it suggests that  $IFN\alpha$  treatment more specifically target the  $JAK2^{V617F/V617F}$  clone in responding patients, as suggested by *in vitro* clonal studies on progenitors from PV.<sup>15</sup> These results suggest a link between the levels of  $JAK2$  signaling and  $IFN\alpha$  response. This may be important to determine the mechanism of action of  $IFN\alpha$  on  $JAK2^{V617F}$  stem cell.





**Figure 2.** Modeling  $JAK2^{V617F}$  clonal architecture in PV patients treated with  $IFN\alpha$ . (a) The calculated values of WT,  $JAK2^{V617F/WT}$  and  $JAK2^{V617F/V617F}$  clones were obtained after quantifying  $JAK2^{V617F}$  and rs12343867 variant by NGS or allele-specific PCR in global granulocytes from 15  $IFN\alpha$ -treated patients over time. Patients were considered as responders except patients P5, P10, P11, P13 as previously established.<sup>13</sup> (b) The percentage of  $JAK2^{V617F}$  allele burdens were calculated in pure homozygous or pure heterozygous patients before the treatment with  $IFN\alpha$  and after molecular response. The duration of the molecular response is also indicated in years (\*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ).

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Leukemia website (<http://www.nature.com/leu>)

## **Use of the 46/1 haplotype to model *JAK2*<sup>V617F</sup> clonal architecture in PV patients: clonal evolution and impact of IFN $\alpha$ treatment.**

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### **SUPPLEMENTAL METHODS:**

#### **Patients.**

We studied samples from twelve 46/1 heterozygous individuals: 9 patients with a *JAK2*<sup>V617F</sup>-positive PV and 3 *JAK2* wild-type (WT) hemochromatosis. 15 patients were studied during PEGASYS (peginterferon alpha 2a) therapy at a weekly dose ranging from 67.5  $\mu$ g to 180  $\mu$ g. Repeated samples were obtained during their clinical survey. Informed consent was obtained from each of them in accordance with the Declaration of Helsinki.

#### **Cell purification and culture.**

CD34<sup>+</sup> and CD3<sup>+</sup> cells were isolated from mononuclear cells by immunomagnetic enrichment and the following fraction (CD90<sup>+</sup>CD34<sup>+</sup>CD38<sup>-</sup>, CD34<sup>+</sup>CD38<sup>-</sup>, CD34<sup>+</sup>CD38<sup>+</sup>) were sorted, cloned at 1 cell /well and cultured in presence of a cocktail of human recombinant cytokines.<sup>1</sup> Fourteen days later, individual colonies were plucked and lysed.

#### **Nucleic acid extraction.**

Genomic DNA from granulocytes, CD34<sup>+</sup>, CD3<sup>+</sup> cells was isolated using DNA QiaAmp Kit (Qiagen, Courtaboeuf, France). Colonies were lysed with proteinase K and 0.2% Tween 20 (Sigma) at 65° for 50 minutes and 95°C for 15 minutes.

### **Next generation sequencing (NGS).**

DNA sample (CD34<sup>+</sup> cells or granulocytes) was amplified with two sets of primers tagged with a unique and identical bar code to generate a library (JAK2\_S3, JAK2\_AS3; rs12343861\_S1, rs12343861\_AS1). Amplicons were sequenced using ion torrent PGM to quantify the *JAK2*<sup>V617F</sup> and rs12343867 allele burdens (average 10 000 reads). For patients prior to treatment with IFN $\alpha$ , genes (*DNMT3A*, *TET2*, *ASXL1*, *SUZ12*, *EZH2*, *ZRSR2*, *SF3B1* (exon 14), *RUNX1*, *IDH1/2*) were amplified using ampliseq technology and libraries were sequenced using ion torrent PGM with a 318 chip (average 100 reads)

### **Basis for the formula for the frequency of homozygous and heterozygous cells:**

The method can only be applied to patients heterozygous for the 46/1 haplotype that is associated with acquisition of *JAK2*<sup>V617F</sup> mutation. It is based on the precise determination of the 9pUPD level by measuring the level of a heterozygous SNP, from the 46/1 haplotype, and thus to calculate the frequency of the *JAK2*<sup>V617F</sup> homozygous clone. Subsequently, the *JAK2*<sup>V617F</sup> burden is determined to calculate the frequency of wild type *JAK2* and *JAK2*<sup>V617F</sup> heterozygous clone.

#### **- 1) How to calculate *JAK2*<sup>V617F</sup>/*JAK2*<sup>V617F</sup> cells:**

As selected patients display germline heterozygous for 46/1 haplotype (rs12343867)

⇒ they should present 50% rs12343867 allele burden.

As rs12343867 and *JAK2*<sup>V617F</sup> recombined simultaneously, rs12343867 is used as a tag to follow mitotic recombination.

Examples:

If a patient presents 100% rs12343867 allele burden; it means that all cells have recombined  $JAK2^{V617F}$  (i.e. 100%  $JAK2^{V617F}/JAK2^{V617F}$  cells).

If a patient present now 75% rs12343867 allele burden; it means that half of cells have recombined  $JAK2^{V617F}$  (i.e. 50%  $JAK2^{V617F}/JAK2^{V617F}$  cells).

=>Formula is: The % of  $JAK2^{V617F}/JAK2^{V617F}$  cells = (rs12343867 allele burden - 50%) x 2

For these calculations we need to quantify global rs12343867 allele burden by Taqman allelic discrimination PCR or by NGS which is more accurate to quantify small differences.

## 2) How to calculate $JAK2^{V617F}/JAK2^{WT}$ cells:

As we know now the % of  $JAK2^{V617F}/JAK2^{V617F}$  cells,

Using the  $JAK2^{V617F}$  allele burden,

=> it possible to determine the  $JAK2^{V617F}/JAK2^{WT}$  cells.

Example: If a patient present 75% rs12343867 allele burden =>50%  $JAK2^{V617F}/JAK2^{V617F}$  cells (see above) and we detect a 60% global  $JAK2^{V617F}$  allele burden.

Calculations are 60% global  $JAK2^{V617F}$  allele burden - 50%  $JAK2^{V617F}/JAK2^{V617F}$  cells

=> We find 10%  $JAK2^{V617F}$  allele burden that represents 20% of  $JAK2^{V617F}/JAK2^{WT}$  cells.

=>Formula is: the % of  $JAK2^{V617F}/JAK2^{WT}$  cells = [% of  $JAK2^{V617F}$  allele burden - % of  $JAK2^{V617F}/JAK2^{V617F}$  cells) x 2

For these calculations we need to quantify global  $JAK2^{V617F}$  allele burden by Taqman allelic discrimination PCR or NGS that is more accurate.

## 3) How to calculate $JAK2^{WT}/JAK2^{WT}$ cells:

As we know now the % of  $JAK2^{V617F/V617F}$  cells and the % of  $JAK2^{V617F/WT}$  cells, we can easily deduced the % of  $JAK2^{WT/WT}$  cells.

=>Formula is: the % of  $JAK2^{WT/WT}$  cells =  $100 - (\% \text{ of } JAK2^{V617F/V617F} + \% \text{ of } JAK2^{V617F/WT} \text{ cells})$

### ***JAK2*<sup>V617F</sup> and rs12343867 quantification by real-time polymerase chain reaction.**

TaqMan allelic discrimination assays were used to determine the allele burdens of *JAK2*<sup>V617F</sup> and rs12343867 (C\_31941689 assay) (ABI 7500, Applied biosystem, Invitrogen). The region encompassing *JAK2*<sup>V617F</sup> and rs12343867 SNP was pre-amplified by PCR (with JAK2\_S2 and JAK2\_AS2) for single colony DNAs (Table S1) and used to determine the mutational status of each clone. For controls, DNA from WT, heterozygous and homozygous *JAK2*<sup>V617F</sup> and rs12343867 patients were used. Therefore, we quantified allele burdens for granulocytes, CD34<sup>+</sup> and CD3<sup>+</sup> cells with the measurement of angles as shown in Figure S3. Of note, colonies genotyping only display heterozygous or homozygous rs12343867 and wild-type, heterozygous or homozygous *JAK2*<sup>V617F</sup>.

### **SUPPLEMENTAL FIGURES :**

#### **Figure S1: 46/1 allele is not responsible for frequent homologous recombination event:**

CD34<sup>+</sup> cells from hemochromatosis patients were cloned at one cell/well and cultured for 14 days in serum-free medium in the presence of cytokines. DNA was extracted from each colony and was subjected to rs12343867 C or T allele discrimination (representative from 3 patients). Black circles stand for positive controls and crosses stand for colonies from the hemochromatosis patient. Black squares represent H<sub>2</sub>O.

#### **Figure S2: *JAK2*<sup>V617F</sup> clonal architecture in PV patients in HSC compartments.**

Either CD34<sup>+</sup>CD38<sup>+</sup> or CD34<sup>+</sup>CD38<sup>-</sup> or CD90<sup>+</sup>CD34<sup>+</sup>CD38<sup>-</sup> cells from PV patients were cloned and cultured for 14 days in serum-free medium in the presence of cytokines. DNA was extracted from each colony (average of 100 colonies for CD34<sup>+</sup>CD38<sup>+</sup> and CD34<sup>+</sup>CD38<sup>-</sup> and 62 colonies for CD90<sup>+</sup>CD34<sup>+</sup>CD38<sup>-</sup>) and was subjected to *JAK2* Taqman allelic discrimination. The observed values of WT, *JAK2*<sup>V617F/WT</sup> and *JAK2*<sup>V617F/V617F</sup> clones were represented.

**Figure S3: Representation of rs12343867 and JAK2V617F allele discrimination from one patient.**

Allele burdens were quantified for rs12343867 and JAK2V617F from granulocytes, CD34<sup>+</sup>, CD3<sup>+</sup> cells or CD34<sup>+</sup>CD38<sup>+</sup> colonies with the measurement of angles. Of note, colonies genotyping display either heterozygous or homozygous rs12343867 and wild-type or heterozygous or homozygous *JAK2*<sup>V617F</sup>. Positive standards are indicated by filled circles.

**Figure S4: Sequencing by NGS of mutated genes associated with JAK2V617F in patients before IFN $\alpha$  treatment**

DNAs from patients before IFN $\alpha$  treatment were sequenced by NGS on *DNMT3A*, *TET2*, *ASXL1*, *SUZ12*, *EZH2*, *ZRSR2*, *SF3B1* (exon 14), *RUNX1*, *IDH1/2*. Three novel unannotated variants of *TET2* were found. Two of them were located in exon 11 leading to an amino acid change in the DSBH domain. ND stands for not determined.

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rs12343867 allele discrimination in one hemochromatosis patient

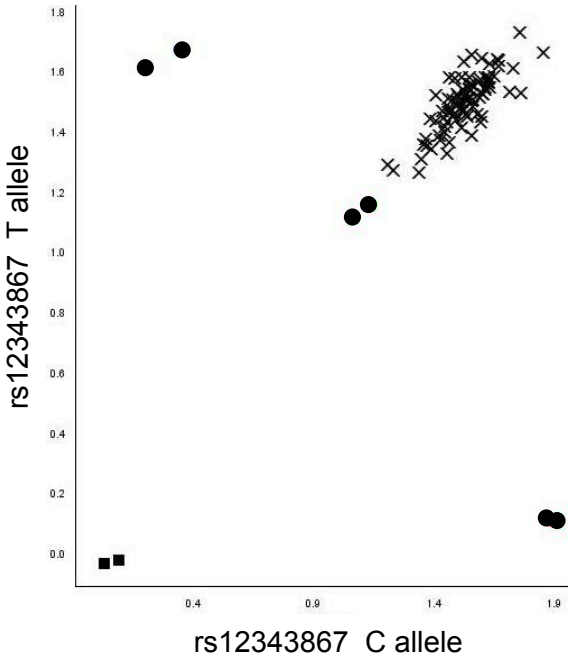


Figure S1: Hasan *et al.*

## JAK2V617F clonal architecture in HSC compartments

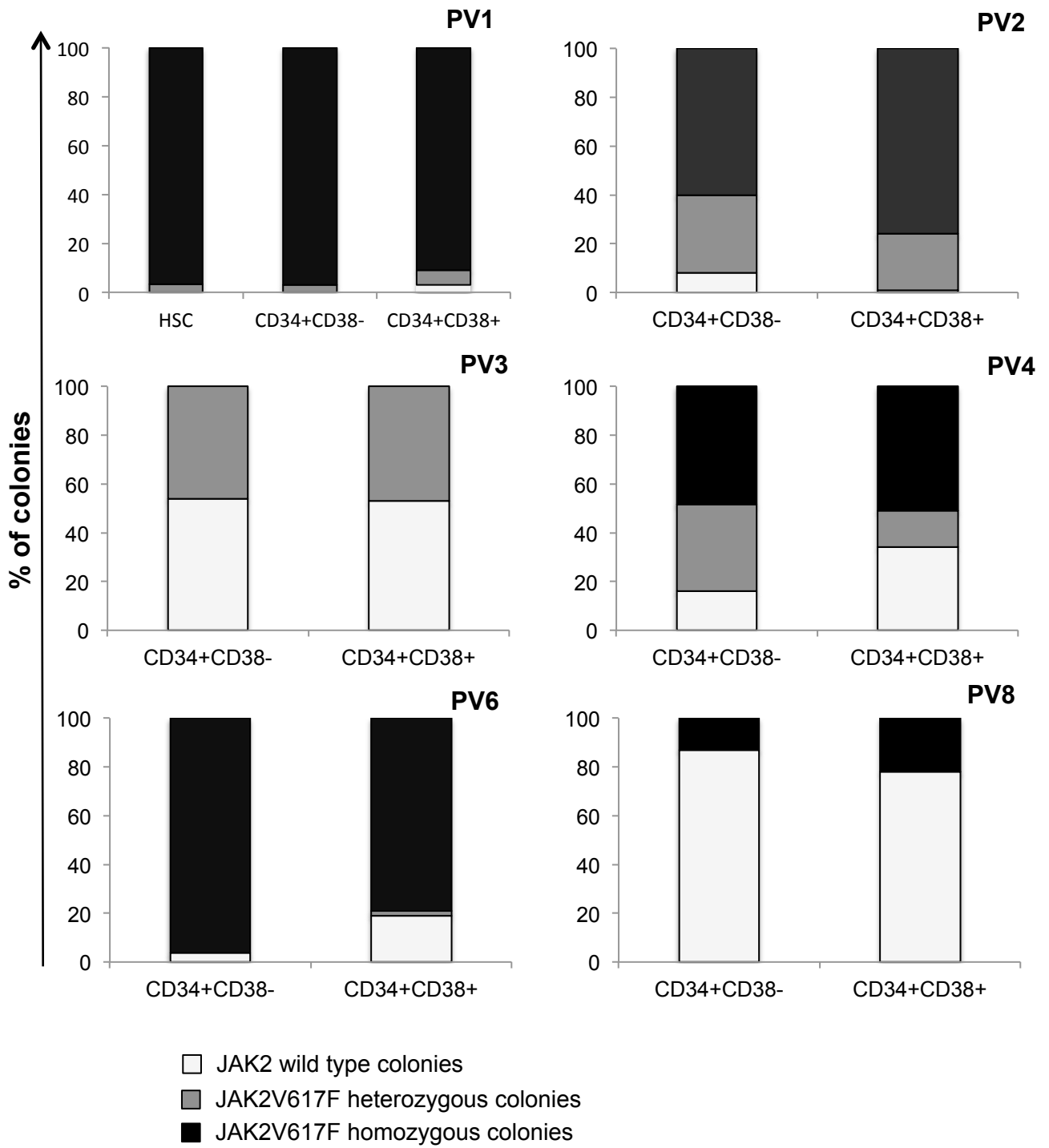
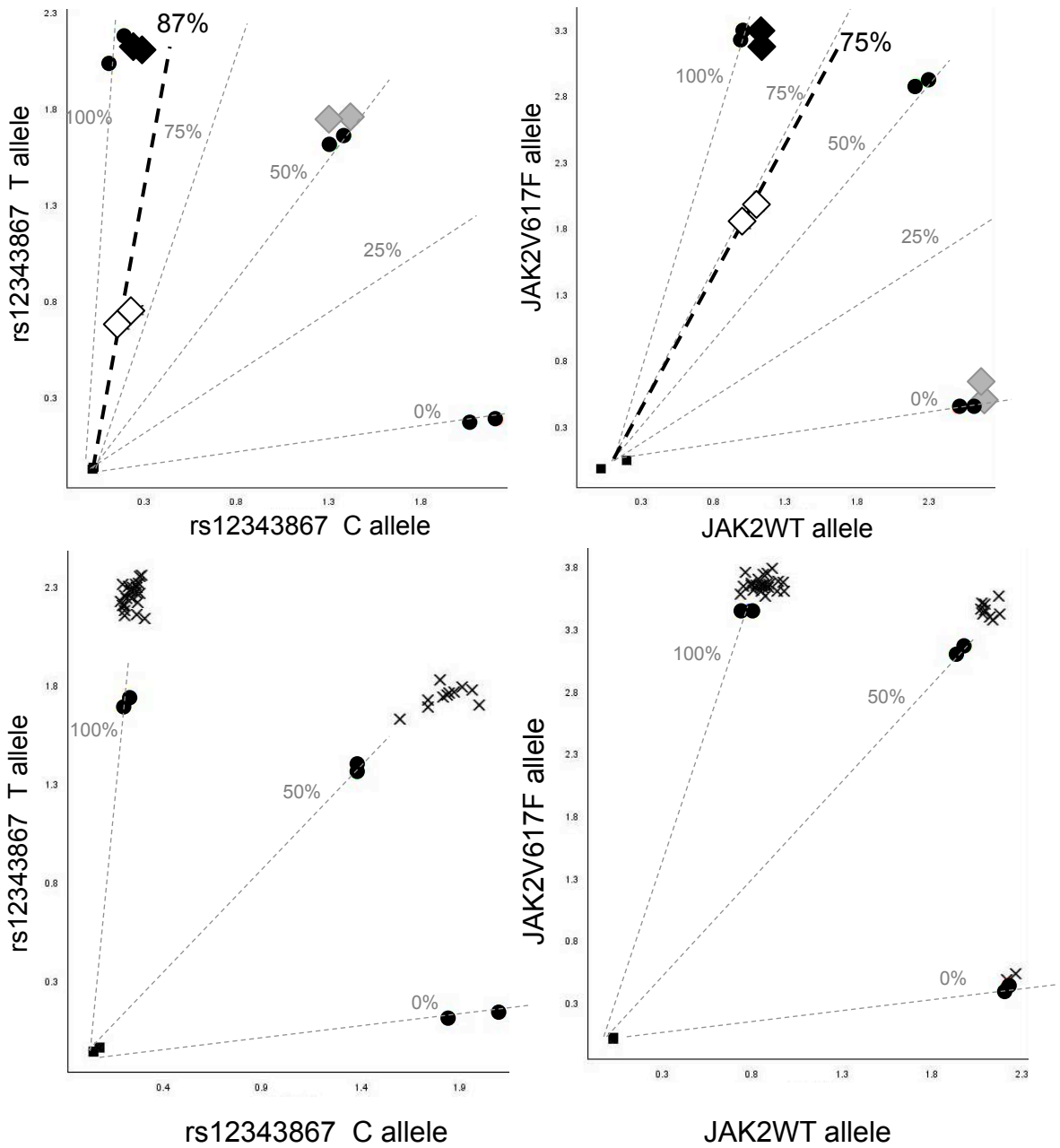


Figure S2: Hasan *et al.*

Representation of rs12343867 and JAK2<sup>V617F</sup> allele discrimination from one patient



- PNN
- CD34+
- CD3+
- CD34+CD38+ colonies X
- Positive standards
- H<sub>2</sub>O

Figure S3: Hasan *et al.*

MUTATED  
GENES

Patient number	DNMT3A	TET2	ASXL1	SUZ12	EZH2	ZRSR2	SF3B1(exon14)	RUNX1	IDH1/2	U2AF1(except exon 2)
PV1	-	-	-	-	-	-	-	-	-	-
PV2	-	-	-	-	-	-	-	-	-	-
PV3	-	-	P779L (CCT->CTT) ; S41P (TCC->CCC)		-	-	-	-	-	-
PV4	-	-	-	-	-	-	-	-	-	-
PV5	-	-	-	-	-	-	-	-	-	-
PV6	-	P1885L (CCT->CTT)	-	-	-	-	-	-	-	-
PV7	-	-	-	-	-	-	-	-	-	-
PV8	-	-	-	-	-	-	-	-	-	-
PV9	-	-	-	-	-	-	-	-	-	-
PV10	-	-	-	-	-	-	-	-	-	-
PV11	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PV12	-	E452A (GAG->GCC)	-	-	-	-	-	-	-	-
PV13	-	G1647A (GGT->GCT)	-	-	-	-	-	-	-	-
PV14	-	-	-	-	-	-	-	-	-	-
PV15	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Figure S4: Hasan *et al.*

## Primers used

### TaqMan® allele discrimination assay

JAK2_F1	AAGCTTTCTACAAGCATTGTTT
JAK2_R1	AGAAAGGCATTAGAAAGCCTGTAGTT
JAK2V617F-VIC (probe)	TCTCCACAGACACATAC
JAK2V617F-FAM (probe)	TCCACAGAAACATAC
JAK2_F2	TGCTGAAAGTAGGAGAAAGTGC
JAK2_R2	GCAAGGTGCAATAAAATGAGG

### Next generation sequencing (NGS):

JAK2_F3	AAGCAGCAAGTATGATGAGCAA
JAK2_R3	TGCTCTGAGAAAGGCATTAGAA
rs12343861_F1	TGAAGACAAAGCATATAAATGATA
rs12343861_R1	TTAAAGCATGGGGTACGATT

## **DISCUSSION AND PERSPECTIVES**

## 1. Can one mutation explain three phenotypes?

Although the discovery of JAK2<sup>V617F</sup> have provided the key insights into the pathophysiology of MPN it remains unclear how one mutated allele can generate three clinically distinct, though related diseases i.e., ET, PV and PMF. Our first goal of study was to decipher the JAK2<sup>V617F</sup> pleiotropy in MPN phenotype.

We have developed a KI heterozygous JAK2<sup>V617F</sup> mouse model, which expressed JAK2<sup>V617F</sup> at physiological levels. This heterozygous JAK2<sup>V617F</sup> expression was sufficient to develop a full human PV-like disease with erythrocytosis, thrombocytosis, leukocytosis, splenomegaly and endogenous erythroid colonies (EEC), a hallmark of PV. As the disease evolved, mice developed severe splenomegaly and accumulation of the collagen and reticulin fibers in both spleen and BM. Thus, for the first time we could demonstrate that heterozygous expression of JAK2<sup>V617F</sup> is sufficient to develop human PV-like disorder with its evolution towards secondary myelofibrosis. However, in our model endogenous heterozygous JAK2<sup>V617F</sup> expression did not result in ET phenotype as expected from clinical studies.

There can be two hypotheses to explain these results.

1. First, the disease phenotype, ET or PV, maybe related to different levels of mutated JAK2 kinase i.e., low JAK2<sup>V617F</sup> expression favors thrombocytosis and high levels favor erythrocytosis while sustained levels develop fibrosis.

Our KI mice expressed JAK2<sup>V617F</sup> and JAK2 WT at a ratio of 1:1 in BM and developed PV. In a JAK2<sup>V617F</sup> TG mouse model presented by Shide and colleagues VF/WT mRNA ratio of ~ 0.2:1 resulted in an ET-like disease and > 0.4:1 resulted in PV like disease<sup>155</sup>. This suggests that JAK2<sup>V617F</sup> expression five times as less as WT JAK2 is required to develop ET. Another JAK2<sup>V617F</sup> TG murine model developed by Tiedt *et al.* displayed ET at mutant to WT ratio of 0.45:1 and PV at the ratio 1:1<sup>171</sup>. These studies suggest that low level of mutated kinase is required to develop ET and equal amounts to develop PV. Noteworthy in the later model is the use of human JAK2<sup>V617F</sup> in contrast to its mouse counterpart in Shide's models and ours. The requirement of 0.2 and 0.45 mutant to WT ratios or less to obtain an ET phenotype when mouse and human JAK2<sup>V617F</sup> genes are used respectively, points towards the probable non-competitiveness of human gene in mouse setting. In fact, mouse JAK2<sup>V617F</sup> is shown to induce erythrocytosis more effectively than human JAK2<sup>V617F</sup> in RV BMT mouse model but this could only be an indication as expression of transgene can not be controlled in such settings<sup>171</sup>.

Akada *et al.* investigated JAK2<sup>V617F</sup> heterozygous and homozygous expression in KI mouse model. Strikingly, their heterozygous KI mice expressed mutant JAK2 mRNA as less as half (0.53) of WT JAK2 in BM and displayed a PV phenotype in accordance with TG model of Shide *et al.* However, human JAK2<sup>V617F</sup> heterozygous expression resulted in ET-like phenotype in one KI murine model<sup>173</sup> in accordance with low activity of hJAK2<sup>V617F</sup> in mouse settings<sup>201</sup>. The JAK2<sup>V617F</sup> gene dosage hypothesis is consistent with our previous RV BMT model where a secondary low JAK2<sup>V617F</sup> expresser group developed transient thrombocytosis compared to high expresser groups that developed PV-like disease<sup>172</sup>.

2. A second possible explanation is that JAK2<sup>V617F</sup> alone is not sufficient to induce ET and another genetic event is required for its development. This is consistent with the clinical studies where the JAK2<sup>V617F</sup> allelic ratio does not correspond to clonal hematopoiesis in many ET patients<sup>52</sup>. In compliance with this notion is the retroviral model developed by Zaleskas *et al.* where the presence of proviral JAK2<sup>V617F</sup>-GFP in megakaryocyte was not sufficient to induce thrombocytosis in two different strains of mice<sup>180</sup>.

Overtime, PV phenotype in our JAK2<sup>V617F</sup> KI mice transformed into secondary myelofibrosis with the accumulation of reticulin fibers depicting grade III fibrosis in spleen and BM. Fibrosis is thought to occur due to elevated fibrogenic cytokine levels such as transforming growth factor beta-1 (TGFβ-1) and basic fibroblast growth factor (bFGF) in BM. Circulating platelets and megakaryocytes from myelofibrotic patients are shown to express excessive amounts of TGFβ-1 and bFGF<sup>404</sup> and consistent with this, transgenic TPO<sup>high</sup> murine model developed myelofibrosis with the elevation of plasma TGFβ-1 levels<sup>405</sup>. JAK2<sup>V617F</sup> in our mouse model induced qualitative and quantitative changes in megakaryocytic lineage manifested by megakaryocytic hyperplasia, dysmegakaryopoiesis (giant platelets in peripheral blood), neutrophilic emperipoiesis and clustered megakaryocyte with multilobulated nucleus in BM and spleen. Although, we did not measure plasma cytokine level, it is tempting to speculate that JAK2<sup>V617F</sup> expression altered hematopoietic environment by the releasing cytokines from these pathologic megakaryocytes and thus contributed to the progression of PV towards secondary MF. Secondary transplantations from JAK2<sup>V617F</sup> RV BMT primary donors in myelofibrotic phase into secondary WT recipients resurrected polycythemic features and not fibrotic features pointing towards the time required by bad seeds (JAK2<sup>V617F</sup> cells) to transform the soil (hematopoietic environment) in the development of myelofibrosis<sup>180,199</sup>.

### 1. Does JAK2<sup>V617F</sup> modulate HSC activity?

MPN are clonal HSC disorders and JAK2<sup>V617F</sup> is present in Lin-CD34+CD38-CD90+ cells in PV patients<sup>49</sup> however its impact on HSC is largely unknown, in particular if JAK2<sup>V617F</sup> is sufficient for disease emergence or not. There is evidence in clinical studies that the effect of JAK2<sup>V617F</sup> is limited because in PV the clonal amplification mainly takes place during differentiation with a minority of JAK2<sup>V617F</sup> HSC<sup>170,189</sup>, which is very unusual for a clonal disorder targeting an HSC. Therefore, we were interested to investigate the effect of JAK2<sup>V617F</sup> on HSC using our KI mouse model.

Our results show that the expression of JAK2<sup>V617F</sup> causes marked expansion in immuno-phenotypically defined LSK and SLAM cells, the later being the most enriched in LT-HSC activity<sup>5</sup>, and this can be explained by their increased cell cycle and less apoptotic status as compared to WT cells. Our results are compatible with the findings of G. Mohi<sup>143</sup> but contrast with ones of AR Green<sup>173</sup>.

1. One hypothesis to explain this expansion is the use of JAK2 by cytokine receptors like the one of IFN-γ, Mpl and G-CSF on HSC surface.

Shao and group have shown that in mice interferon gamma (IFN-γ) treatment expanded the LSK pool in-vitro and as well as in-vivo and activated these cells through canonical IFNGR1-STAT1 pathway<sup>406</sup>.



Interestingly, this expanded pool had more myeloid differentiation potential as compared to lymphoid lineage. Goodell and colleagues using a chronic infection mouse model demonstrated that IFN- $\gamma$  exposure not only increased multipotent progenitors (MPP) and short-term hematopoietic stem cells (ST-HSC) but also long-term hematopoietic stem cell (LT-HSC) and markedly promoted their proliferation but may compromise their long-term reconstituting potential<sup>407</sup>. However, contrary to these findings, recently Bruin *et al.* demonstrated that interferon gamma (IFN- $\gamma$ ) inhibits the in-vitro mouse HSC (SLAM) proliferation by preventing quiescent cells to enter into cycling state. Functional studies demonstrated that IFN- $\gamma$  inhibited Tpo-induced STAT5 activation by inducing SOCS-1 expression and eventually resulting in deregulation of cyclin D1 and p57, two important genes implicated in HSC proliferation<sup>400</sup>. Mpl receptor is shown to play a vital role in the HSC production, quiescence and activity in Tpo<sup>-/-</sup> and Mpl<sup>-/-</sup> murine models<sup>236,399</sup>. Likewise, G-CSF receptor is shown to be involved in the direction of primitive hematopoietic progenitor to myeloid committed progenitors<sup>401</sup>. Constitutive JAK2<sup>V617F</sup> activation downstream to these receptors may result in altered signaling and thus modulation of HSC compartment.

2. Another possible reasoning can be the recent investigations that have highlighted the previously unrecognized intra-nuclear role of JAK2 in cell lines and mouse embryonic stem cells (mESC) that could possibly regulate HSC.

Green and co-workers have demonstrated that JAK2 is present in the nucleus of WT and JAK2<sup>V617F</sup> bearing cell line as well as in CD34+ cells from PV patients. Activated JAK2 signaling results in more abundant H3Y41 phosphorylation and the exclusion of HP1 $\alpha$  from the *lmo2* oncogene promoter thereby increasing its expression and thus contributing to leukemogenesis<sup>165</sup>. A step forward, they demonstrated that Nanog, a pluripotency regulator, was required for factor independence of JAK2<sup>V617F</sup> expressing mESC and inhibition of JAK2 reduced Nanog levels by reducing H3Y41 phosphorylation and concomitantly increased in HP1 $\alpha$  levels at the Nanog promoter indicating towards JAK2 as an important mediator of ESC self-renewal by direct signaling to chromatin. However, if this mechanistic stands true in HSC is yet to be elucidated<sup>408</sup>. In addition to these findings, Fan Liu and co-workers have demonstrated that PRMT5, a methyltransferase that dimethylates arginine residues in H2, H3 and H4 histone proteins, is implicated in negative regulation of hematopoietic stem/progenitor cell expansion and erythroid differentiation from CD34+ in-vitro. Activated JAK2 (JAK2V617F and JAK2K539L) binds more effectively with PRMT5 and hinders in its methyltransferase activity thus contributing in myeloproliferation<sup>166</sup>.

These studies strongly suggest that JAK2 is capable of regulating HSC compartment to drive MPN pathogenesis not only through cytokine receptor mediated pathways but also through intranuclear localization.

Interestingly, our JAK2<sup>V617F</sup> KI mice demonstrated an age dependent increase in myeloid progenitors (CMP, GMP and MEP), LSK and SLAM compartments with one-month-old mice having comparable compartment size to their age-matched littermates and 6-months-old mice with more expanded hematopoietic stem and progenitor compartment (HSPC) as compared to 3-months-old mice. This gradual increase in HSPC maybe due to

accumulation of additional genetic events with the progression of disease that may further contribute to clonal emergence on HSPC level. A very recent investigation has indicated that patients harboring mutations in *TET2* along with *JAK2*<sup>V617F</sup> presented with high *JAK2*<sup>V617F</sup> allele burden (66.7%) as compared to patients harboring only *JAK2*<sup>V617F</sup> mutation (38.7%) in granulocytes suggesting the role of additional event in the expansion of *JAK2*<sup>V617F</sup> clone. To visualize any other probable disease progression-related genetic event we are currently performing CGH array in old KI mice. To purify the genetically altered clone with acquired additional emergence potential, we transplanted whole BM cells from 8-11 months old *JAK2*<sup>V617F</sup> KI mice to lethally irradiated WT mice and collected DNA from KI lineage marker negative (Lin<sup>-</sup>) cells of secondary recipients 3 months post BMT.

*JAK2*<sup>V617F</sup> alone is sufficient to initiate MPN in mouse models but the growing evidences of the presence of other mutations such as *TET2*, *DNMT3a*, *CBL*, and *IDH* in MPN<sup>319</sup> have questioned the exact role of *JAK2*<sup>V617F</sup> in disease emergence. Therefore, we re-examined in our KI mouse model if *JAK2*<sup>V617F</sup> renders HSC a competitive advantage over WT cells thereby contributing in the clonal emergence of disease.

Results show that in our 30% *JAK2*<sup>V617F</sup> KI chimeric model early HSC (LSK and SLAM) and matured myeloid cells (GR1+) have out competed their WT counterparts by the end of 34 weeks. Moreover, in limiting dilution *JAK2*<sup>V617F</sup> KI chimeric groups, grafts containing atleast 50 SLAM cells (30% KI) or more developed an MPN with 100% penetrance. Mice transplanted with 17 SLAM cells (10% KI group) demonstrated only 50% penetrance of disease with doubled mature KI myeloid and early HSC. However, it was intriguing how same graft size could give disease in just 50% of recipients.

1. This can be related to cell cycle status of these *JAK2*<sup>V617F</sup> positive KI cells. We have demonstrated that our KI cells are more cycling than WT cells and this may hinder their migration towards BM niche as shown for human CD34+ cells in S/G2/M phase which encounter a selective transplantability problem as compared to cells in G0 phase<sup>409</sup>.
2. Theoretically, 1 out of every 2.1 phenotypically defined WT SLAM cells harbors long-term engraftment potential<sup>5</sup>. However, in our preliminary experiments/experimental settings this population is even rarer, close to 1/60 cells, therefore that would mean that if 34 *JAK2*<sup>V617F</sup> SLAM graft generate MPN with 100% penetrance, 1 *JAK2*<sup>V617F</sup> HSC is sufficient to generate the disease. A mathematical model has also suggested that clonal dominance and persistence of 1 neoplastic HSC cell would be rare event and will require ~2.5 years that is more than a life time of mouse<sup>410</sup> necessitating the injection of several HSC to generate the disease in mouse life time.

Altogether, our results suggest that as few as 34 *JAK2*<sup>V617F</sup> positive SLAM cells, close to one functional HSC, are sufficient to initiate and sustain a long-term MPN with 100% penetrance. Our competitive assay results are compatible with Mullally's KI model<sup>196</sup>. However, they employed direct method to measure the HSC activity by grafting pure immuno-phenotypically defined LSK population for competitive graft experiments. We have also performed the same sophisticated experiment to investigate *JAK2*<sup>V617F</sup> LSK activity in 50% competitive settings and observed comparable chimerism in mature myeloid compartment by the end of 20 weeks. Strikingly KI

lymphoid compartment presented less than or equal to 10% contribution in contrast to expected 50%. We could not compare KI lymphoid chimerism with Mullally's KI mice, as chimerism in lymphoid compartment was not discussed. But this raised the possibility that the immuno-phenotypically sorted LSK cells were comprised of heterogeneous HSC population with more myeloid-biased as compared to lymphoid-biased cells<sup>411</sup>.

Our results contradict the results obtained by Li *et al.*<sup>173</sup> where they observe that JAK2<sup>V617F</sup> eliminated HSC competitive activity. This maybe attributed to a non-competitiveness of human JAK2<sup>V617F</sup> or to possible nuclear translation of JAK2<sup>V617F</sup> from their KI cDNA construct<sup>412</sup> and accumulation of JAK2<sup>V617F</sup> in nucleus, resulting in deregulation of HSC activity<sup>165,166</sup>.

The long-term reconstitution from a small numbers of JAK2<sup>V617F</sup> HSC cells also points towards their increased self renewal and a formal proof of increased self renewal capacity could only be obtained by performing serial transplantation assays. We plan to perform this experiment to investigate if JAK2<sup>V617F</sup> preserves self-renewal capacity of HSC. Also, as we observed age-dependent gradual increase in HSPC compartment in KI mice, transplantations from donors of different age will answer if this increase in HSPC also includes some cell intrinsic changes resulting in more aggressive and/or accelerated phenotypes in secondary recipient mice.

### 3. Therapeutic potential of IFN- $\alpha$ in treatment of MPN

JAK2<sup>V617F</sup> is present in LT-HSC in MPN patients and is sufficient to sustain the disease through out the lifetime of patient. Elimination of these JAK2<sup>V617F</sup> bearing MPN-propagating cells is imperative to eradicate the disease.

JAK inhibitors have demonstrated palliative virtue in the resolution of MPN phenotype. However, to date in clinical trials and in animal models, no inhibitor has been proven to achieve promising molecular remissions and thus the eradication of disease. In contrast, because of its antiproliferative and myelosuppressive properties, IFN $\alpha$  has a history of more than 20 years to treat hematological malignancies by a mechanism yet largely unknown. It has been largely used in CML before the discovery of Glivec and in rare cases can induce a complete remission. In contrast to the Glivec, this complete remission can stand after treatment arrest suggesting that it may target the leukemic stem cell. Especially, recent investigations highlighting its promising hematological, histological and molecular responses nominate<sup>327,394,402</sup> IFN $\alpha$  as a potent drug capable of disease eradication at HSC level and argue a thorough investigation to understand its potential and mechanism-behind to cure MPN.

We exploited our 30% JAK2<sup>V617F</sup> KI competitive model to mimic human disease with residual normal polyclonal hematopoiesis. IFN $\alpha$  exposure to low and high JAK2<sup>V617F</sup>-positive disease burden mouse groups resulted in correction of hematocrit, leukocytosis and platelet numbers, as already observed in early studies in MPN patients, in both groups without the development of anemia or leukopenia but with thrombocytopenia. Thrombocytopenia can be attributed to the antiproliferative effect of IFN $\alpha$  on megakaryopoiesis and its effects on proplatelets formation<sup>99,368</sup>.

IFN $\alpha$  treatment resulted in the decrease of all hematopoietic lineages composing WBC specially B-cells regardless of WT or KI origin. Of note, IFN $\alpha$  treatment cancelled out KI granulocytes chimerism, but not KI lymphoid chimerism in low disease- and high-disease burden groups respectively, pointing towards the selective elimination of malignant cells in peripheral blood<sup>332</sup>. Although, splenomegaly was corrected in both groups, low-disease burden group demonstrated a better response. Histological investigation from low disease burden group demonstrated that IFN $\alpha$  induced the correction of the distorted splenic architecture with the disappearance of

clustered megakaryocytes as compared to the vehicle treated group. By the end of 12 weeks, vehicle treated mice did not develop myelofibrosis so the effect of IFN $\alpha$  treatment on fibrosis was not studied. Importantly, the onset of response to IFN $\alpha$  was more rapid and pronounced in low- as compared to high-disease burden group. These results are compatible with the clinical study suggesting that IFN $\alpha$  treatment at the early stage of disease, over an extended period, when the marrow was not completely fibrotic or osteosclerotic, results in a regression of splenomegaly and an improvement of marrow architecture<sup>394</sup>.

Overall, these observations suggested that IFN $\alpha$  treatment could achieve hematological, histological and complete molecular responses (CMR) as observed in patients but this does not exclude the presence of residual dormant malignant clone in BM. Patients who sustained CMR for long time after the cessation of treatment did harbor minor malignant clones in their bone marrow<sup>391</sup>. Therefore, to check the effect of IFN $\alpha$  treatment on MPN-propagating cell first we analyzed the HSPC pool in low-disease burden group and found that it was greatly reduced after 13 weeks of treatment but not disappeared pointing towards the longer period of treatment required to acquire CMR as suggested in clinical studies<sup>327,332</sup>. Also, it could have been possible that these residual cells are in cycle and have lost their reconstitution capacity<sup>370,371</sup>. So to check if these residual cells could give secondary disease (in some way synonym of relapse in patients) we transplanted whole BM from IFN $\alpha$  treated mice to lethally irradiated mice. Hematological parameters remained within physiological range from the beginning of transplant or rapidly corrected depending upon initial disease burden. Blood malignant granulocyte chimerism rapidly diminished or was not present after secondary transplantation from high- or low-disease burden groups respectively, suggesting that IFN $\alpha$  treated the disease by targeting JAK2<sup>V617F</sup> positive cells at HSC levels.

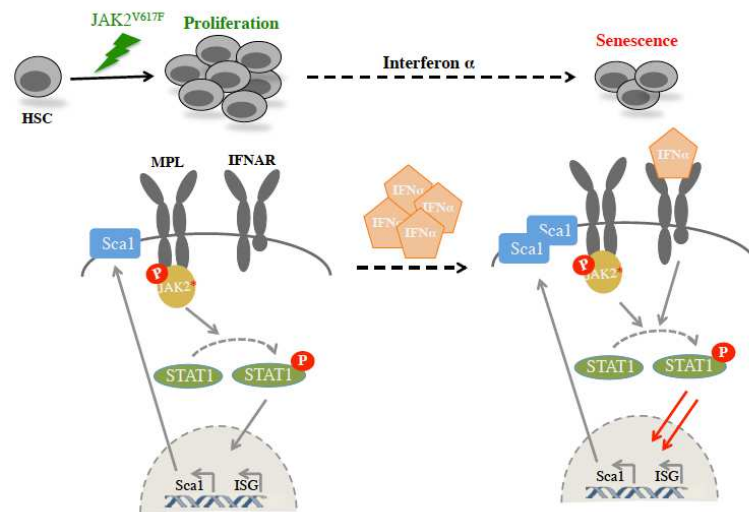
To get insight into the mechanism behind IFN $\alpha$  treatment we opted two common actions attributed to IFN $\alpha$ : anti-proliferation and apoptosis. As expected, our KI SLAM and LSK cells were more cycling at basal levels as compared to WT cells. Short term IFN $\alpha$  treatment forced the quiescent cells to enter into cycling state in both LSK and SLAM compartment from WT mice<sup>370,371</sup> but very few in LSK and no effect in SLAM from KI origin. This suggested that first, IFN- $\alpha$  could not increase the cycling of already cycling HSCP and second, that eradication of leukemic stem cells by IFN $\alpha$  is not only related to induction of cell cycling, but also by other mechanisms which remain to be characterized. We then investigated the apoptotic status of short termed IFN- $\alpha$  treated LSK and SLAM cells. KI myeloid progenitor, LSK and SLAM cells were less apoptotic than their WT counterparts at basal levels and IFN $\alpha$  treatment induced apoptosis in WT and KI cells in the spleen, but not in BM. Induction of apoptosis in the spleen maybe an underlying mechanism for the correction of splenomegaly and other pathologic features after IFN $\alpha$  treatment in our mice. Same results in spleen are also observed in a contemporary JAK2<sup>V617F</sup> KI murine model treated with IFN $\alpha$ <sup>403</sup>.

We hypothesize ‘immunomodulation’ and ‘JAK2<sup>V617F</sup> priming’ as mechanisms to eradicate the malignant clones after exposure of IFN $\alpha$ .

1. IFN $\alpha$  could be involved in modulating immune response to eradicate the malignant clones<sup>413</sup>. Indeed, we have noticed the appearance of a minor but significant T-cell population (CD3+Gr1+) in mice treated with IFN $\alpha$  and its disappearance with the cessation of treatment. This population was not unique to malignant origin but equally present in KI and WT cells further emphasizing on a global

immune response of IFN $\alpha$  in treated mice. Moreover, up-regulation of specific antigens in PV patients is reported in response to IFN $\alpha$  treatment<sup>358</sup>. The role of cytotoxic T-cells in the achievement of molecular response is yet to be explored. IFN $\alpha$  treatment of immunodeficient mice transplanted with JAK2<sup>V617F</sup> KI malignant cells will help us verify if this population is involved in the achievement of molecular response.

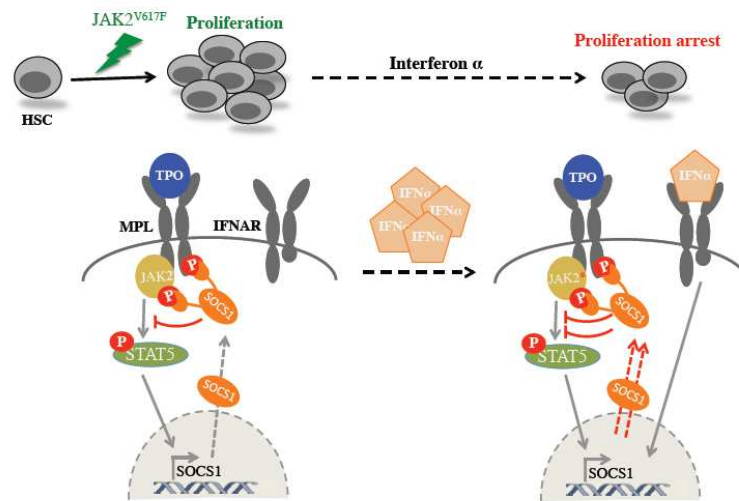
2. Within myeloid malignancies IFN $\alpha$  treatment is shown to be beneficial in disorders involving abnormal kinase pathways such as CML, hypereosinophilic syndrome, systemic mastocytosis and Ph- MPNs. Molecular analysis of ET or PV patients treated with IFN $\alpha$  revealed that patients harboring mutations other than JAK2<sup>V617F</sup> such as DNMT3a and TET2 are mostly poor responders to IFN $\alpha$ <sup>414</sup>. Moreover, IFN $\alpha$  treatment in patients bearing TET2 mutated clones along with JAK2<sup>V617F</sup> clones resulted in the elimination of JAK2<sup>V617F</sup> clones leaving behind TET2 mutated clone<sup>391</sup>. All these observations strongly suggest that IFN $\alpha$  exploits JAK-STAT pathway, already activated by JAK2<sup>V617F</sup>, to exert its therapeutic effects. STAT1 is shown to be over phosphorylated in response to IFN $\alpha$ <sup>370,371</sup> and it is a downstream mediator of IFN $\alpha$  signaling. Constitutive JAK2 activation is supposed to elevate STAT1 signaling (as observed in our KI mice which display increased levels of Sca-1, a direct target of STAT1) resulting in over signalization. Further activation of STAT signaling through IFN $\alpha$  exposure may force the cells to enter in senescence or apoptosis<sup>415</sup>.



**Figure 35. JAK2<sup>V617F</sup> priming hypothesis 1**

3. Other hypothesis that explains JAK2<sup>V617F</sup> priming through JAK-STAT pathway is by inducing SOCS-1 expression, a negative regulator of JAK-STAT signaling. IFN $\alpha$  is shown to inhibit Tpo-induced JAK-STAT pathway by inducing SOCS-1 expression in megakaryocytic progenitor cells<sup>99</sup>. Tpo receptor signalization is vital to HSC regulation<sup>399</sup>. Over signalization by additive effects of IFN $\alpha$  and JAK2<sup>V617F</sup> is assumed to inhibit JAK-STAT pathway, by expressing SOCS-1, more selectively and effectively in

KI cells as compared to WT cells. Our preliminary results using human CD34+ cells also suggest that antiproliferative effect of IFN $\alpha$  exploits SOCS proteins.



**Figure 36. JAK2<sup>V617F</sup> priming hypothesis 2**

In a recent study it has been shown that JAK2-inhibitor-persistent JAK2<sup>V617F</sup> cells remain dependent on JAK2 protein<sup>416</sup>. Therefore, deregulating JAK2 signaling will preferentially eliminate these cells as compared to WT cells. IFN $\alpha$  treatment of JAK2<sup>V617F</sup>-positive cell lines will shed light if these mechanisms are important to JAK2<sup>V617F</sup> molecular remission.

#### 4. JAK2<sup>V617F</sup> clonal architecture

The JAK2<sup>V617F</sup> allele burden is usually measured on granulocytes. One of the characteristics of the MPN such as ET and PV is that the JAK2<sup>V617F</sup> does not totally dominate and even after long time during disease evolution normal clones are not only present but also participate to polyclonal hematopoiesis. Thus, in one patient homozygous, heterozygous and WT cells can be present and the JAK2<sup>V617F</sup> burden is the reflection of this composition.

To follow the natural evolution of the disorder and the effects of different new treatments (JAK2 Inhibitors, interferon- $\alpha$  and combination of targeted therapies) it is important to have a test which allows a simple measurement of the different JAK2<sup>V617F</sup> sub-clones in different populations. The most interesting technique will be the direct measurement in individual cells for example granulocytes, CD34+CD38+ and CD34+CD38- using the new technologies of PCR coupled to cell sorting such as the Fluidigm© technology, though it requires specialized platform.

JAK2<sup>V617F</sup> is preferentially acquired on 46/1 JAK2 haplotype and is in complete linkage disequilibrium with SNP rs12343867. As 9pUPD includes whole 46/1 haplotype the recombination frequency of rs12343867 reflects the JAK2<sup>V617F</sup> homozygosity. Using this strategy we have compared the frequencies of JAK2<sup>V617F</sup> clones, calculated by exploiting JAK2<sup>V617F</sup> and rs12343867 allele burden in CD34+ cells obtained from NGS with the CD34+CD38+ derived clones obtained experimentally. 7 out of 9 PV patients demonstrated comparable

frequencies using both techniques suggesting that this simple modeling can be used to construct the clonal architecture without working on uni cellular levels.

Two patients (patient no 5 and 7) have produced more JAK2 WT colonies than expected from NGS based calculations suggesting that the acquisition of another event in these patients that resulted in in-vitro inhibition of JAK2<sup>V617F</sup> heterozygous and homozygous clones proliferation. We are currently performing whole exome sequencing from CD3, granulocyte and CD34+ cells of these patients to identify any such event. A recent preliminary result in one these patients suggests the acquisition of a HOX mutation, which may induce some self renewal mitosis in hematopoietic progenitors at the expense of differentiation mitosis and of derivation of colonies.

CD34+CD38- colonies genotyped from 3 hemochromatosis subjects did not reveal 46/1 homozygous clones. Likewise, in all PV patients studied, rs12343867 homozygous clones without the acquisition of JAK2<sup>V617F</sup> were not evidenced. This result suggests that the 46/1 *per se* does not favor the 9pLOH and mitotic recombination. These observations suggest that JAK2<sup>V617F</sup> might play a role in mitotic recombination. Consistent with this, Plo *et al.* have demonstrated that JAK2<sup>V617F</sup> induces genetic instability and stimulate HR. In contrast to these studies, HR is shown to antedate the acquisition of JAK2<sup>V617F</sup> in 2 PV patients reported recently<sup>417</sup>. However this seems to be rare. One interesting question will be whether this pre-JAK2 LOH is the reflection of a pre-leukemic clone for example, acquisition of TET2 before the appearance of JAK2<sup>V617F</sup>.

An excellent application of this modeling is to measure a drug's therapeutic potential on different JAK2<sup>V617F</sup> clones. We have applied these calculations on serial samples of peripheral granulocytes from PV patients treated with interferon- $\alpha$  (IFN- $\alpha$ ). Results show that IFN- $\alpha$  preferentially targeted JAK2<sup>V617F</sup> homozygous clones in IFN- $\alpha$  responding patients. Our results are in line with those presented by Hoffman from in-vitro studies<sup>367</sup>.

Preferential targeting of homozygous clone by IFN- $\alpha$  could be explained by the priming hypothesis either due to a higher signaling by JAK2<sup>V617F</sup> homozygous cells or to a higher dependence of the JAK2<sup>V617F</sup> homozygous clone to JAK2 signaling and thus leading to a greater sensitivity to the silencing of this pathway, both hypotheses having been developed above in discussion.

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## **LIST OF PUBLICATIONS**



## **Articles**

1. Hasan S, Cassinat B, Droin N, Le Couedic JP, Favale F, Monte-Mor B, Lacout C, Fontenay M, Dosquet C, Chomienne C, Solary E, Villeval JL, Casadevall N, Kiladjian JJ, Vainchenker W, Plo I. *Use of the 46/1 haplotype to model JAK2<sup>V617F</sup> clonal architecture in PV patients: clonal evolution and impact of IFN $\alpha$  treatment.* 2013, Leukemia.
2. Hasan S., Lacout C., Marty C., Cuignet M., Solary E., Vainchenker W., Villeval JL. *JAK2<sup>V617F</sup> expression in mice amplifies early hematopoietic cells and gives them a competitive advantage that is hampered by IFN $\alpha$ .* 2013, Blood.
3. Marty C., Lacout C., Martin A., Hasan S., Jacquot S., Birling M. C., Vainchenker W., Villeval JL. *Myeloproliferative neoplasm induced by constitutive expression of JAK2<sup>V617F</sup> in knock-in mice.* 2010, Blood.

## **Protocols**

1. *Isolation of human blood progenitor and stem cells from peripheral blood by magnetic beads.* Bio-Protocol, Salma Hasan and Isabelle Plo-Azevedo, 2012.
2. *Protein translation study – Label protein with Methionine in cells.* Bio-Protocol, Salma Hasan and Isabelle Plo-Azevedo, 2012.

**LIST OF COMMUNICATIONS**

1. American Society of Hematology (ASH) – Poster (2013)
  - Use of the 46/1 haplotype to model JAK2<sup>V617F</sup> clonal architecture in PV patients: clonal evolution and impact of IFN $\alpha$  treatment
2. Société Française d'Hématologie (SFH) – Poster (2013)
  - Modélisation de l'architecture clonale de JAK2<sup>V617F</sup> chez les patients atteints de PV grâce à l'haplotype 46/1: évolution des clones et impact du traitement par IFN $\alpha$
3. American Society of Hematology (ASH) – Poster (2012)
  - 46/1 haplotype permits to follow JAK2 homologous recombination: Modeling JAK2<sup>V617F</sup> clonal architecture in PV patients
4. Club Hématopoïèse et Oncogénèse (CHO) – Poster (2012)
  - 46/1 haplotype permits to follow JAK2 homologous recombination: Modeling JAK2<sup>V617F</sup> clonal architecture in PV patients
5. Société Française d'Hématologie (SFH) – Oral (2012)
  - a. JAK2<sup>V617F</sup> induit une amplification des cellules souches hématopoïétiques (CSH) entraînant la dominance clonale des Néoplasmes Myéloprolifératives (NMP); son blocage par l'interféron  $\alpha$  (IFN $\alpha$ )
  - b. Rôle de l'insuffisance en *IKZF1* dans les Néoplasmes Myéloprolifératives (NMP) développé dans un modèle de souris Knock-in (KI) pour JAK2<sup>V617F</sup>
6. American Society of Hematology (ASH) – Oral (2011)
  - JAK2<sup>V617F</sup> promotes stem cell amplification driving MPN clonal dominance in mice and IFN $\alpha$  prevents this effect
7. Club Hématopoïèse et Oncogénèse (CHO) – Poster (2011)
  - Development of a conditional JAK2<sup>V617F</sup> KI mouse model mimicking human myeloproliferative neoplasms (MPNs): role of *IKZF1* deficiency.



## RESUME

### English

This work concerns malignant myeloid hemopathies called classical BCR-ABL-negative Myeloproliferative Neoplasms (MPN) and include Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF). They result from the transformation of a multipotent hematopoietic stem cell (HSC) with hyperproliferation but no blockade of differentiation. The most common molecular defect is the acquired point mutation  $JAK2^{V617F}$  resulting into the activation of the cytokine receptor/JAK2 pathway. We have developed a mouse constitutive and a conditional  $JAK2^{V617F}$  knock-in (KI) mouse models. These animals developed a disease mimicking human PV evolving into secondary MF. They also displayed an age dependent increase in the total numbers of early hematopoietic cells (phenotype LK, LSK and SLAM: LSK/CD48-/CD150+). Using *In vivo* competitive repopulation assays we demonstrated that cells from KI origin outcompeted their WT counterparts and that a low number of  $JAK2^{V617F}$  KI SLAM cells propagates the disease. These results show that the sole  $JAK2^{V617F}$  mutation, without any additional mutations, is sufficient for disease phenotype and emergence. Using this KI mouse model, we tested the effect of interferon- $\alpha$  (IFN $\alpha$ ) treatment on MPN development. We found that IFN $\alpha$  treats the disease phenotype by blocking the propagation of early  $JAK2^{V617F}$  cells and eradicates disease-initiating cells, showing that IFN $\alpha$  could cure the disease in mice, as shown in some PV patients. Finally, we developed a new method combining the measurement of 46/1 SNPs and  $JAK2^{V617F}$  allele burdens in blood predicting the frequency of normal, heterozygous and homozygous  $JAK2^{V617F}$  clones in PV patients. This study suggested that IFN $\alpha$  preferentially targets the homozygous  $JAK2^{V617F}$  clone in PV patients suggesting a link between the levels of JAK2 signaling and the success of the IFN $\alpha$  response.

**KEYWORDS:** Myeloproliferative Neoplasms,  $JAK2^{V617F}$ , HSC, Interferon-  $\alpha$ , haplotype 46/1

### Français

Ce travail concerne des hémopathies myéloïdes malignes appelés Néoplasmes Myéoprolifératifs (NMP) qui incluent les Polyglobulies de Vaquez (PV), les Thrombocythémies Essentielles (TE) et les Myéofibroses Primaires (MFP). Ces maladies résultent de la transformation d'une cellule souche hématopoïétique (CSH) avec hyperprolifération mais sans blocage de différenciation. Leur défaut moléculaire le plus fréquent est la mutation  $JAK2^{V617F}$  résultant dans l'activation de la signalisation des récepteurs aux cytokines utilisant JAK2. Au cours de ce travail, nous avons développé un modèle murin « Knock-In » (KI) constitutif et conditionnel pour la mutation  $JAK2^{V617F}$ . Ces animaux développent une maladie mimant la PV humaine évoluant vers la MF secondaire. Ces animaux présentent augmentation en fonction de l'âge du nombre de cellules immatures (phénotypes Lin-, LSK et SLAM: LSK/CD48-/CD150+). Dans un système compétitifs *in vivo* nous montrons que les cellules KI ont un avantage prolifératif dès le stade CSH et qu'un faible nombre de CSH peuvent déclencher la maladie. Ces résultats suggèrent que la mutation  $JAK2^{V617F}$  seule est suffisante pour (1) le phénotype et (2) l'émergence de ces maladies. Nous avons aussi testé l'effet de l'interféron- $\alpha$  (IFN $\alpha$ ) sur le développement des NMP en utilisant ces souris  $JAK2^{V617F}$  KI. Nous montrons que l'IFN $\alpha$  traite le phénotype de la maladie en bloquant la propagation des cellules KI dès le stade immature avec éradication des cellules souches néoplasiques, entraînant comme chez certains patients PV une rémission hématologique et aussi moléculaire. Enfin, en combinant l'analyse quantitative de l'haplotype 46/1 et de la mutation  $JAK2^{V617F}$  sur les cellules sanguines nous développons une nouvelle méthode prédictive de la fréquence des clones hétérozygotes et homozygotes  $JAK2^{V617F}$  chez les patients PV. Cette étude suggère que l'IFN $\alpha$  cible préférentiellement le clone homozygote  $JAK2^{V617F}$  et que sa réponse est fonction de l'intensité de la signalisation JAK2.

**MOTS CLÉS :** Néoplasies Myéoprolifératifs,  $JAK2^{V617F}$ , CSH, Interféron-  $\alpha$ , haplotype 46/1