MASTERARBEIT | MASTER'S THESIS

Titel | Title

Characterizing the Role of STAT5 Proteins in JAK2 V617F - Positive Myeloproliferative Neoplasms

verfasst von | submitted by

Nikoleta Kavaja BSc

angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien | Vienna, 2024

Studienkennzahl lt. Studienblatt | Degree programme code as it appears on the student record sheet:	UA 066 865
Studienrichtung lt. Studienblatt | Degree programme as it appears on the student record sheet:	Masterstudium Molecular Biology
Betreut von | Supervisor:	Dr. Richard Moriggl

Acknowledgments

I would like to thank Univ.-Prof. Dipl.-Ing. Dr.rer.nat. Richard Moriggl for allowing me to be part of his research lab and for sharing his scientific knowledge and input during the project discussion sessions.

Expressing my gratitude to my fantastic group leader Assist. Prof. Dr. Heidi Neubauer for giving me the opportunity to work on this project. I'm truly thankful for your constant support and insightful feedback and for having a chance to learn from such a committed and passionate scientist.

I'm extremely grateful to my incredible, highly motivated supervisor Marie Ploderer who unselfishly shared her knowledge and skills with me throughout the whole project. Thank you for your patience and for the continuous guidance and support. Thank you for teaching me to work independently, to think scientifically and for encouraging me to continue in the moments when everything felt overwhelming. I could have not imagined a better and more understanding supervisor.

I would like to thank all the lab members for their help and support whenever I needed it. I am very happy that I got a chance to learn from all of you and to experience what it's like to be a part of the team where there is great collegiality and mutual respect. I really enjoyed my time spent in the lab.

I owe my family the deepest gratitude for their constant motivation and unconditional support in life and for always encouraging me to continue despite difficult challenges that life brought us. Without you by my side I could not have achieved any of this. Special thanks to my brother who taught me the most important things in life for unfortunately a short time we got to spend together.

A huge thank you goes to my dear friend Ana, who helped me countless times throughout this journey.

Table of Contents

1. Introduction

1.1. Myeloproliferative Neoplasms

Myeloproliferative neoplasms (MPNs) are clonal hematopoietic malignancies that develop due to the overproliferation of differentiated (mature) cells of the myeloid lineage, caused by oncogenic mutations occurring in hematopoietic stem cells (HSCs) in the bone marrow (BM) (Nangalia and R. Green, 2017). HSC are multipotent cells which through regulated processes of differentiation, self-renewal and maturation develop into all blood cell types. Throughout the process of myelopoiesis, HSC and multipotent progenitors (MPPs) showing myeloid lineage commitment properties mature into cells of myeloid lineage, such as monocteyes, erythrocytes, megakaryocytes, and granulocytes comprising neutrophils, eosinophils and basophils (Lee and Hong, 2019). MPNs comprise seven heterogeneous diseases: polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), chronic myelogenous leukemia (CML), chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia-not otherwise specified (CEL-NOS), and MPN-unclassifiable (MPN-U). The classical MPN subtypes are split into two subcategories: Philadelphia chromosome negative (Ph-), which comprises PV, ET, PMF, and the Philadelphia chromosome positive (Ph+) CML (Barbui *et al.,* 2018). The philadelphia chromosome is formed as a result of a genetic translocation of the *ABL1* (Abelson murine leukemia 1) gene from chromosome 9 to chromosome 22 where it fuses with the *BCR* (breakpoint cluster) gene. The result of this translocation is the aberrant production of the oncogenic BCR-ABL1 fusion protein. It is found in cells of the myeloid, erythroid and megakaryocytic lineage in the BM and its presence is indicative of CML, AML (acute myeloid leukemia), or ALL (acute lymphoblastic leukemia). This fusion protein activates several signaling pathways, including the JAK2-STAT5 pathway, leading to uninhibited cell proliferation and survival (Haider and Anwer, 2023).

1.1.1. Disease characteristics, manifestation and risks

The three classical Ph- MPN subtypes, although differing phenotypically, share disease symptoms such as fatigue, fever, night sweats, abdominal problems, weight loss, cytopenia,

bleeding, bone pain, and concentration issues (Geyer and Mesa, 2017). Within this group, PV is the most prevalent and it is characterized by erythrocytosis (an increase in red blood cells; RBCs), leukocytosis (an increase in white blood cells; WBCs), and thrombocytosis (increased platelet levels), or by a combination of these. On the other hand, both ET and PMF are characterized by an elevated megakaryocytic proliferation in the BM, followed by an elevated platelet count. Additionally, BM fibrosis and extramedullary hematopoiesis are distinguishing and specific characteristics for PMF (Duangnapasatit *et al.,* 2015). Splenomegaly, increased hematocrit and both arterial and venous thrombosis are also very common clinical symptoms of all three Ph-MPNs (Duangnapasatit *et al.,* 2015). Age and history of thrombotic disorders are considered the key factors influencing the probability of thrombosis incidence in MPN patients. Surprisingly, extreme thrombocytosis (platelets > 1000 x 10^9/ L) has been linked to an enhanced risk of bleeding, which could be due to an acquired von Willebrand syndrome (Tefferi and Barbui, 2020). MPN patients also have an increased risk of progression to secondary AML. PV and ET patients are both at risk of progression to PMF, while PV and PMF can progress to AML (Geyer and Mesa 2017).

1.1.2. Mutations in MPNs

1.1.2.1. Disease driving mutations

PV, ET and PMF arise from hematopoietic stem cells that acquire genetic aberrations. The most common driver mutations found in these diseases occur in the *JAK2, CALR* or *MPL* genes. These mutations induce cytokine-independent or cytokine-hypersensitive signaling and lead to a myeloproliferative phenotype by constitutive activation of downstream signaling pathways, including the JAK-STAT pathway, and a subsequent overproduction of myeloid cells (Shammo and Stein, 2016). The discovery of the phenotypic driver mutations was a huge progress in MPN research, which greatly helped and simplified the diagnostic process and made the distinguishment between MPN subtypes easier, but also influenced therapy development. The most prevalent of these driver mutations, the $JAK2^{V617F}$ mutation, can be found in all three classical MPNs. As it is the most relevant variant for our study, it will be further discussed in greater detail below. The two other frequently mutated genes, *CALR,* encoding an endoplasmic reticulum chaperone protein Calreticulin, and *MPL (Myeloproliferative Leukemia virus),* the gene

encoding the thrombopoietin receptor (TPOR), can be found mutated in ET and PMF. The most prevalent mutations in the *MPL* gene are W515L (Tryptophan to Leucine substitution), and W515K (Tryptophan to Lysine substitution). The phenotypic effects of these mutations in comparison to JAK2^{V617F} include decreased hemoglobin, increased platelet levels, higher serum EPO (erythropoietin) levels, endogenous megakaryocytic but not erythroid colony growth, and reduced BM erythroid and overall cellularity. The mutations are not linked with thrombosis, major hemorrhaging, or fibrotic transformation (Beer *et al.,* 2008). The MPN mutational profile determines the phenotypic differences in disease, as well as prognostic variations (Grabek *et al.,* 2020), (Shammo and Stein, 2016). The presence of a particular driver mutation alone, or the combination of a particular driver mutation with additional non-driver mutations, greatly influences the course of the disease and the overall survival. For example, ET patients with CALR mutations have better predictions for overall survival than JAK2^{V617F}-mutated patients. Also, better overall survival has been reported for MF patients with CALR mutations than for JAK2^{V617F} or MPL-mutated patients. The presence of 2 or more non-driver mutations greatly decreases overall survival and increases the risk of AML transformation (Shammo and Stein, 2016). Nevertheless, there are MPN cases where none of the so-called disease driver mutations are found and they are characterized as triple negative (TN) patients. Characterization of this group of MPN patients by whole and targeted exome sequencing revealed numerous alternative mutations. These infrequent somatic mutations are usually found in alternative exons of *JAK2* and *MPL*, as well as in epigenetic modifying genes and regulators of cytokine signaling. It is interesting that the majority of these mutations also lead to increased JAK2-STAT5 signaling, characteristic of MPN patients harboring the disease driver mutations in *JAK2, CALR*, and/or *MPL* genes (Stephen E. Langabeer, 2016).

1.1.2.2. The JAK2^{V617F} mutation

The discovery of the JAK2^{V617F} mutation in 2005 (Baxter *et al.,* 2005; James *et al.,* 2005; Jones *et al.,* 2005; Kralovics *et al.,* 2005) revolutionized the diagnosis of MPNs, and its presence is one of the main diagnostic criteria for these diseases. It can be found in ~95% of PV, and in around 50-60% of ET and PMF patients. Prior to the JAK2^{V617F} mutation discovery, Ph- MPNs were diagnosed by complete blood counts and by BM morphology analysis. The latter is still, together with the peripheral blood mutation screening for the JAK2^{V617F} mutation, considered the most

reliable diagnostic criteria for MPNs. In a small percentage of PV patients, which are negative for this mutation, usually an alternative mutation in the *JAK2* exon 12 is found. This mutation causes slightly different clinical manifestations, but it doesn't differentially influence the clinical outcome compared to JAK2^{V617F} mutated patients (Shammo and Stein, 2016).

The JAK2^{V617F} mutation is a gain of function mutation that is caused by a G to T nucleotide swap. It is located at nucleotide 1849 in exon 14 of the *JAK2* (*Janus Kinase 2*) gene, which is located on chromosome 9 in humans. It leads to an activating point mutation in the JH2 domain (jak homology domain 2) of the JAK2 protein. The structural domains of the JAK2 protein will be described in more detail in one of the following chapters. The substitution of Valine to Phenylalanine at codon 617 disrupts the inhibitory interaction of the JH1 domain thereby resulting in a constitutively active JAK2 protein. This leads to a constant firing of the JAK2-STAT5 signaling pathway as well as other pathways, followed by an uncontrolled differentiation and proliferation of the hematopoietic stem cells (HSCs) (James *et al.,* 2005). JAK2 mediates signaling through its downstream effectors, which are STATs (STAT1, STAT3, and STAT5), MAPK, and PI3K-AKT pathways. Increased STAT signaling leads to increases in proliferation and survival effectors, such as Bcl-xL (Grabek *et al*., 2020), (Shammo and Stein, 2016).

The study of Levine *et al*., 2005 demonstrated the constitutive activity of the JAK2^{V617F} tyrosine kinase and the increased proliferation and survival of HSCs harboring this mutation. Experiments performed using Ba/F3 cells engineered to express EPOR (erythropoietin receptor) in the presence of the JAK2^{V617F} mutation showed the cytokine-independent growth of the cells. EPO-hypersensitivity was observed too, which was not the case in JAK2 wildtype (wt) cells. In the same study, they used small molecule inhibitor against JAK2 protein, JAK inhibitor I Calbiochem to reduce JAK2 activating phosphorylation. This also led to decreases in phosphorylation and activation of its downstream effector STAT5, resulting in apoptotic cell death of human JAK2^{V617F}-positive HEL cells (Levine *et al.,* 2005).

1.1.2.3. Other somatic mutations in MPNs

Besides the three disease driving mutations, many other additionally acquired mutations are present in MPNs. These can affect tumor suppressors (*TP53)*, transcriptional regulators (*RUNX1)*, spliceosome complex components (*SRSF2, U2AF1, SF3B1*), DNA methylation

modifiers (*DNMT3A, TET2, IDH1 and IDH2)* and chromatin modifier genes (*ASXL1, EZH2).* These mutations can be acquired either before or after the acquisition of the driver mutation and can affect the course of the disease differently (Grabek *et al.,* 2020).

1.1.3. MPN treatment

MPNs are a heterogeneous group of diseases and the diversity of MPN patients and the differences in their genetic background influence the response to therapy, but also the choice of treatment option. The combination of disease driver mutations with other additional coexisting mutations affects the phenotype, development and severity of the disease (Grabek *et al.,* 2020) (Shammo and Stein, 2016). At present, molecular profiling is still not in use as a standard method to inform treatment decisions. Factors that are taken into consideration when choosing the therapeutic approach are the MPN subtype, the severity of symptoms and the risk category.

The first line treatments for PV include phlebotomy, to decrease hematocrit levels to <45%, and low-doses of aspirin once daily. The patients are split into either the low-risk (age <60 and no history of thrombosis) or the high-risk (age >60 and history of thrombosis) group. For low-risk patients, aspirin doses twice a day are advised, while patients belonging to the high-risk group are subjected to Hydroxyurea (HU) treatment, which is a cytoreductive drug used to control blood cell counts and for reducing the risk of thrombosis. In cases of HU intolerance or resistance, pegylated interferon alpha (IFNα) is advised for younger (<65 years) patients, and busulfan treatment is indicated for older patients. If there is no clinical response to these treatments, targeted therapy in the form of the JAK1/JAK2 inhibitor Ruxolitinib, is used. This can greatly improve the symptoms of the patients but does not represent a curative treatment (Tefferi and Barbui, 2020).

ET patients are categorized as either very low-risk (age <60, no history of thrombosis and *JAK2* unmutated), low-risk (age <60, no history of thrombosis and *JAK2* mutated), intermediate-risk (age >60, no history of thrombosis and *JAK2* unmutated) and high-risk (age >60 with *JAK2* mutation or history of thrombosis) patients. Aspirin remains the first line treatment for ET in the lower risk groups. Depending on the presence or absence of cardiovascular risk factors in the very low, low, and intermediate risk groups, the doses of aspirin are adjusted. The treatment of high-risk ET patients corresponds to that of high-risk PV patients, with HU as a first line choice,

followed by either IFNα, or busulfan in cases of HU-resistance or -intolerance. Ruxolitinib is recommend as the last treatment option (Tefferi and Barbui, 2020), (Brachet-Botineau *et al.,* 2020).

The beneficial role of IFNα in the treatment of Ph- MPN diseases has already been known for a while and IFNα is FDA approved for the treatment of hairy cell leukemia, AIDS-related Kaposi's sarcoma, and chronic myelogenous leukemia (CML) (How and Hobbs, 2020). Still, mechanisms underlying the IFN response in patients remain the topic of investigation. The study of Saleiro *et al.,* 2022, uncovered the pathway behind the IFN suppressive effects on MPN malignancies. The group has shown that PKCδ (protein kinase C delta), an upstream regulator of the ULK1-p38-MAPK cascade, is important for the IFN response in primary MPN cells. They also demonstrated that the levels of ULK1 and p38 MAPK define the clinical response to IFN treatment. Conversely, the same study showed that IFNα-induced caspase-dependent activation of ULK1-interacting ROCK1/2 in normal and malignant HSCs results in a negative feedback loop, thereby suppressing the IFN response (Saleiro *et al.,* 2022).

However, the only curative treatment for MPNs so far is allogeneic stem cell transplantation (ASCT), but it is only performed on patients suffering from PMF and secondary MF. Despite improvements in overall survival and disease-free survival, there is also evidence of excessive mortality in patients receiving ASCT (Grabek *et al.,* 2020).

Considering patients' resistance to currently available and non-curative therapies, there is a need for the development of novel targeted therapeutics, which should be supported by further investigation and understanding of the mechanisms behind the pathology of the MPN diseases.

1.2. The JAK-STAT pathway

The crosstalk between cells and the extracellular environment is essential for processes determining cell fate like proliferation, differentiation, and apoptosis. A number of extracellular signals, including cytokines, hormones and growth factors, elicit different signaling cascades inside the cell, thereby controlling gene expression and maintaining cellular homeostasis. Nevertheless, various mechanisms can lead to the deregulation of this signaling and subsequently to cancer. One signaling pathway crucial for hematopoiesis, among other things, is

the JAK-STAT pathway, a highly conserved pathway which, if deregulated, can cause diverse hematopoietic cancers (Fasouli and Katsantoni, 2021), (Figure 1).

1.2.1. JAK-STAT signaling

The JAK-STAT pathway consists of a cell membrane receptor, janus kinases (JAKs) and signal transducers and activators of transcription (STATs). The family of JAK proteins comprises JAK1, JAK2, JAK3 and TYK2 (Tyrosine kinase 2), while the family of STAT proteins includes 7 members: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 (O'Shea *et al.,* 2017).

The signaling starts by ligand binding to the cell surface receptor. The transmembrane receptors most commonly associated with JAK-STAT signaling are cytokine (interleukin-, interferon-, growth factor-receptors) and hormone (erythropoietin receptor, EPO-R, thrombopoietin receptor, TPO-R, involved in erythropoiesis and thrombopoiesis, respectively) receptors. The binding of the ligand to the receptor induces a dimerization of the receptor. This dimerization brings the receptor-associated JAKs in close proximity to each other and allows them to phosphorylate each other on critical tyrosine residues. This activates them and leads them to phosphorylate the cytoplasmic tail of the receptor, generating the docking sites for the downstream effectors within the pathway, the STATs. JAKs then also tyrosine phosphorylate and activate the STATs. Activated STATs then form parallel homo- or heterodimers and translocate to the nucleus, where they act as transcription factors and regulate genes responsible for HSC proliferation, differentiation, migration, and apoptosis (Bousoik and Montazeri Aliabadi, 2018), (Figure 1). JAK1, JAK3, and TYK2 are important for the development and function of the immune system, whereas JAK2 plays a key role in hematopoiesis (Xue *et al.,* 2023).

Due to its importance, the JAK-STAT pathway needs to be tightly regulated and both positive and negative regulators contribute to the maintenance of proper signaling. STAMs (signal transfusing adapter molecules) and SH2 domain-containing proteins belong to the co-activators of the pathway (Able *et al.,* 2017). There are three main groups of negative JAK-STAT pathway regulators. SOCS (suppressor of cytokine signaling) proteins are repressors of STAT signaling and their expression is also controlled by activated STAT dimers, thus forming a negative feedback loop. They inhibit the signaling by either binding the phosphorylated cytokine receptor and thereby preventing STAT recruitment, or by inhibiting JAK kinase activity through direct

interaction with the JAK or its receptor. Other negative regulators of STAT signaling are protein inhibitors of activated STATs (PIAS), which interact with STAT dimers directly and block their binding to DNA. They can also lead to protein ubiquitination and consequent proteasomal degradation. Protein tyrosine phosphates (PTPs) also block the signaling by dephosphorylating STATs or JAKs (Hu *et al.,* 2021).

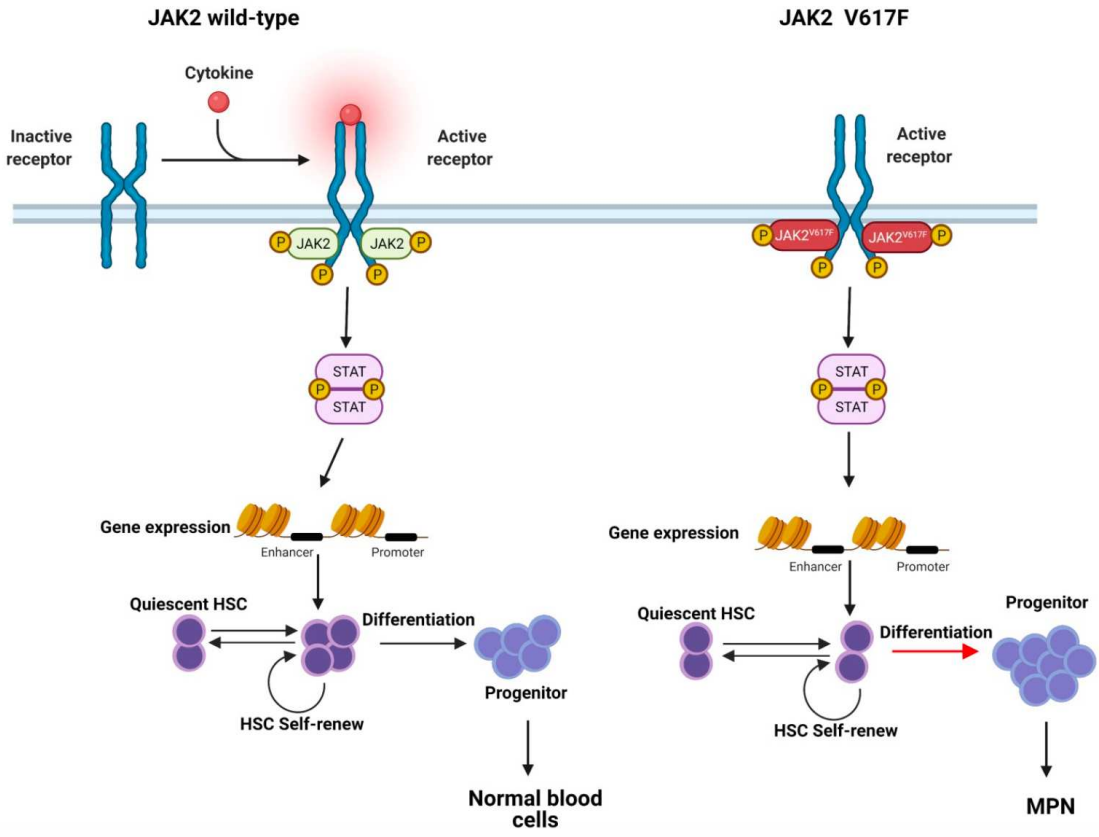


Figure 1. *JAK2-STAT5 signaling in normal (left) and malignant (right) hematopoiesis (Gou et al., 2022).*

1.2.2. The JAK family of proteins

JAKs are intracellular receptor-associated tyrosine kinases. Each member (JAK1, JAK2, JAK3, TYK2) of the JAK family of proteins consists of seven Jak homology (JH) domains as well as conserved tyrosine phosphorylation sites which are important for its activation. The JH1 domain

is a tyrosine kinase domain at the carboxyl terminus of the protein, and it is important for the protein kinase activity (Hu *et al.,* 2021). The JH2 was previously thought to be a pseudo kinase domain without catalytic activity. Nevertheless, it proves to be a Serine/Threonine kinase regulating the activity of the whole JAK protein by interacting with the JH1 domain and inhibiting its tyrosine phosphorylation (O'Shea *et al.,* 2017). The Src-homology 2 (SH2) domain is composed of JH3 and a part of the JH4 domain. The other part of the JH4 domain, together with JH5, JH6 and JH7 domains, make up the FERM (four-point-one, ezrin, radixin, moesin) domain at the N-terminus of the protein. The SH2 domain of the JAK protein recognizes phosphotyrosine motifs on cytokine receptors and interacts with another phosphorylated JAK protein. The FERM domain of the JAK protein is important for the interaction with the cytokine receptor itself (Hu *et al.,* 2021) (Figure 2).

The main member of the JAK family of interest for this study is the JAK2 protein. It is important for myeloid cell development by transducing signals from the three myeloid receptors, including EPOR (erythropoietin receptor), TPOR-MPL (thrombopoietin receptor MPL), and GM-CSFR (granulocyte colony stimulating factor receptor), to the inside of the cell (Kollmann *et al.,* 2019). JAK2 also becomes activated upon binding of IL-3 and IL-5 interleukins to the receptors IL-3R and IL-5R (Able *et al.,* 2017), (Wang and Bunting 2013).

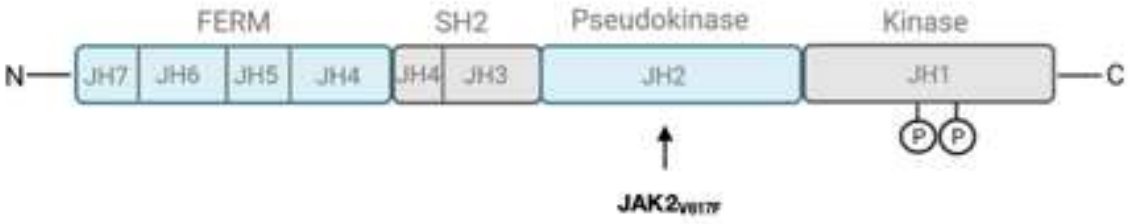


Figure 2. *The structural domains of the JAK2 protein with the activating tyrosine phosphorylation residues in the JH1 domain and the gain of function (GOF) JAK2^{V617F} mutation in the JH2 domain* (adapted from Hu *et al.,* 2021).

1.2.3. The STAT family of proteins

STATs are signal transducers and transcription factors in the JAK-STAT pathway. STAT activation via tyrosine phosphorylation results in the formation of parallel homo- or heterodimers through the interplay between their SH2 domains and phosphotyrosine motifs. Activated parallel dimers are imported in the nucleus to regulate target genes (Bousoik and Montazeri Aliabadi, 2018). All seven members of the STAT family (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6) have 6 conserved functional domains. The N-terminal domain is an oligomerization domain enabling tetramer formation, as well as the binding of STATs to other protein regulators. These can be co-factors, negative regulators of STAT proteins (protein inhibitors of activated STATs, PIAS), and receptors. The coiled-coil domain plays a role in the nuclear import/export. The following DNA binding domain binds DNA to facilitate transcription and is linked to the SH2 domain through the linker domain. The SH2 domain of STAT proteins is responsible for recruitment of STATs to tyrosine phosphorylated cytokine receptors and for STAT-STAT interactions (Xue *et al.,* 2023). The transactivation domain (TAD) at the C-terminal end interacts with other proteins involved in transcriptional regulation (Hennighausen and Robinson, 2008) (Figure 3). After exerting their function in the nucleus, STATs become dephosphorylated and exported back to the cytoplasm.

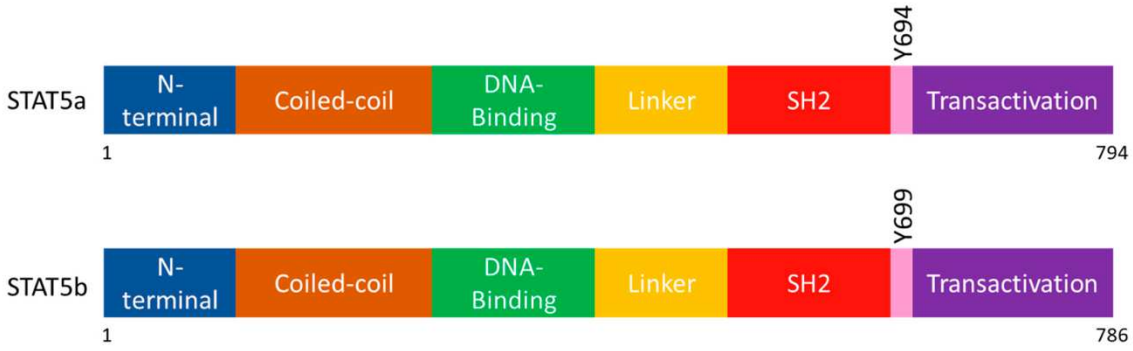


Figure 3. *The structural domains of the STAT5 proteins with the tyrosine phosphorylation residue within the TAD domain, important for the activation of the protein* (Hallim *et al.,* 2020).

1.2.4. STAT5A and STAT5B

One of the main downstream targets of JAK2 is STAT5. STAT5 encompasses two adjacent genes, *STAT5A* and *STAT5B.* Both genes are transcribed within the same locus also containing the *STAT3* gene, on chromosome #17 and #11 in humans and mice, respectively. The two gene products, STAT5A and STAT5B proteins, share more than 90% similarity at the amino acid level, with most differences present at the C-terminus of the proteins in the TAD. The C-terminus of STAT5A is 12 amino acids longer. The last 20 amino acids of STAT5A and the last 8 amino acids of STAT5B are unique to the specific protein. STAT5A/B can undergo post-translational modifications, like phosphorylation, acetylation, and sumoylation. Phosphorylation of the tyrosine residues 694 and 699 in the TAD domain activate STAT5A and STAT5B, respectively. Some additional phosphotyrosine or phosphoserine residues, as well as acetylation and sumoylation sites, are enabling either enhanced activation and nuclear transport, or are regulating the enhancement or inhibition of phosphorylation of the tyrosine motifs (Kollmann *et al.,* 2019).

1.2.5. The differential roles of STAT5A and STAT5B

STAT5A and STAT5B play both redundant and non-redundant roles in the regulation of growth and development, in the regulation of the immune system, cellular proliferation, differentiation and apoptosis, as well as in the regulation of tumor immunity (Hennighausen and Robinson, 2008). Knock-out studies in mice showed their differential but also common roles in regulation of STAT5 target genes. They are believed to have redundant roles, because of their overlapping genome binding sites (Basham *et al.,* 2008). STAT5A plays a crucial role in mammary tissue and the *Stat5a* gene was originally discovered as a mammary gland factor (MGF). Female mice lacking STAT5A showed impaired mammary gland development and impaired lactogenesis (Liu *et al.,* 1997). A STAT5B knock-out in mice on the other hand leads to growth defects and defects in male specific liver gene expression, highlighting the protein's role in sexual dimorphism of body growth rates (Udy *et al.,* 1997). The regulation of post-natal body growth is dependent on STAT5B-mediated GH (growth hormone) signaling. STAT5B has been shown to regulate *IGF-1* (insulin-like growth factor 1) target gene transcription in hepatocytes and muscles. STAT5B and GH receptor loss of function mutations lead to impairment of STAT5B function, which then leads

to IGF-1 deficiency and consequent growth retardation in humans. STAT5B's unique role in the regulation of body growth is confirmed by the incapability of STAT5A to compensate for the loss of this STAT5B function (Hennighausen and Robinson, 2008). The role of STAT5A/B in B- and NK-cell development has also been reported, since the development of B cells was arrested in the pre-pro-B cell stage of mice lacking *Stat5a/b*, while the absence of *Stat5b* alone, compared to the absence of *Stat5a*, led to an evident decrease in NK-cell numbers. Reduction in CD8+ T cell numbers was also observed in mice lacking STAT5A/B. The absence of STAT5B alone affected CD4+ T cell numbers more than the absence of STAT5A. These observations are in line with increased expression of STAT5B in CD4+ T cells and suggest STAT5B as the more important protein in NK and T cells (Kollmann *et al.,* 2019). These phenotypical differences induced by STAT5A or STAT5B deficiencies demonstrate their non-redundant roles.

Moreover, STAT5A and STAT5B control hematopoietic stem and mature cell lineage differentiation and survival, and therefore represent important regulators of normal and malignant hematopoiesis (Wang and Bunting, 2013). Hematopoiesis is the development of all blood cells from the HSCs in the BM and is a highly regulated process. JAK2-STAT5 signaling plays an important role in maintaining blood cell homeostasis. Different cytokines trigger JAK2-STAT5 pathway activation in different cell types. The signaling starts with JAK2 activation upon stimulation by EPO (erythropoietin), TPO (thrombopoietin), G-CSF, GM-CSF (granulocyte-, and granulocyte-macrophage colony stimulating factor), or different interleukins. Activated JAK2 in turn causes STAT5A/B tyrosine phosphorylation and parallel STAT5A/B dimer formation. These dimers travel to the nucleus where they bind to GAS (gamma-interferon activated sequence) motifs and lead to activation of transcription of STAT5 target genes. STAT5 deficiency can cause developmental issues within both lymphoid and myeloid cell lineages. A loss of STAT5 also impairs the LT (long-term) multi-lineage repopulation capacity of fetal liver and BM HSCs, indicating its role in promoting HSC quiescence and self-renewal (Hennighausen and Robinson, 2008), (Wang and Bunting, 2013).

1.2.6. The roles of STAT5A and STAT5B in cancer

Like healthy cells, cancer cells too depend on signaling pathways for their growth and survival. Deregulation of JAK2-STAT5 signaling is at the root of a variety of hematopoietic and solid cancers. Altered hematopoiesis due to GOF (gain of function) mutations occurring mostly in STAT5B variant, or in JAK2 itself, as well as copy number gains of *STAT5* gene can cause various hematopoietic malignancies (Kollmann *et al.,* 2019). MPNs, as cancers of the hematopoietic compartment, develop due to mutations arising at the onset of hematopoiesis, in the HSCs in the BM. STAT5A/B hyperactivity in MPNs is usually caused by constitutive activation via hyper-activated upstream tyrosine kinases, such as $JAK2^{V617F}$, FLT3-ITD, and BCR-ABL (Funakoshi-Tago *et al.,* 2009), (Choudhary *et al.,* 2007), (Schaller-Schoenitz *et al.,* 2014). STAT5 hyper-activation results in many invasive human cancers, and it is a strong leukemic driver prevailing in AML (acute myeloid leukemia), ALL (acute lymphoblastic leukemia), MPN, CML, B-ALL (B-cell acute lymphoblastic leukemia), and PTCL (peripheral T-cell lymphoma) (Kollmann *et al.,* 2019). STAT5A/B are proto-oncogenes which support carcinogenesis by activating the transcription of anti-apoptotic genes (*Mcl-1, Bcl-2, Bcl2l1, miR15/16, c-Myc*, and *D-type cyclins D1*, *D2* and *D3).* It has been found that STAT5B mutations occur more often in cancer than STAT5A mutations, suggesting STAT5B as the major STAT5 protein involved in oncogenesis. The GOF $STAT5B^{N642H}$ mutation occurring in the SH2 domain of the STAT5B protein has been found in patients of liquid cancers related to T-cell origin (De Araujo *et al.,* 2019). The mutation results in increased and longer tyrosine phosphorylation conferring a gain of function phenotype. The expression of the $STAT5B^{N642H}$ mutant at physiological levels induced a severe T-cell neoplasia in mice, which was not the case with a GOF STAT5A mutant expressed at similar levels. However, GOF mutant STAT5A also resulted in T-cell malignancy, when more highly over-expressed (Maurer *et al.,* 2019). Persistent STAT5A activity results in a multi-lineage leukemia, the expansion of HSCs and increased erythropoiesis (Moriggl *et al.,* 2005), (Schuringa *et al.,* 2004).

In addition to the canonical roles of tyrosine phosphorylated (pY)STAT5, uSTAT5 (unphosphorylated STAT5) has also been found to play a role in transcription (Kollmann *et al.,* 2019). Unphosphorylated STAT5 proteins associate with epigenetic and chromatin remodelers, thereby positively or negatively influencing target gene expression. In colon cancer, a uSTAT5A interaction with HP1α (heterochromatin protein 1α) stabilizes heterochromatin, which was found

to be tumor suppressive. However, this role has not been reported in the hematopoietic system (Kollmann *et al.,* 2019). Some studies also suggest a regulatory role of uSTAT5 in cell differentiation. A study from Szybinski *et al.,* 2019 demonstrates the specific role of uSTAT5B in blocking the differentiation of AML cells. It does so through an interaction with transcriptional regulator histone H3K4 demethylase KDM5C. These findings propose that targeting uSTAT5B, or its interacting partners, could be of interest for future AML therapies (Szybinski *et al.,* 2019).

The role of uSTAT5 has been previously shown in megakaryocyte differentiation too. uSTAT5 has been shown to bind and repress megakaryocyte differentiation genes. However, megakaryocytic differentiation occurs upon TPO stimulation, due to the loss of uSTAT5 and phosphorylation and activated of the protein. The study shows that tyrosine phosphorylation incapable form of STAT5B protein (Y699F STAT5B mutant) when expressed in HPC-7 cell line, a murine hematopoietic stem/progenitor cell line, confers for decreased megakaryocytic differentiation even after TPO stimulation (Park *et al.,* 2015).

Although, as stated above, STAT5A and STAT5B share a lot of similarities, their differential roles in human disease remain under investigation.

1.2.7. STAT1 and STAT3

The two other members of the STAT family of proteins extensively involved in cancer biology are STAT1 and STAT3. Deregulation of the signaling cascades mediated by these transcription factors can lead to a variety of diseases in humans, including cancer.

1.2.7.1. STAT1 and its role in cancer

STAT1 has been shown to be a tumor suppressor and it functions as a downstream effector of IFN signaling, which is important for the immune response against bacterial and especially viral infections. It controls cell growth by regulating the expression of cell-cycle related genes; STAT1 is capable of inhibiting cell growth by stimulating the expression of the Cyclin-dependent kinase inhibitors P21 and P27 or by inhibiting the expression of c-myc. It is involved in apoptosis induction by promoting the expression of some pro-apoptotic factors (apoptotic protein caspase 1, 3 and

11 precursors and Fas). It is also involved in MHC class I antigen presentation, thereby influencing the activation of the immune system (Hu *et al.,* 2021). The STAT1 protein has been identified as an important player in megakaryopoiesis downstream of GATA-1. *Gata-1* deficiency in mice leads to decreased STAT1 expression and defective megakaryocyte maturation, confirming its role especially in the polyploidization and maturation of megakaryocytes (Huang *et al.,* 2007).

However, mice lacking STAT1 were shown to be more susceptible to develop solid tumors, with the lack of STAT1 leading to faster tumor growth (Kovacic *et al.,* 2006). Nevertheless, an opposite, tumor-promoting role of STAT1 has been reported in hematopoietic malignancies. Its tumor promoting role has been shown for hematopoietic cancers, such as T-cell acute lymphoblastic leukemia (T-ALL) and ALK+ anaplastic large cell lymphoma (ALCL).

1.2.7.2. STAT3 and its role in cancer

STAT3 is a multifunctional protein, being involved in metabolism, autophagy, and cancer. The protein also exerts functions in innate immunity (regulating granulopoiesis, DC-dendritic cell development and function, anti-inflammatory signaling), and in adaptive immunity (B- and T-lymphocyte regulation) (Hillmer *et al.,* 2016). The absence of STAT3 in mice is lethal to the embryo, confirming its essential role in normal physiology (Bousoik and Montazeri Aliabadi, 2018). Posttranslational modifications of STAT3 can also influence its function by either increasing or decreasing its activity. CBP/p300-mediated STAT3 acetylation on lysine 685 in the C-terminal domain leads to enhanced DNA binding of STAT3, thereby positively influencing the transcription of target genes. On contrary, SET9-mediated methylation on lysine 49 of already phosphorylated and promoter-bound STAT3 leads to reduction of proteins's transcriptional activity (Orlova *et al.,* 2019). Moreover, STAT3 regulates the expression of mitochondrial genes and respiration in embryonic stem cells (ESC), thereby influencing their growth and maintenance (Hillmer *et al.,* 2016). Non-canonical activation of STAT3 by serine phosphorylation on 727 residue (pS727) is advantageous for a variety of tumors, leading to protein being integrated into the inner mitochondrial membrane. This is followed by oxidative phosphorylation and consequent increase in ATP production responsible for providing additional energy for cancer cell proliferation (Arévalo *et al.,* 2023), (Brachet-Botineau *et al.,* 2020).

In contrast to STAT1, STAT3 is known as a tumor promoter (Wang *et al.,* 2023). STAT3 is frequently mutated and constitutively activated in diverse cancers, both solid and hematological. It supports carcinogenesis by driving cancer cell proliferation, migration, survival, and immune evasion (Wang *et al.,* 2023). This is achieved by STAT3-mediated over expression of its target gene products which include anti-apoptotic proteins (Mcl-1, Bcl-2, Bcl-xL, Survivin) and factors favoring angiogenesis (VEGF). STAT3 is also capable of down-regulating the expression of pro- apoptotic proteins, such as p53, IFNβ, Fas, Fas ligand, and BAX. Persistent STAT3 activity has been reported in cancer cells, but also in cells residing in the tumor microenvironment (TME). The TME-residing cells, such as cancer-associated fibroblasts (CAFs), endothelial cells, smooth muscle cells, tumor infiltrating immune cells and cancer cells themselves suppress the immune system by stimulating pro-inflammatory cytokines, by leading to decreased antigen presentation and by disabling tumor cell killing by the immune system (Zou *et al.,* 2020). Hyperactivation of STAT3 is usually a consequence of GOF mutations, mostly in the SH2 domain of STAT3, or due to elevated signaling from GOF mutations in upstream oncogenic drivers (hyperactive mutated tyrosine kinases, such as $JAK2^{V617F}$ or FLT3-ITD, or fusion proteins, such as BCR-ABL, TEL- JAK2, or TEL-ABL1) (Orlova *et al.,* 2019).

There is some evidence of a regulatory role of uSTAT3 (unphosphorylated STAT3) in the transcription of many pro-oncogenic genes (Bousoik and Montazeri Aliabadi, 2018). uSTAT3 and pYSTAT3 dimers bind similar DNA regions. However, uSTAT3 cooperates together with the NF- κB transcriptional regulator to regulate transcription of a number of genes not regulated by pYSTAT3 (Hillmer *et al.,* 2016). There is also evidence of uSTAT3-mediated inhibition of cancer cell apoptosis. This effect can be reversed by inhibiting the N-domain of the protein. STAT3, as a transcription factor, directly regulates the transcription of its target genes by binding to their promoter regions. Furthermore, activated STAT3 controls gene expression also indirectly by recruiting chromatin remodelers which induce either eu-, or heterochromatin states, thereby either activating or repressing the expression of certain genes (Orlova *et al.,* 2019).

$JAK2^{V617F}$ -mediated signaling activates not only STAT5 but also STAT1 and STAT3 in hematopoietic stem cells. STAT1, STAT3, and STAT5 protein expression imbalance in MPNs with the $JAK2^{V617F}$ mutation results in different phenotypic outcomes. Namely, STAT1 overexpression causes ET-like disease, whereas its depletion causes a PV-like phenotype. The development of myelofibrosis is characterized by a pro-inflammatory and fibrotic

microenvironment and by the expansion of the MPN clone. Here, STAT3 plays a role for both of these processes, because pan-hematopoietic STAT3 deletion has been shown to disrupt the phenotype (Grabek *et al.,* 2020). Zhang *et al.,* 2023 have shown in their study that selective activation of STAT3 and STAT5 dictates the fate of myeloid progenitor cells. The authors report that STAT5 depletion or overexpression affects STAT3 phosphorylation differently. STAT5 overexpression induced decreased STAT3 phosphorylation, whereas STAT5 depletion led to increased phosphorylation and activation of STAT3 (Zhang *et al.,* 2023). All these previous findings are encouraging further investigation. Since STAT5 is the major downstream effector of mutated JAK2, but STAT1 and STAT3 have also been shown to be activated by the JAK2^{V617F} mutation, if and how the lack of STAT5A/B reflects on the levels and activity of STAT1 and STAT3 needs to be investigated.

1.3. Rationale of the master thesis

The three classical MPN subtypes (PV, ET, and PMF) can all be driven by the JAK2^{V617F} mutation which leads to persistent activation of the JAK2-STAT5 pathway. STAT5 comprises two different gene products, STAT5A and STAT5B, and it is not known whether they have differential roles in these diseases. Our lab is investigating the implication of this signaling pathway in MPNs and whether STAT5A and STAT5B contribute differently to these diseases, or whether one of the variants has a more prominent oncogenic role downstream of V617F-mutated JAK2.

STAT5B in a JAK2^{V617F} positive background

Previous work in the lab performed with human MPN cell lines engineered to over-express either human STAT5A or human STAT5B has shown that an increased expression of STAT5B, but not STAT5A, in the JAK2^{V617F} mutation positive setting causes an increase in cell proliferation (M. Ploderer, 2020). This implies a more dominant role of STAT5B downstream of V617F mutated JAK2 in human MPN cells. Another piece of evidence that points towards a more important role of STAT5B in a JAK2^{V617F} positive background is a study from Kollmann *et al.*, 2021. Here the authors showed that STAT5B, but not STAT5A, is present in the nucleus of mouse hematopoietic progenitor cell lines with the JAK2^{V617F} mutation (HPC-7 and HPC-LSKs), whereas both variants are present in the cytoplasm. Additional hints towards a more significant role of

STAT5B in MPNs is patient data from a Rampal *et al.* study, showing higher STAT5B target genes expression in granulocytes of JAK2^{V617F} positive MPN patients than in controls (Rampal *et al.,* 2014). Whether this increased STAT5B expression observed in MPN is a consequence of the JAK2^{V617F} mutation itself remains unclear.

1.3.1. Aim of the master thesis

Based on the data our lab collected which suggests that STAT5B is the more dominant oncogenic sibling downstream of JAK2^{V617F} in human MPN cells, in this study we aimed to get a better insight into the effects of both STAT5 variants in this context. This was achieved by conducting experiments on human MPN and mouse cell lines. The HEL human MPN cell line, previously generated from an erythroleukemia patient, possesses erythroid and monocytic markers as well as megakaryocytic markers of differentiation (Tabilio *et al.*, 1984). The cell line is homozygous for the JAK2^{V617F} mutation mimicking the polycythemia vera/erythroleukemia disease of MPNs, which makes it a very good model for investigation of the mechanisms underlying MPN diseases.

In this study, we:

- confirmed the absence of expression of either STAT5 variant in CRISPR/Cas9 targeted HEL cells harboring the JAK2^{V617F} mutation
- studied the effect of STAT5A or STAT5B knock-out on STAT1 and STAT3 protein expression and their respective activation levels
- analyzed the proliferation of HEL STAT5A/B knock-out cell lines to investigate the effects of these protein variants on MPN cell growth in a JAK2^{V617F} positive background
- studied the effect of STAT5A/B over-expression on the apoptosis rate of HEL cells induced with interferon-alpha
- analyzed the relative gene expression and protein expression levels of STAT5A and STAT5B and the level of activated, phosphorylated STAT5 in a murine hematopoietic/stem progenitor cell line (HPC-7) expressing the JAK2^{V617F} mutation

2. Materials and Methods

2.1. Materials

2.1.1. Reagents and buffers used for qPCR

RNA extraction

TRIzol™ LS Reagent (Thermo Fisher Scientific)

Chloroform (ROTH)

3M Sodium acetate (Thermo Fisher Scientific)

cDNA synthesis kit

RevertAid RT Kit (Thermo Fisher Scientific)

qPCR MasterMix

GoTaq qPCR MasterMix 2x (Promega)

qPCR primers

Table 1: List of primer sequences used for qPCR analysis

Name	Target	Species	Sequence	Origin
mSTAT5A_RT fw	mSTAT5A	mouse	TCCGCAGCACCA GGTAAA	Integrated DNA Technologies
mSTAT5A_RT rev	mSTAT5A	mouse	GGGATTATCCAA GTCAATAGCATC	Integrated DNA Technologies
mSTAT5B_RT fw	mSTAT5B	mouse	ACAACGGCAGCT CTCCAG	Integrated DNA Technologies
mSTAT5B_RT rev	mSTAT5B	mouse	TGGGCAAACTGA GCTTGGATC	Integrated DNA Technologies

mHPRT_RT fw	mHPRT	mouse	AGTGTTGGATAC AGGCCAGAC	Integrated DNA Technologies
mHPRT_RT rev	mHPRT	mouse	CGTGATTCAAAT CCCTGAAGT	Integrated DNA Technologies

2.1.2. Reagents and solutions for Western blot

IP-buffer with inhibitors (added freshly) for protein extraction

25 mM HEPES pH 7.5
25 mM Tris/HCl pH 7.5
150 mM NaCl
10 mM EDTA
0.1% Tween-20
0.5% NP-40
1 mM Na_3VPO_4
1 mM NaF
10 µg/mL Leupeptin
10 µg/mL Aprotinin
1 mM PMSF
1x cOmplete™ Protease Inhibitor Cocktail (Roche)

Loading buffer, 6x

1.6% SDS
20mM Tris, pH 6.8
16% glycerol
0.24 g/mL bromphenol blue
0.04 g/mL Dithiothreitol
in ddH_2O

Stacking gel 5%

5% acrylamide mix
130 mM Tris/HCl, pH 6.8
1% SDS
0.1% Ammonium persulfate (APS)
0.001% TEMED

Running gel 8%

8% acrylamide mix
390 mM Tris/HCl, pH 8.8
1% SDS
0.1% APS
0.06% TEMED
in ddH2O

10x Tris-Glycine buffer

0.25 M Tris
1.92 M Glycine

1x Running buffer

1:10 dilution of 10x Tris/Glycine buffer
0.5% SDS

1x Transfer buffer

1x Tris/Glycine buffer
20% Methanol

10x Tris buffered saline solution (TBS)

500 mM Tris, pH 7.9
1.5 M NaCl (Roth)

1x TBS-T

1:10 dilution of 10x TBS
0.1% Tween® – 20 (Sigma)

Blocking buffer

5% BSA (bovine serum albumine) (Albumine Fraction V Roth) diluted in TBS

Table 2: List of primary antibodies used for Western blot analysis

Antibody	Company	Cat. #	Dilution	Size in kDa	Host species
STAT5A (C-6) X	Santa Cruz Biotechnology	SC271542 X	1:5000	95	mouse
STAT5B (G-2)	Santa Cruz Biotechnology	SC-1656	1:5000	95	mouse
pYSTAT5 Y694	Cell Signaling Technology	#9351S	1:1000	90	rabbit
Actin (C-11)-R	Santa Cruz Biotechnology	SC-1615-R	1:5000	43	rabbit
STAT1	BD Transduction Laboratories	610115	1:1000	84/91	mouse
STAT3	BD Transduction Laboratories	610189	1:1000	92	mouse
pYSTAT1 (Tyr701) (D4A7)	Cell Signaling Technology	#7649S	1:1000	84/91	rabbit
pYSTAT3 (Tyr705)	Cell Signaling Technology	#9131S	1:1000	79/86	rabbit

β-Actin (C4)	Santa Cruz Biotechnology	SC-47778	1:5000	43	mouse

Table 3: List of secondary antibodies used for Western blot analysis

Antibody	Company	Target	Dilution	Host species
IRDye® 680RD Goat anti-Mouse IgG	LI-COR™	anti-mouse	1:10 000	goat
IRDye® 800RCW Goat anti-Rabbit IgG	LI-COR™	anti-rabbit	1:10 000	goat

2.1.3. Reagents and solution used with cell lines

Cultivation medium for HEL cells

Advanced RPMI 1640 Medium (Gibco™)

10% fetal bovine serum, FBS (Gibco™)

1% L-Glutamine (Gibco™)

1% Penicillin-Streptomycin Solution, 100x (Biowest)

Cultivation medium for HPC-7 cells

IMDM 1x (Gibco™)

10% fetal bovine serum, FBS (Gibco™)

1% penicillin-streptomycin solution (100x stock; Biowest)

5% SCF (stem cell factor) in media (kindly gifted by Kollmann group, Vetmeduni Vienna)

74.8 µM monothioglycerol (MTG; Sigma)

Phosphate buffered saline solution (PBS)

PBS (Gibco™)
pH 7.4

Freezing media

90% FBS (Gibco™)
10% DMSO (dimethyl sulfoxide)

MTS cell proliferation assay

PMS, MTS solutions prepared according to the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay protocol (Promega)

Cell apoptosis assay reagents

10 000 U/µl rhIFNalpha-2b (ImmunoTools) Cat#. 11343514
10x AnnexinV binding buffer diluted to 1x Binding buffer in distilled water (BD Biosciences) Cat#.556454
APC AnnexinV (BD Biosciences) Cat#. 550474
FITC AnnexinV (BioLegend) Cat#. 640906
0.5 mg/ml PI (Propidium Iodide) (BioLegend) Cat#. 421301
10 mM and 50 mM Bortezomib (MedChem Express) Cat#. HY-10227

2.2 Methods

2.2.1. Cultivation of human and mouse cell lines

The human (HEL) and mouse (HPC-7) cell lines were cryopreserved and stored at -80°C. For the purpose of the experiments, cells were thawed and washed with PBS (Gibco™) to remove trace amounts of DMSO, a toxic component of the freezing media. Cells were then cultivated at a concentration of 10^6 cells/ml or below in their respective medium (see Materials). Reagents and solutions used with cell lines are stated in 2.1.3. All cells were cultured at 37°C and 5% CO_2. Cells were regularly tested for mycoplasma contamination using a mycoplasma detection kit, MycoAlert™ (Lonza).

2.2.2. Harvesting of cells

Prior to RNA or protein extraction, cells were harvested by centrifugation at 300 x g for 5 min at room temperature. Afterwards, the media was aspirated, and cell pellets were washed twice in 1x cold PBS and centrifuged for 5 min, at 0.4 x g at 4°C after each washing step. Lastly, PBS was aspirated, and cell pellets were snap-frozen in liquid nitrogen and stored at -80°C until needed for analysis.

2.2.3. Freezing of cell lines

To maintain all cell lines in a cell bank, all cell lines were frozen at low passage and at a concentration of approximately $2x10^6$ cells/ml of freezing media (90% FBS + 10% DMSO). Before freezing, the cells were counted by mixing an aliquot of cell suspension with sterile filtered trypan blue solution in a ratio 1:1. 10 µl of the mixture was applied in the Neubauer chamber to count cells visually using an Axiovert 35 Phase Contrast Microscope (Zeiss).

2.2.4. RNA extraction from cell lines

RNA isolation from cell pellets was performed by applying 0.75 ml of TRIzol™ LS Reagent (Thermo Fisher Scientific) to 0.25 ml of the samples (approximately 5-10x10^6 cells/sample). Homogenization was achieved by pipetting up and down to lyse cells, followed by 5 min incubation at room temperature. Afterwards, 150 µl chloroform was added (for 1 ml TRIzol, 200 µl chloroform). Samples were shaken thoroughly for 15 sec and incubated for 2-3 minutes at room temperature. Samples were then centrifuged at 12 000 x g 15 min at 4°C, which resulted in three phases. The RNA in the upper, aqueous phase was carefully taken out and transferred to a fresh tube. Per 1 ml TRIzol used for homogenization, 500 µl 100% isopropanol was added to the aqueous phase (for 0.75 ml TRIzol, 375 µl 100% isopropanol was added). Samples were shaken vigorously and incubated 10 min at room temperature, followed by a centrifugation step at 12 000 x g for 15 min at 4°C. Supernatants were removed and pellets were washed with 1ml 75% ethanol per 1 ml TRIZol. A second washing step was performed. Afterwards, ethanol was removed as much as possible without losing the RNA pellets. Pellets were dried at room temperature and resuspended in 20-50 µl nuclease free water (depending on the size of the pellet) and RNA concentrations of samples were measured with Tecani-control infinite 200 Pro by Nanodrop™. If required, RNA was precipitated with ethanol to increase the purity. Ethanol precipitation was performed by adding 0.1 volumes 3M Sodium acetate and 2.5-3 volumes ice-cold 100% ethanol to the RNA and precipitation was performed overnight at -80°C. The next day precipitated RNA was centrifuged at 16 000 x g, for 30 min, at 4°C. Pellets were washed twice with 0.5 ml ice-cold 75% ethanol, and centrifuged for 10 min, at 4°C each time. Ethanol was removed and pellets were air-dried. Pellets were resuspended in the appropriate volume of nuclease free water and the concentration of RNA was again measured with Tecani-control infinite 200 Pro by Nanodrop™. RNA samples were stored at -80°C until further use.

2.2.5. Protein extraction from cell lines

Protein was isolated in IP-buffer containing freshly added inhibitors, as stated in 2.1.2. Depending on the size of the cell pellet, 50-200 µl IP-buffer with inhibitors was added to the pellets, and samples were rotated for 30 min at 4°C, followed by a subsequent centrifugation for 20 min, at 4°C and 16 000 x g. The supernatants containing the soluble protein were transferred into fresh tubes and protein lysates were snap-frozen in liquid nitrogen and stored at -80°C.

2.2.6. Bradford assay

Concentration of isolated protein was measured by performing a Bradford assay. For setting up the standard curve, BSA (bovine serum albumin) solution was used. BSA solution of a starting concentration of 1 µg/ml was added to 1 ml of 1:5 diluted Bradford reagent (Protein Assay Dye Reagent Concentrate, Bio-Rad), and a dilution series of 5 standards was made with standard concentrations increasing in a range from 1 µg/ml to 16 µg/ml. 1 µl of IP-buffer with inhibitors was added to 1 ml of each standard dilution and to the blank. 1 µl of the protein lysate was added to 1 ml of diluted Bradford reagent. A BioPhotometer plus (Eppendorf) was used to measure the absorbance at a wavelength of 595 nm. A linear equation of the standard curve was used to determine the protein concentration.

2.2.7. Western blot analysis

20 µg of protein was loaded per well. After adding the loading buffer, samples were boiled at 95°C for 5 min, spun down shortly and loaded onto an 8% polyacrylamide gel. A protein ladder (PageRuler™, Thermo Scientific) was loaded as a protein size standard. The electrophoresis was run in 1x running buffer at 70 V for 10 min, with an increase in voltage to 120 V for another hour. For blotting the proteins on a nitrocellulose membrane, a transfer sandwich was built by assembling the polyacrylamide gel with the nitrocellulose membrane with 2 Whatman® papers and fiber pads on top and bottom of the sandwich, previously soaked in 1x transfer buffer. Transfer of the proteins to the membrane was performed in 1x transfer buffer with a magnetic stirrer for 1 h, at 4°C and 340 mA. Subsequently, the membrane was blocked for 1 h in blocking buffer, on a shaker at room temperature, to prevent unspecific antibody binding. Appropriate dilutions of the appropriate primary antibodies were added to the blocking buffer and the membrane and incubated for 1 h on a shaker, at room temperature. Then three washing steps in TBS-T, 5 min each, were performed. Appropriate dilutions of the appropriate secondary antibodies were then applied on the membrane for another hour of incubation under the same conditions. Lastly, 3 washing steps with TBS-T, each 5 min were done before detection of the membrane with the Odyssey® Imaging System. All antibodies, solutions and reagents used for Western blot are stated in 2.1.2.

2.2.8. qPCR analysis

For synthesizing cDNA, 1 µg of previously extracted RNA was used together with the First strand cDNA Synthesis Kit (Thermo Scientific) as described in the manufacturer's protocol. cDNA was diluted 1:5 in nuclease-free water and 2 µl of diluted sample was pipetted together with 8 µl of a qPCR master mix (see Table 5) in a 96-well plate. The CFX96 Touch Real-Time PCR Detection System was used to perform the analysis using the appropriate qPCR program (Table 6). *HPRT* (Hypoxanthine-guanine-phosphoribosyl-transferase) housekeeping gene, was used to normalize Ct values of each sample to the Ct value of this reference gene using the "ΔΔCt-Method".

Table 5: Reaction mix for qPCR analysis

Reagent	Amount for 1 reaction [µl]
GoTaq qPCR MasterMix 2x	5
fw primer 10 µM	0.4
rev primer 10 µM	0.4
nuclease-free water	2.2

Table 6: qPCR program

Temperature [°C]	Time [s]
95	120
95	15 (40 cycles)
60	60 (40 cycles)
Melting curve: 65°C – 95°C, 5 min	

2.2.9. MTS - cell proliferation assay

MTS cell proliferation assays were performed each week in the four weeks directly after HEL STAT5A/STAT5B knock-out cell lines had been thawed. Cells were seeded at a concentration of

60 000 cells/ml or as stated and grown in RPMI culture media containing either 1% FBS or 10% FBS. Cells were seeded in triplicates (100 µl per well) into five 96-well plates and the plates were cultured under standard conditions. MTS and PMS reagents were prepared according to the manufacturer's protocol (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay, Promega). After seeding the cells, 20 µl of MTS-PMS reagent was added to each well of one 96-well plate (day 0), followed by plate incubation at 37°C for 1h and subsequent absorbance measurement at a wavelength of 490 nm. Since metabolically active cells reduce tetrazolium salt MTS to formazan, which is soluble in cell culture medium, the measured absorbance at 490 nm corresponds to the number of metabolically active and thereby viable cells. The monitoring was done every 24 h over 5 days (time points: 0 h, 24 h, 48 h, 72 h, 96 h). Data was analyzed with GraphPad Prism, and two-way ANOVA with Bonferroni correction was used for statistical analysis.

2.2.10. Flow cytometry - cell proliferation assay

Seeding of HEL cells with STAT5A or STAT5B knock-out was done in triplicates in T25 cell culturing flasks. Prior to cell number quantification using a CytoFLEX (Flow Cytometer, BECKMAN COULTER), cells were shortly resuspended and 500 µl of the cell suspension was taken out each day for the measurement. Approximately 20 µl of the cell suspension was counted by flow cytometry. Statistical analysis was done with GraphPad Prism using two-way ANOVA with Bonferroni comparison.

2.2.11. Automated cell counting - cell proliferation assay

Assessing cell proliferation with an automated cell counter was done by seeding each cell line (HEL STAT5A or HEL STAT5B KOs) in triplicates. Every day, prior to counting, cells were resuspended and 500 µl of the cell suspension was taken out and of this a 10 µl aliquot was mixed in a 1:1 ratio with sterile trypan blue solution for live/dead staining. 10 µl of this mixture was applied onto BioRad slides and counting was performed with a TC20 Automated Cell Counter, BioRad. Statistical analysis was done with GraphPad Prism. Two-way ANOVA with Bonferroni comparison was performed for statistical analysis.

2.2.12. Cell apoptosis assay

Cell lines were seeded in triplicates in a 6-well plate (total volume of 15 ml for triplicates, each well 5 ml) at a concentration of 60 000 cells/ml and were cultured with three different conditions (untreated, 48 h rhIFNalpha-2b and 96 h rhIFNalpha-2b). 96 h after seeding of cells (day 4), cells were harvested, washed, resuspended in 1x binding buffer, and stained with APC-AnnexinV to identify apoptosis undergoing cells, FITC AnnexinV to identify GFP expression and low-level stable over-expression of the respective variant and with PI staining to detect late apoptotic and necrotic cells for flow cytometry analysis. Cell viability and proliferation were quantified. Compensation controls for flow cytometry were included, namely, unstained cells, cells stained with Annexin V only, or PI only, and compensation for the FITC channel (GPF positive cells). 24 h before staining and flow cytometric analysis, the positive control was set up. For this, HEL parental cells were treated with 10 mM or 50 mM Bortezomib and then analyzed with all other samples. All reagents and solutions used for the interferon-induced apoptosis assay are listed in 2.1.3.

2.2.13. Statistics

One-way, or two-way analysis of variance (ANOVA), together with Bonferroni multiple comparison tests, and t-test (unpaired) were applied for statistical analysis of the results, using the GraphPad Prism 5 software.

3. Results

3.1. The effect of STAT5A or STAT5B knock-out on the proliferation rate of a JAK2^{V617F} positive human MPN cell line

We were interested in investigating whether STAT5A and STAT5B contribute differently to MPN disease and if one is more dominant in signaling downstream of JAK2^{V617F} than the other. Our lab has shown previously that an increased expression of STAT5B leads to a proliferative advantage in two human MPN cell lines (HEL and SET-2) positive for the JAK2^{V617F} mutation (M. Ploderer, 2020). Increased expression of STAT5A did not have this effect. We thus wanted to see if the absence of either STAT5 variant would affect the proliferation of HEL cells. For this we used CRISPR engineered cell lines that were previously generated in the lab, which have either a STAT5A or STAT5B knock-out or knock-down (L. Schreiberhuber, 2021, unpublished).

3.1.1. Confirming the absence of STAT5A or STAT5B protein in HEL knock-out cell lines by immunoblotting

To confirm the expected protein levels or lack thereof, we checked the status of our knock-out cell lines prior to the proliferation assay experiments. The protein levels of the two STAT5 variants was determined via Western blot. For each STAT5 variant, three guide RNAs had been previously designed, which induced either a complete knock-out (gRNAs STAT5A_1, STAT5A_3, STAT5B_1), or a knock-down (gRNAs STAT5A_2, STAT5B_2, STAT5B_3) of the respective variant. The EV (empty vector) control cell line was transduced with an empty vector expressessing Cas9 only and does not show any changes in STAT5A or STAT5B levels. The expected pattern of knock-out/-down was confirmed by immunoblotting (Figure 4). The knock-out/-down for each line is specific to either STAT5A or STAT5B and does not affect the level of the other STAT5 variant in the same cell line, as seen by similar protein levels as the control cell line. Hence, we were able to confirm the expected levels of STAT5A and STAT5B in each of the cell lines (Figure 4).

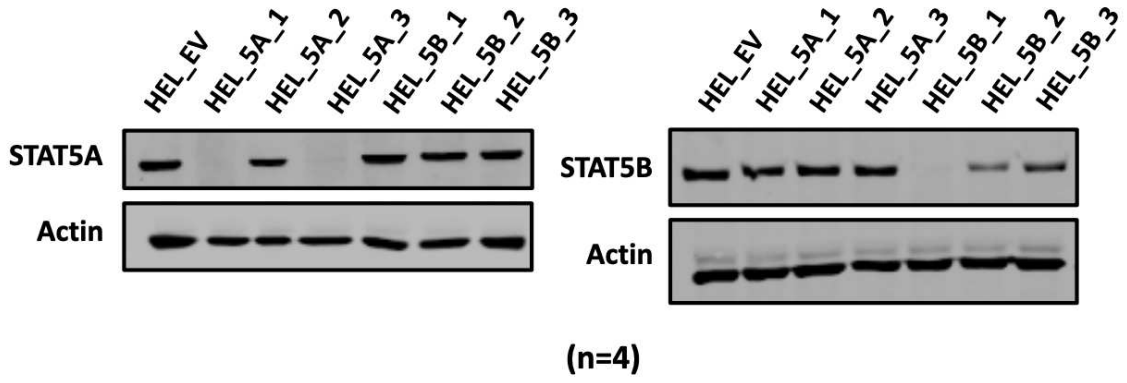


Figure 4. *STAT5A and STAT5B protein levels in CRISPR/Cas9 targeted HEL cells determined via Western blot analysis.* The expected pattern of STAT5A and STAT5B knock-out/-down in HEL cells upon knock-out/-down of STAT5A or STAT5B was observed. Actin was used as loading control. Data shown is representative of four independent experiments (n=4).

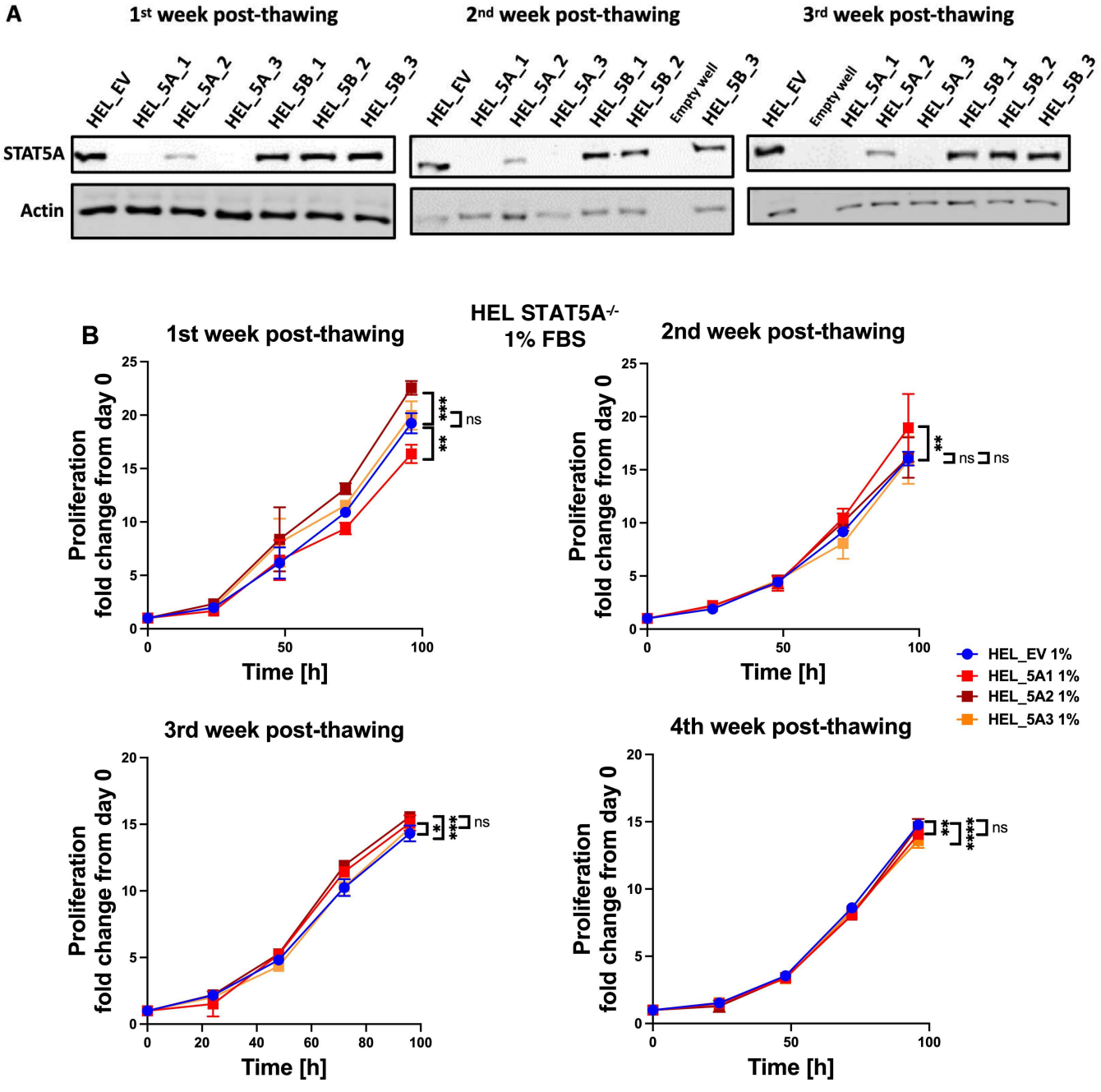
3.1.2. Proliferation of HEL cells upon knock-out of either STAT5A or STAT5B

Since previous data obtained by M. Ploderer with HEL cells engineered to over-express either STAT5A or STAT5B variant showed that an increased expression of STAT5B increases proliferation, we wanted to assess whether decreased or a lack of expression would have the opposite effect. We thus decided to perform an MTS cell proliferation assay on our HEL STAT5A and STAT5B knock-out cell lines, as was done for the over-expressor lines. The MTS proliferation experiments were repeated four times overall. We cultured cells under standard (media with 10% FBS) and low-serum (media with 1% FBS) conditions. Since intracellular signaling pathways could be affected by low-serum cell culture, we wanted to see how this stress condition might affect the proliferation of our cells that lack or have decreased expression of STAT5A/B. Additionally, for the first three MTS assay experiments, side-by-side Western blot analysis was performed to ensure that the cells retained their knock-out or knock-down status (Figure 5A, 6A).

3.1.2.1. Proliferation of HEL cells upon knock-out of STAT5A

In the low-serum culture condition (1% FBS), cells that lack or have decreased STAT5A expression showed very inconsistent proliferation rate trends. The complete STAT5A knock-out (gRNA_STAT5A_1) induced decreased proliferation of the cells in the first and in the fourth MTS assay, but an increased proliferation in the second and in the third MTS assay, compared to the empty vector cells. The other gRNA that also knocked-out STAT5A (gRNA_STAT5A_3), induced increased proliferation of the cells in the first and in the third assay, while in the other two assays (second and fourth MTS assays), the proliferation rate of the cells remained unchanged compared to the control. Cells transduced with the gRNA_STAT5A_2, which led to a knock-down of the variant, show a decrease in proliferation only in the last experiment, while in all previous repeats their proliferation was unaffected (Figure 5B).

Cells cultured in standard condition media (10% FBS) also showed diverse proliferation rate trends. The lack of STAT5A induced by the gRNA_STAT5A_1 resulted in a decreased proliferation in the first and in the second MTS assay, in an increased proliferation in the third assay, whereas no change in proliferation rate was observed in the fourth assay, when compared to the empty vector control cells. On the other hand, cells transduced with the other knock-out guide RNA (gRNA_STAT5A_3) showed an increase in proliferation twice (first and third MTS assays), a decrease in proliferation once (second MTS assay), and no change in the proliferation rate in the fourth experiment. Cells transduced with the knock-down gRNA_STAT5A_2 showed no change in cell proliferation in two out of four assays (first and fourth MTS assays), while in the second assay, the proliferation rate was decreased and in the third assay, it was increased, in comparison to the empty vector cell line (Figure 5C). This rather variable data and inconsistent proliferation rate trends of knock-out/-down cell lines across the experiments did not allow for a conclusion on the effect that reduced/absent STAT5A has on cell proliferation in a $JAK2^{V617F}$ positive background. We hypothesized that these inconsistencies in the proliferation rate of cells in both standard and low-serum culture conditions might be due to technical issues during the experimentation or to the assay method not being appropriate for this experimental setup.


A
1st week post-thawing
2nd week post-thawing
3rd week post-thawing
HEL_EV
HEL_5A_1
HEL_5A_2
HEL_5A_3
HEL_5B_1
HEL_5B_2
HEL_5B_3
Empty well
STAT5A
Actin
B
HEL STAT5A-/-
1% FBS
1st week post-thawing
2nd week post-thawing
3rd week post-thawing
4th week post-thawing
Proliferation
fold change from day 0
Time [h]
ns
HEL_EV 1%
HEL_5A1 1%
HEL_5A2 1%
HEL_5A3 1%

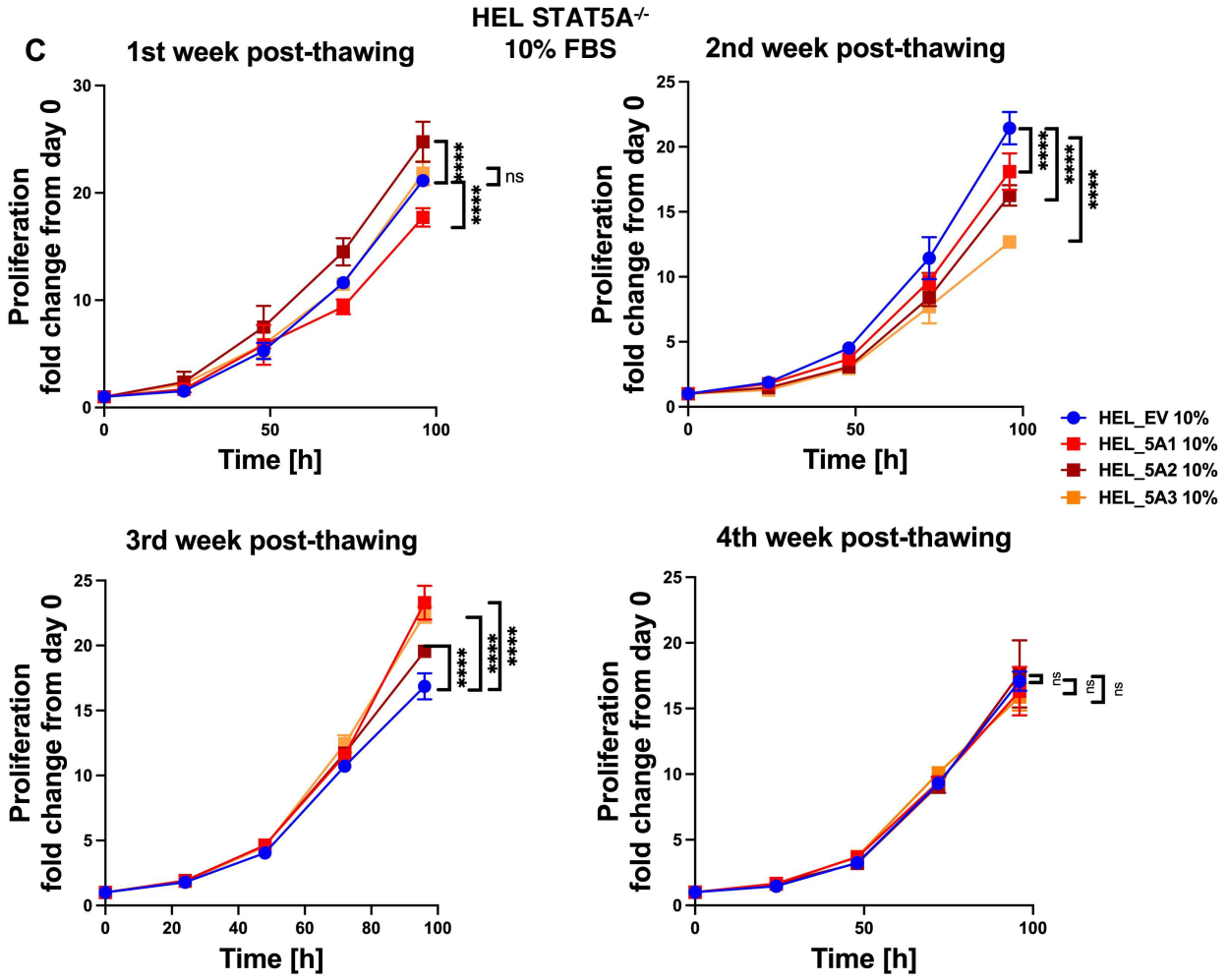
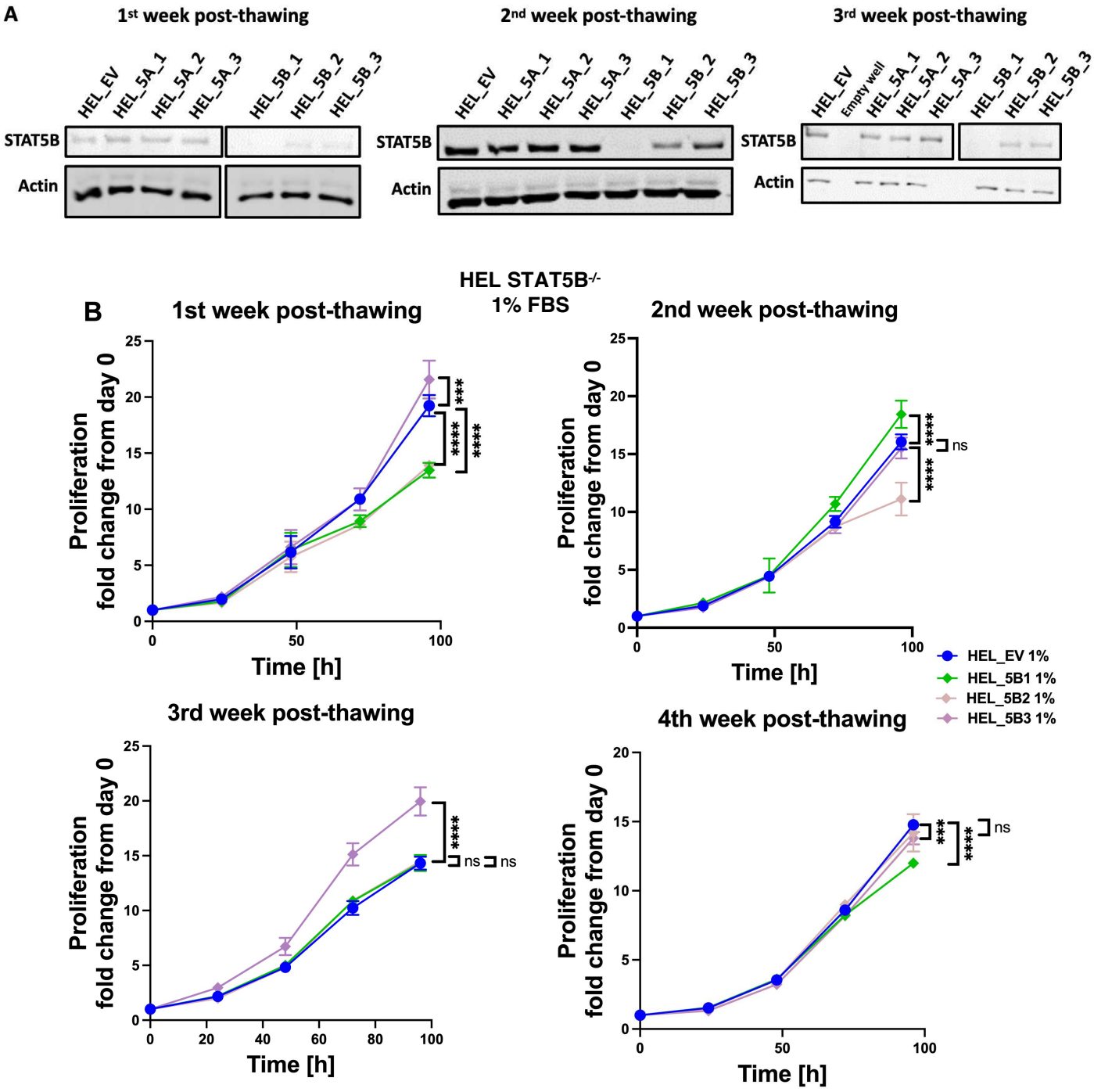


Figure 5. *Western blot analysis and MTS proliferation assays of HEL cells targeted with CRISPR/Cas9 against STAT5A.* **(A)** STAT5A protein levels in HEL cells upon knock-out/-down of STAT5A determined via Western blot. The Western blots were performed using cells after the indicated amount of culture post-thawing. Actin was used as loading control. **(B)** Proliferation of HEL cells upon knock-out/-down of STAT5A in a culture media with 1% FBS. **(C)** Proliferation of the same cell lines in a culture media with 10% FBS. Graphs are showing the growth curves of each cell line within 96 h of culture determined via MTS assay. Fold change was calculated relative to the basal absorbance level from day 0. The MTS experiments were performed in parallel to the Western blot analyses. Error bars indicate the standard error of the mean. For statistical analysis a two-way ANOVA with Bonferroni comparisons was performed. The graphs show p-values for comparisons with EV. ns = p-value > 0.05 ** = p-value < 0.01 *** = p-value < 0.001 **** = p-value < 0.0001.

3.1.2.2. Proliferation of HEL cells upon knock-out of STAT5B

STAT5B knock-out cells (gRNA_STAT5B_1) cultured in media supplemented with 1% FBS showed decreased proliferation in two out of the four experiments (first and fourth MTS assays). An increase in cell proliferation was observed in the second MTS assay, while in the third experiment, no difference in cell proliferation rates could be seen between STAT5B knock-outs and the empty vector control. The STAT5B knock-down cells transduced with the gRNA_STAT5B_2 showed a decrease in cell proliferation in the first and in the second assay, whereas in the third and in the fourth repeats of the experiment, the growth curves of the cells didn't significantly differ from the empty vector control. The other knock-down gRNA_STAT5B_3 led to an increase in proliferation in the first and in the third assay, no change in the proliferation in the second assay, and a decrease in the proliferation in the last assay, when compared to the control (Figure 6B).

In growth media supplemented with 10% FBS, a decrease in proliferation of the gRNA_STAT5B_1 transduced knock-out cells was observed in the MTS assays performed in the first and in the second week after thawing of fresh cells. This trend was lost in the third and in the fourth week. However, the gRNA_STAT5B_2 induced knock-down of the variant led to a decrease in cell proliferation in the first, second and fourth MTS assays, but to an increase in cell proliferation in the third assay. The other STAT5B knock-down by gRNA_STAT5B_3 decreased cell proliferation in the first experiment, but it increased proliferation in the two experiments that followed (second and third MTS assays), whereas in the last repeat it didn't induce a significant change in the proliferation rates of cells, when compared to the control cell line (Figure 6C).


A
1st week post-thawing
2nd week post-thawing
3rd week post-thawing
HEL_EV
HEL_5A_1
HEL_5A_2
HEL_5A_3
HEL_5B_1
HEL_5B_2
HEL_5B_3
Empty well
STAT5B
Actin
B
HEL STAT5B-/-
1% FBS
1st week post-thawing
2nd week post-thawing
3rd week post-thawing
4th week post-thawing
Proliferation
fold change from day 0
Time [h]
***
****
ns
HEL_EV 1%
HEL_5B1 1%
HEL_5B2 1%
HEL_5B3 1%

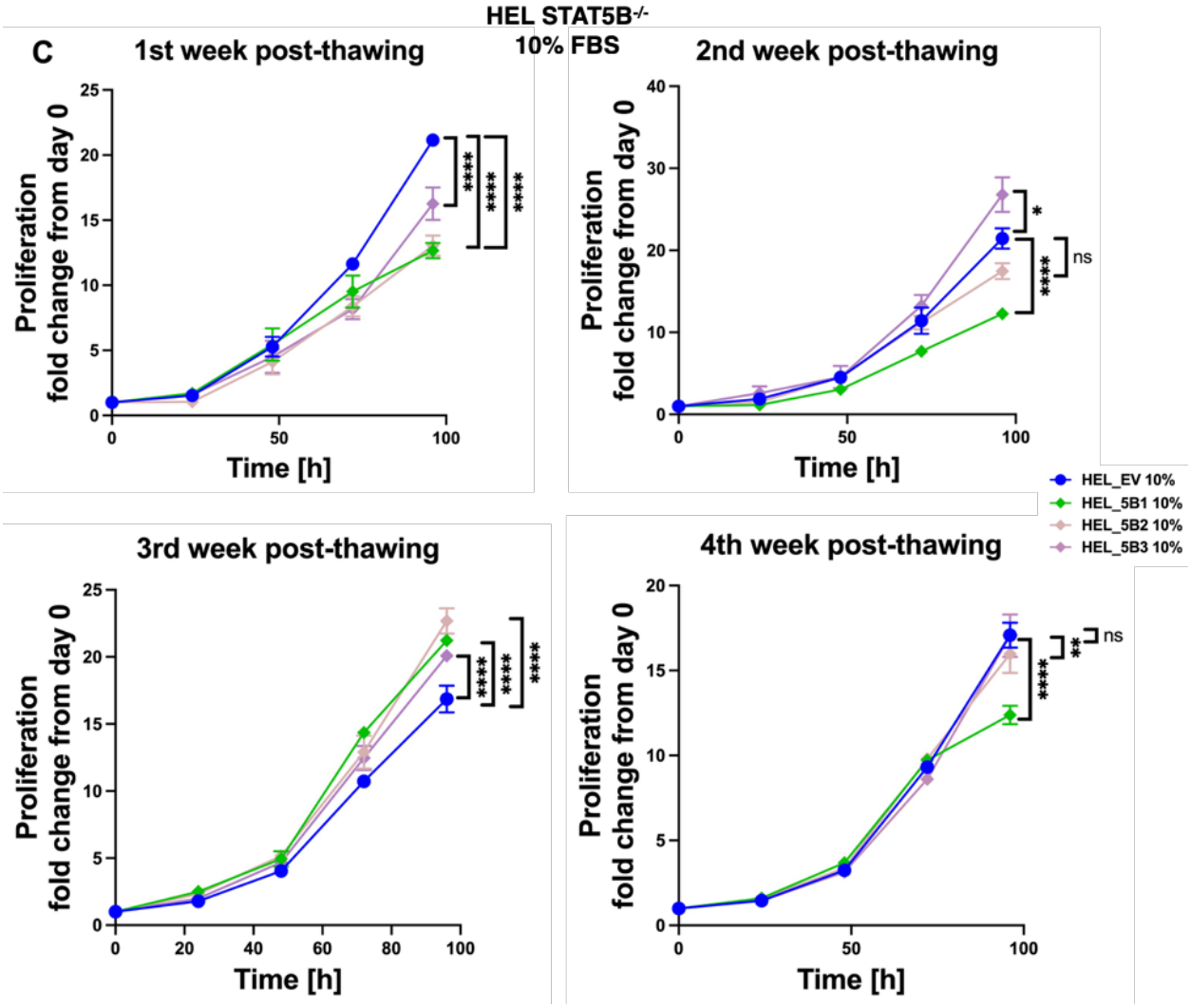

HEL STAT5B-/-
10% FBS
C
1st week post-thawing
2nd week post-thawing
3rd week post-thawing
4th week post-thawing
Proliferation
fold change from day 0
Time [h]
HEL_EV 10%
HEL_5B1 10%
HEL_5B2 10%
HEL_5B3 10%
****
*
ns
**

Figure 6. *Western blot analysis and MTS proliferation assays of HEL cells targeted with CRISPR/Cas9 against STAT5B.* **(A)** STAT5B protein levels in HEL cells upon knock-out/-down of STAT5B determined via Western blot. The Western blots were performed using cells after the indicated amount of culture post-thawing. Actin was used as loading control. **(B)** Proliferation of HEL cells upon knock-out/-down of STAT5B in a culture media with 1% FBS. **(C)** Proliferation of the same cell lines in a culture media with 10% FBS. The graphs are showing the growth curves of each cell line within 96 h of culture determined via MTS assay. Fold change was calculated relative to the basal absorbance level from day 0. The MTS experiments were performed in parallel to the Western blot analyses. Error bars indicate the standard error of the mean. For statistical analysis a two-way ANOVA with Bonferroni comparisons was performed. The graphs show p-values for comparisons with EV. ns = p-value > 0.05 * = p-value < 0.05 ** = p-value < 0.01 *** = p-value < 0.001 **** = p-value < 0.0001.

We thus concluded that knocking-out STAT5B in HEL cells resulted most frequently in decreased cell proliferation when cells were grown in media with 10% FBS, but this effect did not persist over time and was lost already in the third week after the cells had been thawed (Figure 6C). The reason behind this might be a compensatory mechanism, which allowed the cells to adapt to the loss of STAT5B after being in culture for a prolonged period of time. The absence of STAT5A affected the proliferation differently in each of the repeats of the MTS assay monitored and thus it is difficult to draw conclusions from these data (Figure 5B, C). Since MTS assay reagents can be affected by the presence of FBS in the media (Huang *et al.,* 2004), the assay can display variability between experiments due to the low number of cells seeded and small area for growth in 96-well plates, and to ensure that no technical error or problem was the cause of the inconsistent results, we wanted to validate our results by employing alternative ways of assessing proliferation. For this, we next focused on optimization of these methods.

3.1.2.3. Validation and optimization of the proliferation assay

To test whether the variable results from the MTS assays were due to technical issues (experimental or methodological) or were accurate and inherent to the cell lines, we aimed to perform two other methods of monitoring cell proliferation. We included counting with an automated cell counter and the quantification of cell numbers via flow cytometry, in addition to again measuring via MTS assay. Thus, cells were freshly thawed, cultured as previously

described, and their proliferation rate was determined using all three methods side-by-side in the first week directly prior to thawing.

3.1.2.3.1. Validation of the proliferation assay using HEL cells with knock-out of either STAT5A or STAT5B

A comparison of MTS, flow cytometry and automated cell counter methods for measuring proliferation rates of low-serum cultured cells (media with 1% FBS) showed that all STAT5A knock-out/-down cell lines displayed increased proliferation when analyzed with flow cytometry and an automated cell counter, whereas all cell lines showed decreased proliferation by MTS assay, compared to control cells (Figure 7A). When monitoring cell proliferation under standard conditions (media with 10% FBS), all three methods showed increased cell proliferation for STAT5A knock-out/-down cell lines compared to the control (Figure 7B).

On the other hand, all three methods showed decreased proliferation of cells lacking STAT5B (gRNA_STAT5B_1) and grown under low-serum culture conditions (media with 1% FBS), although when analyzed with the automated cell counter the observed decrease in proliferation was not statistically significant when compared to the empty vector cell line, due to high technical variance (Figure 7C). The other two STAT5B knock-down (gRNA_STAT5B_2, gRNA_STAT5B_3) cell lines showed decreased proliferation in the MTS assay, whereas an increase in proliferation rate of these cells was observed via flow cytometry and automated cell counting (Figure 7C). Surprisingly, the proliferation of cells cultivated under standard conditions (media with 10% FBS) monitored via MTS assay showed that even in the first week post-thawing, a lack of STAT5B resulted in increased proliferation (Figure 7D), which doesn't correlate with our previous MTS data of decreased proliferation in the absence of STAT5B in the first and in the second week post-thawing (Figure 6C). Flow cytometry analysis showed decreased proliferation of cells lacking STAT5B (gRNA_STAT5B_1), whereas automated cell counter analysis shows no significant deviation in the proliferation rate in comparison to the empty vector control (Figure 7D). Both STAT5B knock-down cell lines (guides RNA_STAT5B_2, RNA_STAT5B_3) displayed enhanced proliferation in the MTS assay and flow cytometry analysis, while showing unaffected proliferation when analyzed with the automated cell counter (Figure 7D).

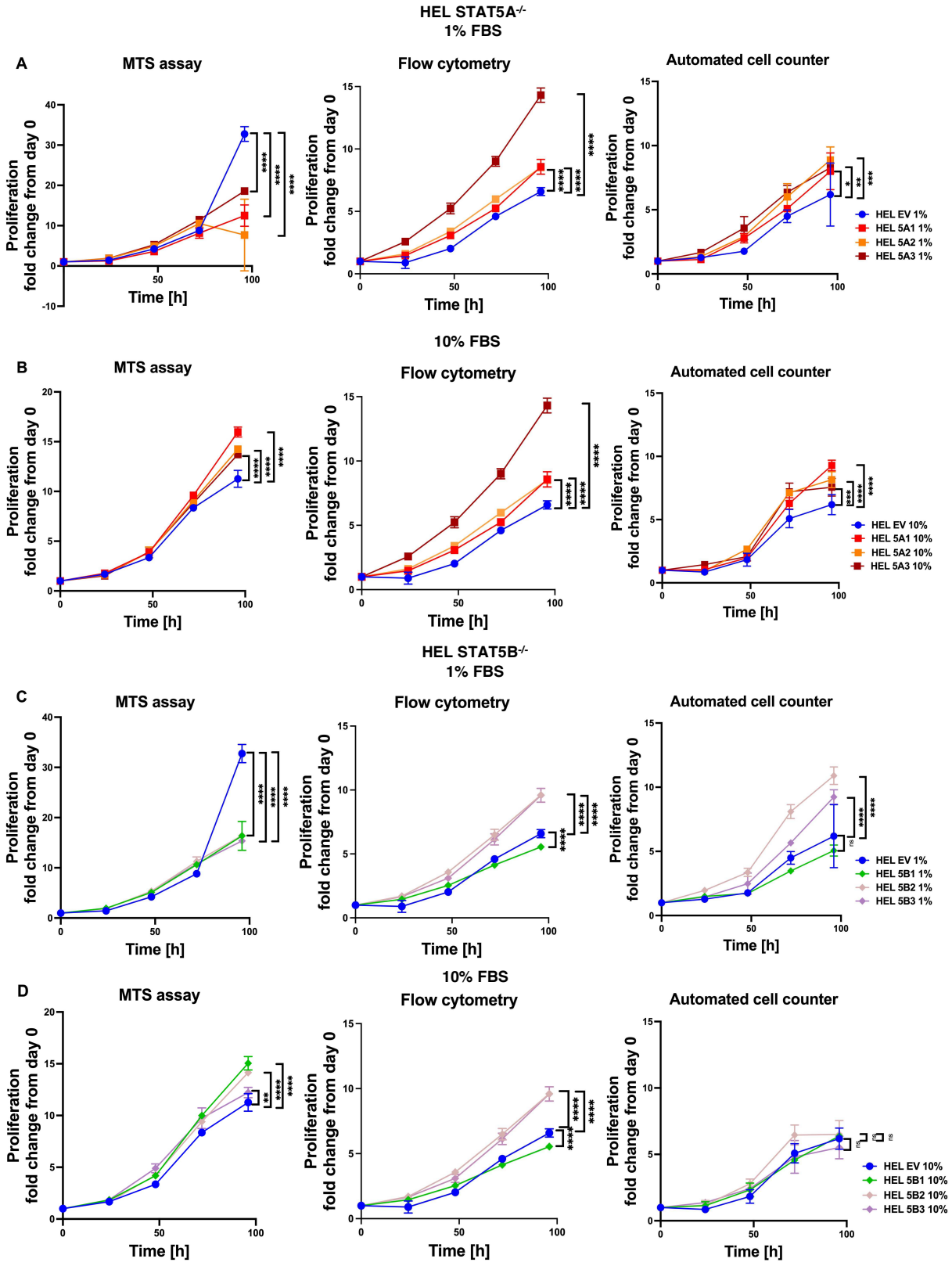

HEL STAT5A-/-
1% FBS
A
MTS assay
Flow cytometry
Automated cell counter
Proliferation
fold change from day 0
Time [h]
HEL EV 1%
HEL 5A1 1%
HEL 5A2 1%
HEL 5A3 1%
10% FBS
B
HEL EV 10%
HEL 5A1 10%
HEL 5A2 10%
HEL 5A3 10%
HEL STAT5B-/-
1% FBS
C
HEL EV 1%
HEL 5B1 1%
HEL 5B2 1%
HEL 5B3 1%
10% FBS
D
HEL EV 10%
HEL 5B1 10%
HEL 5B2 10%
HEL 5B3 10%

Figure 7. *Proliferation rate of CRISPR/Cas9 targeted HEL cells determined via MTS assay, an automated cell counter and cell number quantification via flow cytometry.* **(A)** Proliferation of HEL cells upon knock-out/-down of STAT5A in culture media with 1% FBS. **(B)** Proliferation of the same cell lines in culture media with 10% FBS. **(C)** Proliferation of HEL cells upon knock-out/-down of STAT5B in culture media with 1% FBS. **(D)** Proliferation of the same cell lines in culture media with 10% FBS. The growth curves of cell lines within 96 h are shown. Fold change was calculated relative to the basal cell counts/absorbance from day 0. Error bars indicate the standard error of the mean. For statistical analysis a two-way ANOVA with Bonferroni comparisons was performed. The graphs show p-values for comparisons with EV. ns = p-value > 0.05 * = p-value < 0.05 ** = p-value < 0.01 *** = p-value < 0.001 **** = p-value < 0.0001.

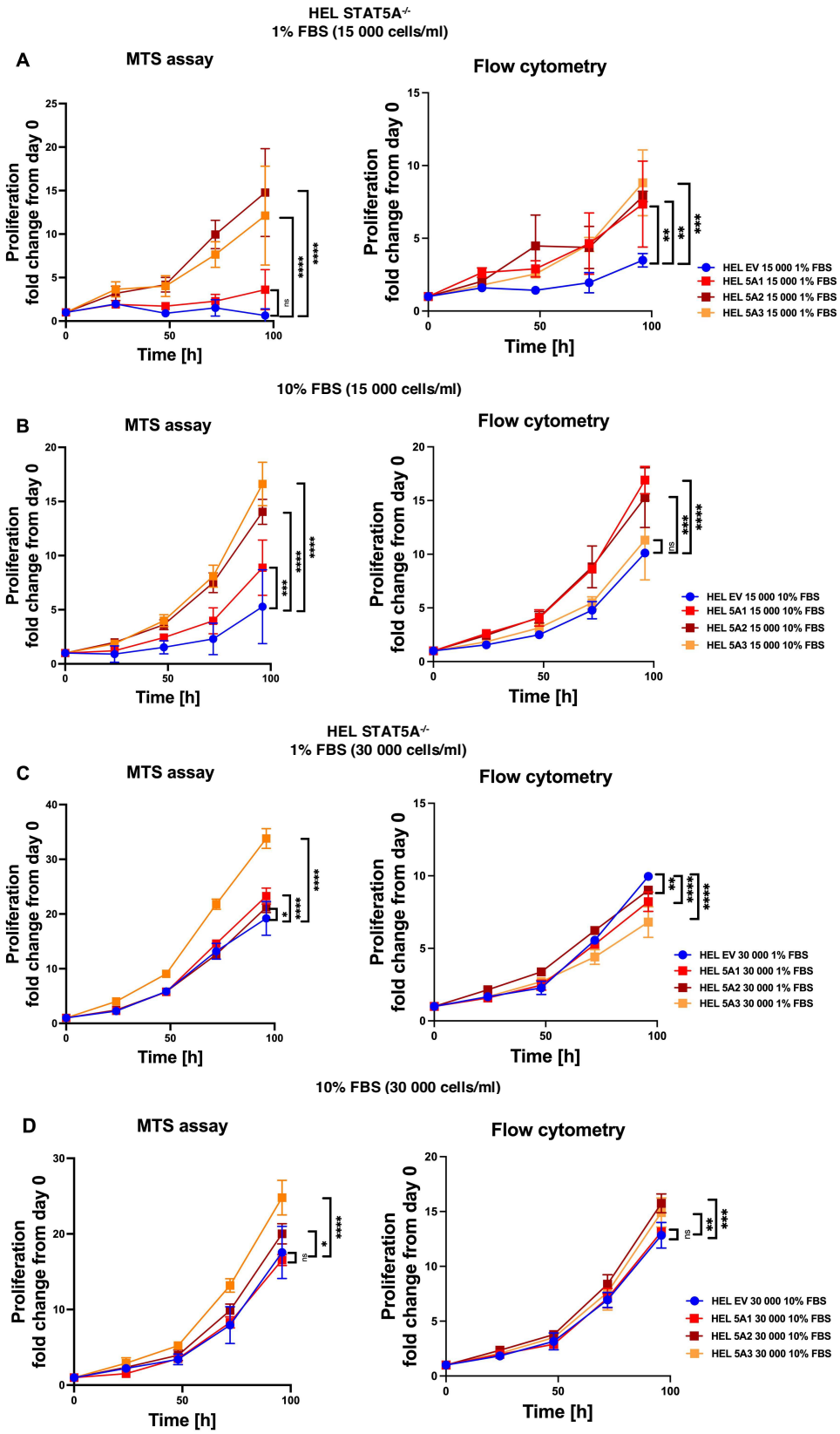
Overall, our data show that variability among results is maintained despite employing different methods for proliferation quantification. It is difficult to conclude which method is the most suitable and reliable, since in some cases the same cell line showed different proliferation rates when analyzed by a different method, as is the case with cells with STAT5B knock-out induced by the guide RNA_STAT5B_1 (Figure 7D). Although, comparing the methods so far reveals that results obtained via MTS and flow cytometry are often similar to each other for both low-serum culture and standard cell culture conditions (Figure 7). This implies that the MTS assay method is likely to be reliable for measuring cell proliferation in our cells and that the variable data obtained in Figures 5 and 6 is unlikely to be due to the choice of method.

3.1.2.3.2. Validation of the proliferation assay using HEL cells with knock-out of either STAT5 variant with different seeding concentrations

In our next attempt to reveal reliable differences between STAT5A and STAT5B in MPN cell proliferation, we excluded the automated cell counter, since among the three methods employed it yielded the highest variability between technical replicates. However, we continued with both MTS assay and flow cytometric analysis for the comparison of proliferation rates and decided to try different seeding concentrations of cells in culture media containing either 1% FBS or 10% FBS, as used before. In previous experiments, we seeded cells at 60 000 cells/ml, but we wanted to see whether stressing the cells even more by reducing cell numbers from the beginning of the experiment would further enhance any effects caused by the reduction/loss of either STAT5 protein and would therefore provide more robust results. Seeding concentrations of

15 000 cells/ml and 30 000 cells/ml were then tested to determine whether the proliferation rates might be influenced by cell numbers in culture, and whether lower seeding concentrations would help reduce the variability of the data.

The proliferation rates of STAT5A or STAT5B knock-out/-down cells gained via MTS or flow cytometry did not differ much between the assays, when comparing cells transduced with the same guide. We saw that the complete loss of STAT5A or its decreased expression (all STAT5A knock-out/-down cell lines) and the complete loss of STAT5B (gRNA_STAT5B_1) led to a significantly decreased proliferation only when cell proliferation rates were analyzed via flow cytometry and only when cells were grown in low-serum media with 1% FBS and with a seeding concentration of 30 000 cells/ml (Figure 8C, G). In all other cases, both assays showed either increased or unchanged proliferation of all STAT5A/B knock-out/-down cells when compared to the empty vector control, regardless of the culture conditions (Figure 8). Overall, enhanced proliferation of the cells was observed more often than unchanged proliferation, when comparing different culture conditions (1% vs 10% FBS; and seeding concentrations of 15 000 cells/ml vs 30 000 cells/ml) and the two methods (MTS assay vs flow cytometry) (Figure 8). The empty vector control cell line was the slowest to grow and the combination of low-serum culture media together with lower seeding concentration of cells negatively affected the proliferation strongly (Figure 8), indicating that this condition is not suitable.


HEL STAT5A-/-
1% FBS (15 000 cells/ml)
A
MTS assay
Flow cytometry
Proliferation
fold change from day 0
Time [h]
HEL EV 15 000 1% FBS
HEL 5A1 15 000 1% FBS
HEL 5A2 15 000 1% FBS
HEL 5A3 15 000 1% FBS
10% FBS (15 000 cells/ml)
B
MTS assay
Flow cytometry
HEL EV 15 000 10% FBS
HEL 5A1 15 000 10% FBS
HEL 5A2 15 000 10% FBS
HEL 5A3 15 000 10% FBS
HEL STAT5A-/-
1% FBS (30 000 cells/ml)
C
MTS assay
Flow cytometry
HEL EV 30 000 1% FBS
HEL 5A1 30 000 1% FBS
HEL 5A2 30 000 1% FBS
HEL 5A3 30 000 1% FBS
10% FBS (30 000 cells/ml)
D
MTS assay
Flow cytometry
HEL EV 30 000 10% FBS
HEL 5A1 30 000 10% FBS
HEL 5A2 30 000 10% FBS
HEL 5A3 30 000 10% FBS

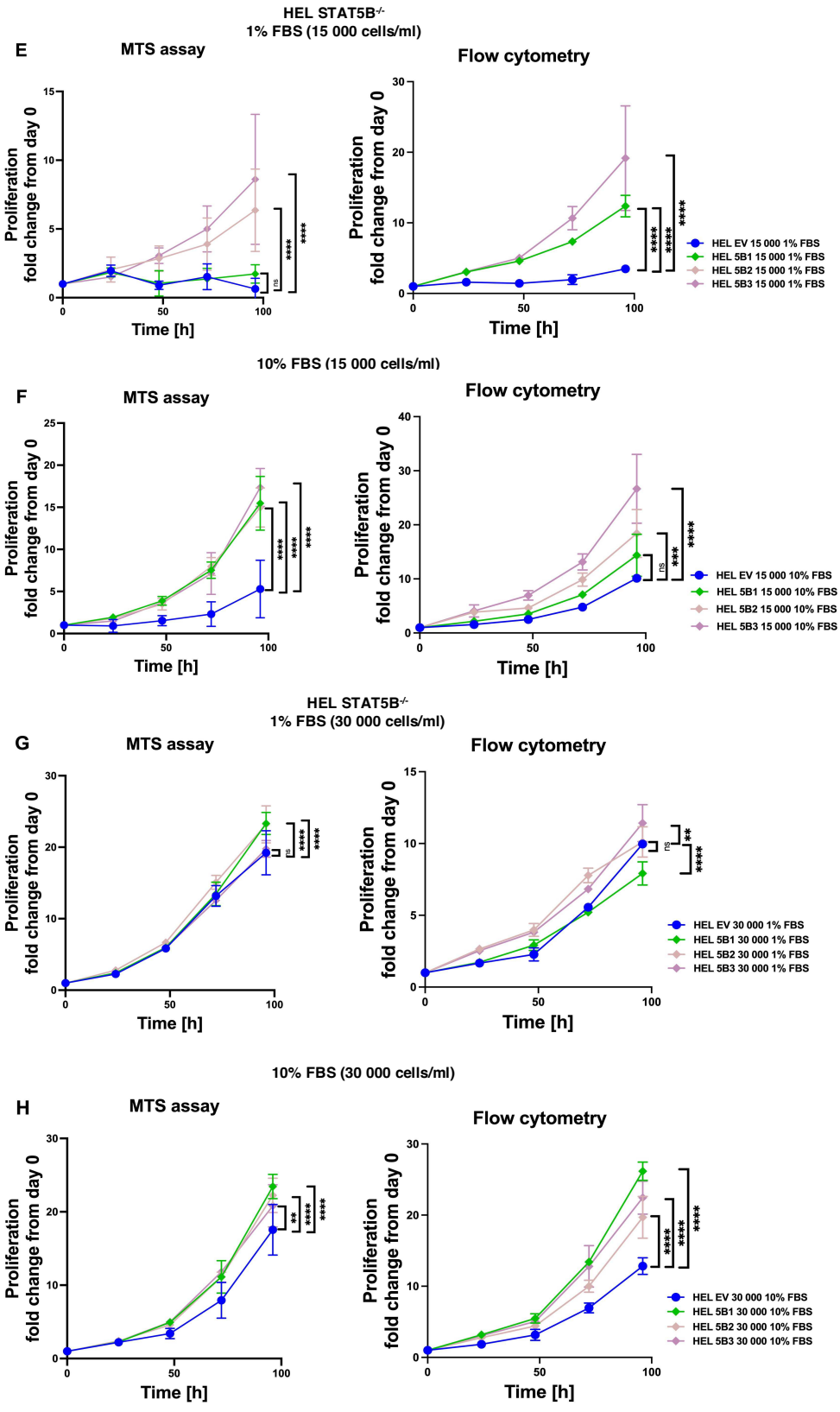

HEL STAT5B-/-
1% FBS (15 000 cells/ml)
E
MTS assay
Flow cytometry
Proliferation
fold change from day 0
Time [h]
HEL EV 15 000 1% FBS
HEL 5B1 15 000 1% FBS
HEL 5B2 15 000 1% FBS
HEL 5B3 15 000 1% FBS
10% FBS (15 000 cells/ml)
F
MTS assay
Flow cytometry
HEL EV 15 000 10% FBS
HEL 5B1 15 000 10% FBS
HEL 5B2 15 000 10% FBS
HEL 5B3 15 000 10% FBS
HEL STAT5B-/-
1% FBS (30 000 cells/ml)
G
MTS assay
Flow cytometry
HEL EV 30 000 1% FBS
HEL 5B1 30 000 1% FBS
HEL 5B2 30 000 1% FBS
HEL 5B3 30 000 1% FBS
10% FBS (30 000 cells/ml)
H
MTS assay
Flow cytometry
HEL EV 30 000 10% FBS
HEL 5B1 30 000 10% FBS
HEL 5B2 30 000 10% FBS
HEL 5B3 30 000 10% FBS

Figure 8. *Proliferation rate of CRISPR/Cas9 targeted HEL cells determined via MTS assay and cell number quantification via flow cytometry.* Proliferation of HEL cells upon knock-out/-down of STAT5A/STAT5B. Cells grown in culture media with either 1% FBS **(A, C, E, G)**, or 10% FBS **(B, D, F, H)** and with seeding concentrations of either 15 000 cells/ml **(A, B, E, F)**, or 30 000 cells/ml **(C, D, G, H)**. The growth curves of cell lines within 96 h are shown. Fold change was calculated relative to the basal cell numbers/absorbance level from day 0. Error bars indicate the standard error of the mean. For statistical analysis a two-way ANOVA with Bonferroni comparisons was performed. The graphs show p-values for comparisons with EV. ns = p-value > 0.05 * = p-value < 0.05 ** = p-value < 0.01 *** = p-value < 0.001 **** = p-value < 0.0001.

MTS aassay						
1% FBS (60 000 cells/ml)						
	HEL STAT5A_1	HEL STAT5A_2	HEL STAT5A_3	HEL STAT5B_1	HEL STAT5B_2	HEL STAT5B_3
1st week post-thawing	down **	up ***	unchanged ns	down ****	down ****	up ***
2nd week post-thawing	up **	unchanged ns	unchanged ns	up ****	down ****	unchanged ns
3rd week post-thawing	up *	up ***	unchanged ns	unchanged ns	unchanged ns	up ****
4th week post-thawing	down **	unchanged ns	down ****	down ****	unchanged ns	down ***
10% FBS (60 000 cels/ml)						
	HEL STAT5A_1	HEL STAT5A_2	HEL STAT5A_3	HEL STAT5B_1	HEL STAT5B_2	HEL STAT5B_3
1st week post-thawing	down ****	up ****	unchanged ns	down ****	down ****	down ****
2nd week post-thawing	down ****	down ****	down ****	down ****	unchanged	up *
3rd week post-thawing	up ****	up ****	up ****	up ****	up ****	up ****
4th week post-thawing	unchanged ns	unchanged ns	unchanged ns	down ****	down **	unchanged ns
Optimization						
1% FBS (60 000 cells/ml)						
	HEL STAT5A_1	HEL STAT5A_2	HEL STAT5A_3	HEL STAT5B_1	HEL STAT5B_2	HEL STAT5B_3
MTS assay	down ****	down ****	down ****	down ****	down ****	down ****
Flow cytometry	up ****	up ****	up ****	down ****	up ****	up ****
Automated cell counting	up *	up ***	up **	unchanged ns	up ****	up ****
10% FBS (60 000 cells/ml)						
MTS assay	up ****	up ****	up ****	up ****	up ****	up **
Flow cytometry	up ****	up ****	up ****	down ****	up ****	up ****
Automated cell counting	up ****	up ****	up ****	unchanged ns	unchanged ns	unchanged ns
Optimization						
1% FBS (30 000 cells/ml)						
MTS assay	up ****	up *	up ****	up ****	up ****	unchanged ns
Flow cytometry	down ****	down **	down ****	down ****	unchanged ns	up **
10% FBS (30 000 cells/ml)						
MTS assay	unchanged ns	up *	up ****	up ****	up ****	up **
Flow cytometry	unchanged ns	up ***	up **	up ****	up ****	up ****
1% FBS (15 000 cells/ml)						
MTS assay	unchanged ns	up ****	up ****	unchanged ns	up ****	up ****
Flow cytometry	up **	up **	up ***	up ****	up ****	up ****
10% FBS (15 000 cells/ml)						
MTS assay	up ***	up ****	up ****	up ****	up ****	up ****
Flow cytometry	up ****	up ***	unchanged ns	unchanged ns	up ***	up ****

Figure 9. *Summary of all cell proliferation assay results of CRISPR/Cas9-targeted HEL cells determined with different methods (MTS assay, flow cytometry, automated cell counter) and under different conditions (standard 10% FBS vs low-serum 1% FBS culture media; seeding concentrations of 30 000 cells/ml vs 15 000 cells/ml).*

3.2. The effect of STAT5A or STAT5B knock-out on STAT1 and STAT3 protein and activation levels in JAK2^{V617F} positive HEL cells

STAT5 is not the only downstream effector activated by V617F-mutated JAK2 tyrosine kinase. STAT1 and STAT3 also get activated by JAK2^{V617F} in hematopoietic cells and are thus important in MPNs (Grabek *et al.,* 2020), (Zhang *et al.,* 2023). We were interested in investigating whether the knock-out of STAT5A or STAT5B influences the expression and activation of STAT1 and STAT3. We hypothesized that they are responsible for the increased proliferation observed in our proliferation assay experiments with HEL STAT5B knock-out cell lines, as a potential compensation mechanism. However, our Western blot analysis showed no changes in STAT1 or STAT3 total protein or activation levels upon knock-out of either STAT5 variant (Figure 10). Therefore, it seems that STAT1 and STAT3 do not compensate for the loss of either STAT5A or STAT5B.

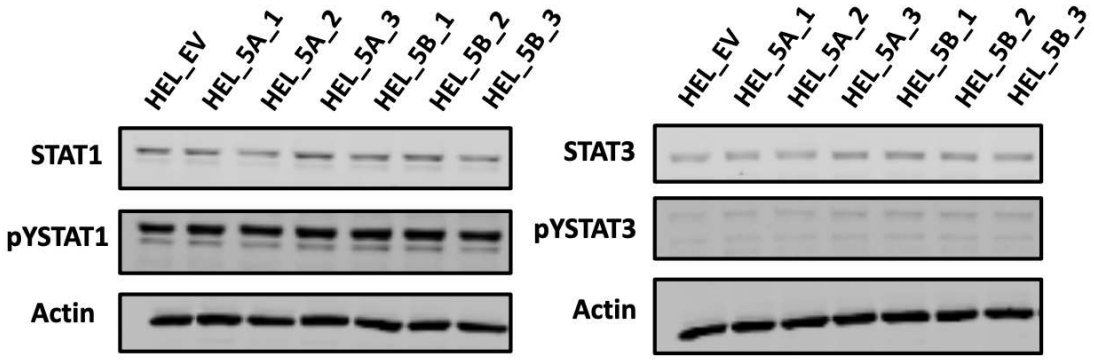


Figure 10. *STAT1 and STAT3 protein levels and levels of activated, phosphorylated STAT1 and STAT3 in CRISPR/Cas9 targeted HEL cells with gRNAs inducing either STAT5A or STAT5B knock-outs/-downs determined via Western blot analysis.* Actin was used as loading control. Data shown is representative of four independent experiments (n=4).

3.3. The effect of STAT5A or STAT5B over-expression on the apoptosis rate of JAK2^{V617F} positive HEL cells

HEL cells were previously engineered in the lab to over-express either STAT5A or STAT5B, or the GFP-positive empty vector only. The over-expressed proteins are tagged with a FLAG-tag at the C-terminus. To ensure stable over-expression, cells were FACS-sorted for GFP expression and low-level stable over-expression of the respective variant was previously confirmed by FLAG and STAT5 Western blot analysis (M. Ploderer, 2020).

We were interested to see whether the increased expression of either STAT5 variant in the JAK2^{V617F} positive background not only leads to increased proliferation, as previously shown, but also influences the apoptosis rate of HEL cells. Many clinical trials indicate the suppressive effect of IFNα on MPN malignancies by inducing apoptosis (How and Hobbs, 2020). We were thus wondering whether IFNα treatment affects the viability of our HEL cells over-expressing STAT5A or STAT5B downstream of JAK2^{V617F}. To study this, we treated our cells with human IFNα-2b and assessed the apoptosis rate.

3.3.1. The effect of hSTAT5A or hSTAT5B over-expression on the IFN-induced apoptosis rate of JAK2^{V617F} positive HEL cells

To perform an apoptosis assay with our HEL hSTAT5A or hSTAT5B over-expressor cells, we used the same protocol as was used in the Saleiro *et al.,* 2022 study, where an increased apoptosis rate was observed upon IFNα treatment in two MPN cell lines (HEL and SET-2), both harboring the JAK2^{V617F} mutation. We treated cells with 1000 U/ml human IFNα-2b for 48 h and 96 h, as was done in the publication (Saleiro *et al.,* 2022). An untreated control and positive control treated for 24 h with Bortezomib, a proteasome inhibitor capable of apoptosis induction, were included. Bortezomib application for the treatment of various solid and liquid cancers has been reported (Ghansah *et al*., 2021). Apoptosis was measured by AnnexinV and PI staining via flow cytometry.

Our data from the first apoptosis experiment showed a significantly higher apoptotic rate of positive control cells treated with Bortezomib, as expected, while apoptotic levels of cells treated with IFNα-2b were extremely low, indicating a very weak effect of IFNα-2b on cell viability (Figure 11A). We thus hypothesized that the IFNα-2b concentration might be too low to induce apoptosis and hence, we increased it to 10 000 U/ml and 100 000 U/ml. Upon repeating the experiment, this still did not suffice to strongly induce apoptosis in the cells (Figure 11B, C).

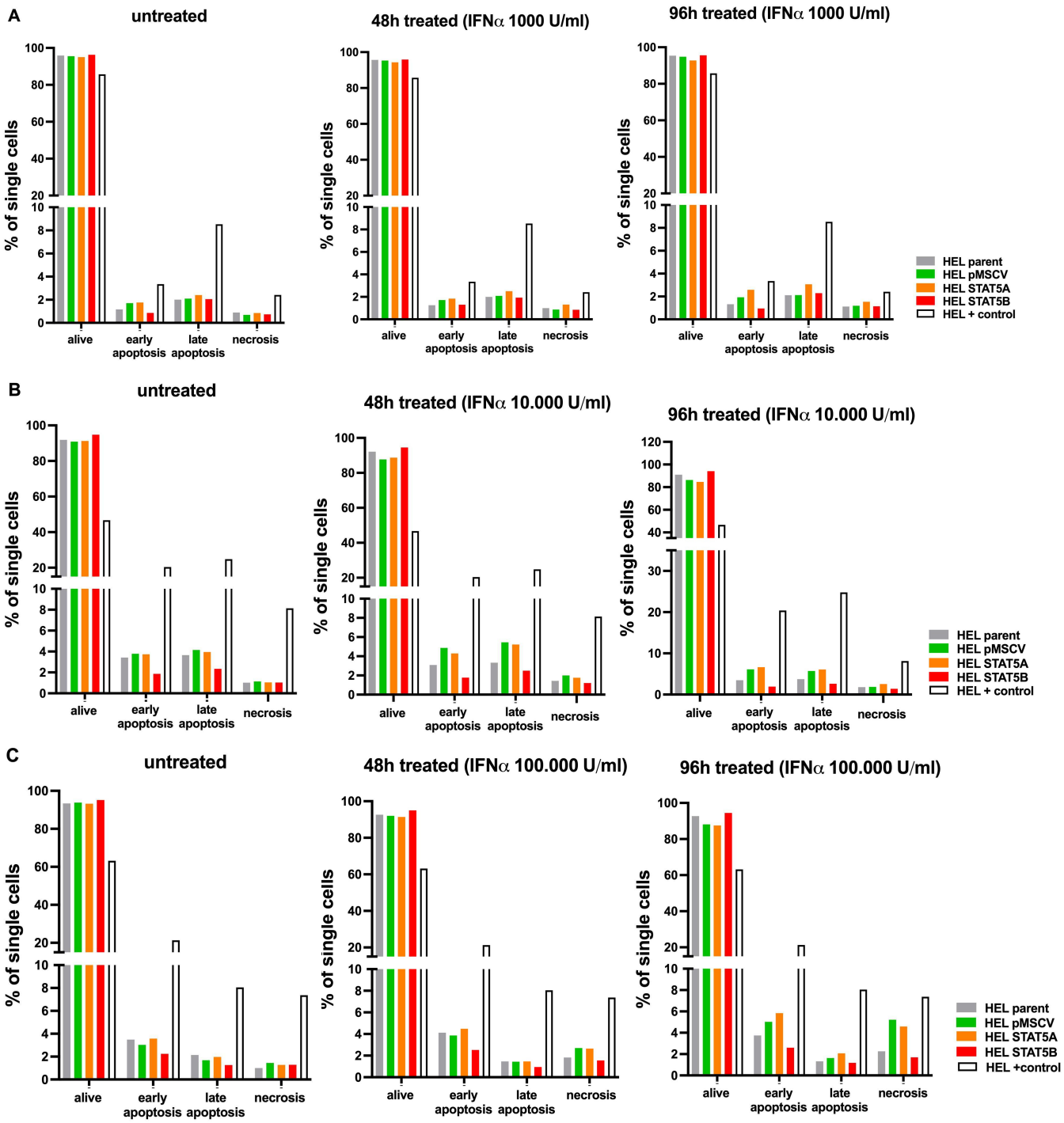

A
untreated
48h treated (IFNα 1000 U/ml)
96h treated (IFNα 1000 U/ml)
% of single cells
alive
early apoptosis
late apoptosis
necrosis
HEL parent
HEL pMSCV
HEL STAT5A
HEL STAT5B
HEL + control
B
untreated
48h treated (IFNα 10.000 U/ml)
96h treated (IFNα 10.000 U/ml)
C
untreated
48h treated (IFNα 100.000 U/ml)
96h treated (IFNα 100.000 U/ml)
HEL +control

Figure 11. *Apoptosis rates of hSTAT5A and hSTAT5B over-expressor HEL cells induced with IFNα and determined with AnnexinV-PI staining for apoptosis with flow cytometric analysis.* **(A)** Treatment with 1000 U/ml IFNα 48 h or 96 h before flow cytometric analysis. **(B)** Treatment optimization with increased IFNα concentration of 10 000 U/ml 48 h or 96 h before flow cytometric analysis. **(C)** Second assay optimization with further increased IFNα concentration of 100 000 U/ml 48 h or 96 h before flow cytometric analysis. Control: HEL cells treated with 10 uM **(A)** and 50 uM **(B, C)** Bortezomib 24 h before flow cytometric analysis. The graphs were made in GraphPad Prism and show the percentage of single cells in viable state, early apoptotic, late apoptotic and necrotic state. Data shown is representative of one independent experiment (n=1).

Data obtained from one technical replicate do not allow for conclusion on the effect of IFNα on the apoptosis rates of HEL cells over-expressing either STAT5A or STAT5B. Although, the results from this experiment suggest the potential trend of STAT5B over-expression leading to a decreased induction of apoptosis when compared to the empty vector control and STAT5A over-expression (Figure 11). This effect was the strongest in early apoptosis though. To confirm the results, repetition of the experiment and further optimization to induce stronger apoptosis in these cells would be needed but were not performed within this study due to time constraints.

3.4. Assessing STAT5A/B levels in a JAK2^{V617F} positive murine hematopoietic precursor cell line (HPC-7)

The previous study has shown an increased *STAT5B* target genes expression in MPN patients with the JAK2^{V617F} mutation (Rampal *et al.,* 2014) which led us to the question of whether this increased expression of *STAT5B* in MPN patients is induced as a downstream consequence of the hyperactive JAK2^{V617F} mutant itself, or if there are some other mechanisms involved. To test this, we needed a model with and without the JAK2^{V617F} mutation, so our human MPN cell lines were not suitable. We instead chose to use HPC-7 wild type and HPC-7 JAK2^{V617F} transduced murine cells. We determined the relative *Stat5a* and *Stat5b* mRNA expression and protein levels via qPCR and Western blot analysis, respectively. Based on the patient data, we expected to see either increased or unchanged STAT5B levels in the JAK2^{V617F} positive background compared to JAK2^{wt} cells.

3.4.1. *STAT5A* and *STAT5B* mRNA levels in JAK2^{V617F} positive HPC-7 cells

We performed qPCR analysis to determine gene expression of both STAT5 variants in HPC-7 wt and in JAK2^{V617F} positive cells. We observed a strong and significant decrease in relative mRNA expression of both *STAT5A* and *STAT5B* variants in the JAK2^{V617F} positive cells compared to wild type cells (Figure 12).

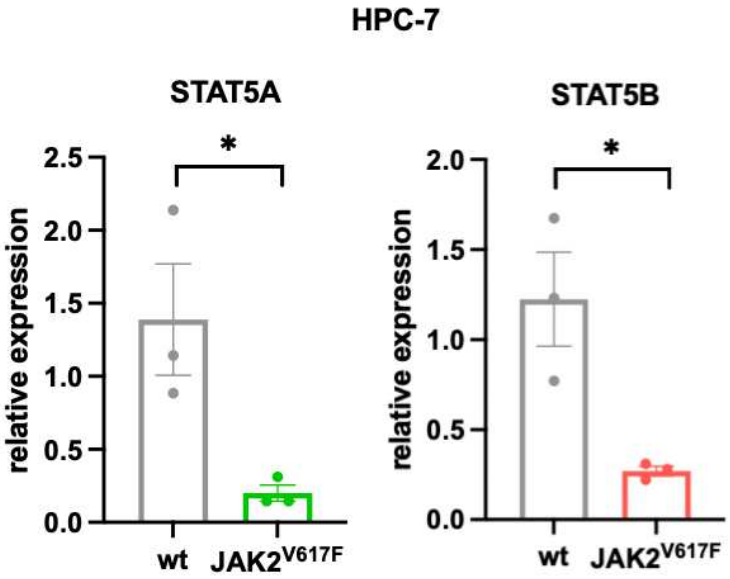


Figure 12. *Relative mSTAT5A and mSTAT5B mRNA expression in JAK2^{V617F} positive HPC-7 cells determined by qPCR analysis. The gene expression of the two STAT5 variants was calculated relative to the housekeeping gene HPRT and is presented as its x-fold using the ΔΔCt method. The error bars represent the standard error of the mean. For statistical analysis a t-test (unpaired) was performed. The graphs were made in GraphPad Prism and show p-values for comparison with HPC-7 wild type cells. * = p-value < 0.05. Data shown is representative of three independent experiments (n=3).*

3.4.2. STAT5A and STAT5B total protein and activation levels in JAK2^{V617F} positive HPC-7 cells

To confirm our results of the previous gene expression experiments at the protein level and investigate STAT5 activation, we performed Western blot analysis of the HPC-7 lines. HEL cells were used as a positive control, since we previously saw consistent protein expression of both STAT5 variants in this cell lines (Figure 4). Our data showed a slight decrease in STAT5A protein levels in the mutated compared to non-mutated HPC-7 cells. As observed in our qPCR analysis, STAT5B protein levels were strongly decreased in the JAK2^{V617F} positive background. As expected, a pYSTAT5 signal was present in the HPC-7 JAK2^{V617F} cells, but not in the HPC-7 wt cell line (Figure 13). Activity levels of the individual STAT5 variants cannot be determined due to a lack of protein-specific pY antibodies.

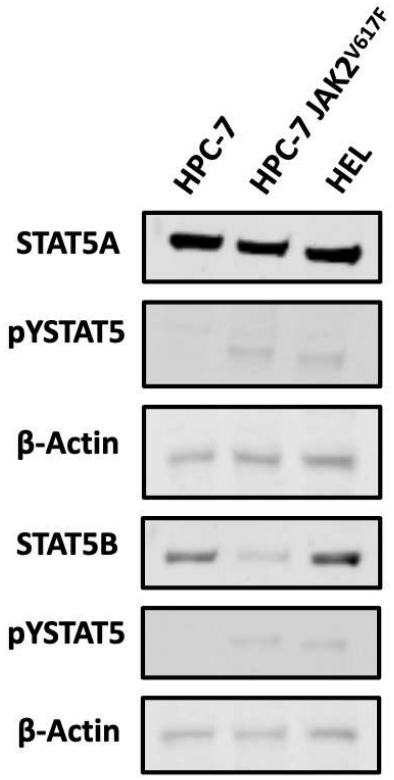


Figure 13. *STAT5A and STAT5B protein levels and levels of activated, phosphorylated STAT5 in JAK2^{V617F} positive HPC-7 cells determined via Western blot analysis.* pYSTAT5 levels showing the activation of total STAT5 protein are indicated by tyrosine phosphorylation at either 694 (STAT5A), or 699 (STAT5B) residues. β-Actin was used as loading control. Data shown is representative of four independent experiments (n=4).

4. Discussion and Outlook

The classical Philadelphia chromosome negative MPNs (PV, ET, and PMF) are clonal hematopoietic stem cell disorders resulting in abnormal proliferation of mature myeloid cells. Oncogenic mutations in the HSCs of the BM lead to a proliferative advantage of one or more myeloid lineages. The molecular origin and the lineages involved account for significant disease heterogeneity leading to different phenotypic outcomes, frequency and severity of the disease, as well as different responses to available therapy.

The central signaling pathway affected in MPNs is the JAK2/STAT5 pathway. The most commonly found mutation in MPNs is the JAK2^{V617F} mutation, which leads to hyperactivation of the downstream protein STAT5 (James *et al.,* 2005). STAT5 is central to MPN development as it is always found to be activated in MPNs and knocking it out in a murine MPN model abrogates disease development (Yan *et al.,* 2011). What remains to be investigated is whether the STAT5 variants, STAT5A and STAT5B, play equal roles in transmitting oncogenic signaling downstream of the JAK2 mutant or if one is more dominant than the other. What has been found so far is that mutations in cancer occur more frequently in STAT5B, compared to STAT5A, suggesting STAT5B as being more oncogenic (De Araujo *et al.,* 2019), (Pham *et al.,* 2018). In line with this, previous work from our lab suggests that STAT5B might be the more dominant downstream effector of hyperactive JAK2^{V617F} (M. Ploderer, 2020). Our aim for this study was thus to further investigate the differential roles of STAT5A and STAT5B in JAK2^{V617F} positive MPN.

Since the lab previously showed that increased STAT5B expression leads to a proliferative advantage in MPN cell lines, which was not the case for increased STAT5A, we wanted to

investigate if the opposite is also true when the proteins are reduced/deleted. For this we assessed the proliferation of HEL cells, which had been previously engineered to lack or to have a decreased expression of either STAT5A or STAT5B. We hypothesized that decreased or a lack of STAT5B expression would have the opposite effect of an increased expression and would lead the cells to proliferate less. We used growth media both with standard (10% FBS) and low-serum (1% FBS) culture conditions. Our MTS cell proliferation assays performed for this study showed mixed results. We saw the expected outcome in the first two MTS assay replicates after thawing the cells. STAT5B knock-out led to decreased proliferation in the standard culture conditions (Figure 6C). In the low-serum culture condition only the first assay showed the expected, decreased proliferation of cells (Figure 6B). This effect of decreased proliferation upon decreased STAT5B disappeared in the third and fourth repeat of the experiment (Figure 6C). This could be due to some compensatory mechanism that allows cells in culture to adapt to their knock-out/-down status and overcome the associated disadvantage. Another explanation could be that the cells lose their knock-out/-down status over time. However, our Western blot analysis performed in parallel with the first three MTS assays confirmed that the STAT5A and STAT5B levels in the cells remained unchanged over time (Figure 6A). We saw no consistent effect of STAT5A loss on the proliferation rate of the cells both in the standard and low-serum culture conditions (Figure 5B, C).

To ensure that the high variability of our results was not due to technical issues of the MTS assay, we performed additional proliferation experiments using flow cytometry and automated cell counting (Figure 7). The results between different methods were largely consistent leading us to conclude that our MTS data were reliable from the methodology perspective. However, comparing the results from different methods led us to conclude that the cell number quantification via flow cytometry proves to be, due to its very straightforward and time efficient sample preparation and measurement process, a more elegant way to assess proliferation than the MTS assay. Additionally, it appeared to be more reliable, since the error bars between the replicates were very small. Experiment optimization approaches with different seeding concentrations of cells were tried too. Stressing cells by reducing cell numbers was performed with the intention of more robustly revealing the differential effect of STAT5A and STAT5B. We reasoned that STAT5B might be playing a role in helping cells to be more independent from signals of surrounding cells and thus enabling their growth even when they are less dense, but this was not the case. None

of the changed experimental conditions improved the reliability of the results and therefore, didn't allow us to conclude on the effects of STAT5A or STAT5B knock-out/-down on cellular proliferation in the $JAK2^{V617F}$ positive setting (Figure 8). The results we got do not validate our hypothesis that STAT5B knock-out induces decreased proliferation in a $JAK2^{V617F}$ positive background. It could be that our cell lines simply have inconsistent growth rates. Due to time reasons the experiment with lower seeding concentrations was done only once and it should be repeated in the future to validate our results.

$JAK2^{V617F}$ -mediated signaling not only activates STAT5, but also STAT1 and STAT3 in hematopoietic stem cells (Grabek *et al.,* 2020), (Zhang *et al.,* 2023). STAT1, STAT3, and STAT5 protein level imbalances in MPNs with the $JAK2^{V617F}$ mutation result in different phenotypical outcomes. STAT1 is a regulator of megakaryopoiesis and its deficiency in $JAK2^{V617F}$ mice leads to a reduction in megakaryocyte precursors leading to the development of PV (Grabek *et al.,* 2020). STAT3 deficiency in HSCs leads to increased thrombocytosis and decreased overall survival (Grabek *et al.,* 2020). We thus wanted to see whether the absence of either STAT5 variant had an effect on the expression and activation levels of STAT1 and STAT3. We saw no effect of STAT5A/B knock-out/-down on STAT1 or STAT3 protein levels or their activation, as our Western blot analyses showed consistent expression of the respective protein across all HEL knock-out/-down cell lines (Figure 10).

As classical STAT5 transcriptional targets involve anti-apoptotic proteins such as BCL-xL and BCL-2, we wanted to investigate if an increase in either variant would impact apoptosis of HEL cells. Based on previous findings from the lab showing increased STAT5B leading to increased proliferation, we expected those cells to also be more resistant to apoptosis. Since MPN cells were reported to undergo apoptosis upon IFNα exposure, and it is used to treat MPN patients, we decided to use IFNα to induce apoptosis in our cells (How and Hobbs, 2020). We did this by determining the rate of apoptosis of our cell lines with increased STAT5 expression by monitoring the ratio of viable, early and late apoptotic, and necrotic cells using flow cytometry upon IFNα treatment. Surprisingly, we saw little to no effect of the IFNα treatment on our cells even after increasing the concentration to 100 000 U/mL (Figure 11). This leads us to question whether the IFNα used was biologically active enough to induce apoptosis, even though fresh aliquots were prepared for these experiments. Even though the IFNα treatment did not induce strong apoptosis

in our cells, we still observed that cells expressing increased STAT5B had trends of reduced apoptosis levels compared to the STAT5A over-expressing and empty vector control cell lines (Figure 11). However, these experiments need to be repeated with a stronger inducer of apoptosis to be able to draw clear conclusions on the differential effect of STAT5A and STAT5B on apoptosis in HEL cells.

It was previously observed that JAK2^{V617F} positive MPN patients have increased *STAT5B* target genes expression (Rampal *et al.,* 2014) and thus, we wanted to investigate if this is a direct downstream consequence of JAK2 mutant protein signaling or if other mechanisms are involved. We decided to use a hematopoietic/precursor mouse cell line (HPC-7) which transgenically expresses the JAK2^{V617F} mutation and determine its endogenous STAT5A and STAT5B expression levels at the mRNA and protein level. We expected the expression of STAT5B to be either enhanced in the presence of the mutation (if STAT5B expression is enhanced by mutant JAK2) or to remain unchanged (if STAT5B expression is not regulated by the JAK2 mutant). Surprisingly, we saw a decreased relative mRNA expression of both STAT5 variants in the JAK2 mutant positive context, in comparison to the HPC-7 control cells without the mutation (Figure 12). Our protein expression results were in line with these results, showing slightly decreased STAT5A and strongly decreased STAT5B protein levels induced by JAK2^{V617F} (Figure 13). As expected, we only observed a signal for activated total STAT5 in JAK2 mutated, but not in the control cells (Figure 13).

The observed data do not match our hypothesis of increased STAT5B expression in MPN patients being a consequence of the JAK2^{V617F} mutation. Several reasons could be causing these unexpected results. It could be that some other mechanism underlying higher STAT5B levels in patients is involved, or we speculate that high transgenic expression of JAK2^{V617F} might lead to such strong oncogenic signaling that the cells have to downregulate the STAT5 expression as a protective mechanism against too strong and potentially toxic levels of signaling. Another potential issue is that at the time of the study no empty vector HPC-7 control cells were available and the parental cells used were therefore not the most appropriate control for our experiments. The experiment thus should be repeated with proper controls, or another more suitable models with lower or more physiological JAK2^{V617F} expression should be used for future studies. Assessing STAT5A and STAT5B levels in bone marrow cells isolated from mice with a knock-in of the JAK2^{V617F} mutation, and in control littermate mice, could be performed. Looking at the relative

gene expression by qPCR in these isolated mouse cells as well as protein levels will allow us to draw clearer conclusions on whether JAK2^{V617F} drives increased STAT5B expression in patients.

In summary, the contribution of the STAT5 variants in MPNs remains a topic of investigation and the role of each variant downstream of JAK2^{V617F} still needs to be further investigated. In the future, this work could potentially contribute to the development of new, more specific and effective targeted therapies.

5. Bibliography

Able, A., Burrell, J., & Stephens, J. (2017). STAT5-Interacting Proteins: A Synopsis of Proteins that Regulate STAT5 Activity. *Biology*, *6*(4), 20. https://doi.org/10.3390/biology6010020

Arévalo, J., Campoy, I., Durán, M., Nemours, S., Areny, A., Vall-Palomar, M., Martínez, C., Cantero-Recasens, G., & Meseguer, A. (2023). STAT3 phosphorylation at serine 727 activates specific genetic programs and promotes clear cell renal cell carcinoma (ccRCC) aggressiveness. *Scientific Reports*, *13*(1). https://doi.org/10.1038/s41598-023-46628-5

Barbui, T., Thiele, J., Gisslinger, H., Kvasnicka, H. M., Vannucchi, A. M., Guglielmelli, P., Orazi, A., & Tefferi, A. (2018). The 2016 WHO classification and diagnostic criteria for myeloproliferative neoplasms: document summary and in-depth discussion. *Blood Cancer Journal*, *8*(2). https://doi.org/10.1038/s41408-018-0054-y

Basham, B., Sathe, M., Grein, J., McClanahan, T., D'Andrea, A., Lees, E., & Rascle, A. (2008). In vivo identification of novel STAT5 target genes. *Nucleic Acids Research*, *36*(11), 3802–3818. https://doi.org/10.1093/nar/gkn271

Baxter, E. J., Scott, L. M., Campbell, P. J., East, C., Fourouclas, N., Swanton, S., Vassiliou, G. S., Bench, A. J., Boyd, E. M., Curtin, N., Scott, M. A., Erber, W. N., & Green, A. R. (2005). Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *The Lancet*, *365*(9464), 1054–1061. https://doi.org/10.1016/s0140-6736(05)71142-9

Beer, P. A., Campbell, P. J., Scott, L. M., Bench, A. J., Erber, W. N., Bareford, D., Wilkins, B. S., Reilly, J. T., Hasselbalch, H. C., Bowman, R., Wheatley, K., Buck, G., Harrison, C. N., & Green, A. R. (2008). MPL mutations in myeloproliferative disorders: analysis of the PT-1 cohort. *Blood*, *112*(1), 141–149. https://doi.org/10.1182/blood-2008-01-131664

Bousoik, E., & Aliabadi, H. M. (2018). “Do we know Jack” about JAK? A closer look at JAK/STAT signaling pathway. *Frontiers in Oncology*, *8*. https://doi.org/10.3389/fonc.2018.00287

Brachet-Botineau, M., Polomski, M., Neubauer, H., Juen, L., Hédou, D., Viaud-Massuard, M., Prié, G., & Gouilleux, F. (2020). Pharmacological inhibition of oncogenic STAT3 and STAT5 signaling in hematopoietic cancers. *Cancers*, *12*(1), 240. https://doi.org/10.3390/cancers12010240

Choudhary, C., Brandts, C., Schwable, J., Tickenbrock, L., Sargin, B., Ueker, A., Böhmer, F., Berdel, W. E., Müller-Tidow, C., & Serve, H. (2007). Activation mechanisms of STAT5 by oncogenic Flt3-ITD. *Blood*, *110*(1), 370–374. https://doi.org/10.1182/blood-2006-05-024018

De Araujo, E. D., Erdogan, F., Neubauer, H. A., Meneksedag-Erol, D., Manaswiyoungkul, P., Eram, M. S., Seo, H., Qadree, A. K., Israelian, J., Orlova, A., Suske, T., Pham, H. T. T., Boersma, A., Tangermann, S., Kenner, L., Rülicke, T., Dong, A., Ravichandran, M., Brown, P. J., . . . Gunning, P. T. (2019). Structural and functional consequences of the STAT5BN642H driver mutation. *Nature Communications*, *10*(1). https://doi.org/10.1038/s41467-019-10422-7

Duangnapasatit, B., Rattarittamrong, E., Rattanathammethee, T., Hantrakool, S., Chai-Adisaksopha, C., Tantiworawit, A., & Norasetthada, L. (2015). Clinical manifestations and risk factors for complications of Philadelphia Chromosome-Negative myeloproliferative neoplasms. *Asian Pacific Journal of Cancer Prevention*, *16*(12), 5013–5018. https://doi.org/10.7314/apjcp.2015.16.12.5013

Fasouli, E. S., & Katsantoni, E. (2021). JAK-STAT in early hematopoiesis and leukemia. *Frontiers in Cell and Developmental Biology*, *9*. https://doi.org/10.3389/fcell.2021.669363

Funakoshi-Tago, M., Tago, K., Abe, M., Sonoda, Y., & Kasahara, T. (2009). STAT5 activation is critical for the transformation mediated by myeloproliferative disorder-associated JAK2 V617F mutant. *Journal of Biological Chemistry*, *285*(8), 5296–5307. https://doi.org/10.1074/jbc.m109.040733

Geyer, H., & Mesa, R. A. (2017). Approach to MPN symptom assessment. *Current Hematologic Malignancy Reports*, *12*(5), 381–388. https://doi.org/10.1007/s11899-017-0399-5

Ghansah, H., Debreceni, I. B., Fejes, Z., Nagy, B., & Kappelmayer, J. (2021). The proteasome inhibitor bortezomib induces apoptosis and activation in Gel-Filtered human platelets. *International Journal of Molecular Sciences*, *22*(16), 8955. https://doi.org/10.3390/ijms22168955

Gou, P., Zhang, W., & Giraudier, S. (2022). Insights into the Potential Mechanisms of JAK2V617F Somatic Mutation Contributing Distinct Phenotypes in Myeloproliferative Neoplasms. *International Journal of Molecular Sciences*, *23*(3), 1013. https://doi.org/10.3390/ijms23031013

Grabek, J., Straube, J., Bywater, M., & Lane, S. W. (2020a). MPN: The Molecular Drivers of Disease Initiation, Progression and Transformation and their Effect on Treatment. *Cells*, *9*(8), 1901. https://doi.org/10.3390/cells9081901

Haider MZ, Anwer F. Secondary Polycythemia. 2023 May 8. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 Jan–. PMID: 32965904.

Halim, C. E., Deng, S., Ong, M. S., & Yap, C. T. (2020). Involvement of STAT5 in oncogenesis. *Biomedicines*, *8*(9), 316. https://doi.org/10.3390/biomedicines8090316

Hennighausen, L., & Robinson, G. W. (2008b). Interpretation of cytokine signaling through the transcription factors STAT5A and STAT5B. *Genes & Development*, *22*(6), 711–721. https://doi.org/10.1101/gad.1643908

Hillmer, E. J., Zhang, H., Li, H. S., & Watowich, S. S. (2016). STAT3 signaling in immunity. *Cytokine & Growth Factor Reviews*, *31*, 1–15. https://doi.org/10.1016/j.cytogfr.2016.05.001

How, J., & Hobbs, G. (2020). Use of interferon Alfa in the treatment of myeloproliferative neoplasms: Perspectives and review of the literature. *Cancers*, *12*(7), 1954. https://doi.org/10.3390/cancers12071954

Hu, X., Li, J., Fu, M., Zhao, X., & Wang, W. (2021). The JAK/STAT signaling pathway: from bench to clinic. *Signal Transduction and Targeted Therapy*, *6*(1). https://doi.org/10.1038/s41392-021-00791-1

Huang, K. T., Chen, Y. H., & Walker, A. M. (2004). Inaccuracies in MTS assays: major distorting effects of medium, serum albumin, and fatty acids. *BioTechniques*, *37*(3), 406–412. https://doi.org/10.2144/04373st05

Huang, Z., Richmond, T. D., Muntean, A. G., Barber, D. L., Weiss, M. J., & Crispino, J. D. (2007). STAT1 promotes megakaryopoiesis downstream of GATA-1 in mice. *Journal of Clinical Investigation*, *117*(12), 3890–3899. https://doi.org/10.1172/jci33010

James, C., Ugo, V., Couédic, J. L., Staerk, J., Delhommeau, F., Lacout, C., Garçon, L., Raslova, H., Berger, R., Bennaceur-Griscelli, A., Villeval, J. L., Constantinescu, S. N., Casadevall, N., & Vainchenker, W. (2005). A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*, *434*(7037), 1144–1148. https://doi.org/10.1038/nature03546

Jones, A. V., Kreil, S., Zoi, K., Waghorn, K., Curtis, C., Zhang, L., Score, J., Seear, R., Chase, A. J., Grand, F. H., White, H., Zoi, C., Loukopoulos, D., Terpos, E., Vervessou, E., Schultheis, B., Emig, M., Ernst, T., Lengfelder, E., . . . Cross, N. C. P. (2005). Widespread occurrence of the JAK2 V617F mutation in chronic myeloproliferative disorders. *Blood*, *106*(6), 2162–2168. https://doi.org/10.1182/blood-2005-03-1320

Kollmann, S., Grundschober, E., Maurer, B., Warsch, W., Grausenburger, R., Edlinger, L., Huuhtanen, J., Lagger, S., Hennighausen, L., Valent, P., Decker, T., Strobl, B., Mueller, M., Mustjoki, S., Hoelbl-Kovacic, A., & Sexl, V. (2019). Twins with different personalities: STAT5B—but not STAT5A—has a key role in BCR/ABL-induced leukemia. *Leukemia*, *33*(7), 1583–1597. https://doi.org/10.1038/s41375-018-0369-5

Kollmann, S., Grausenburger, R., Klampfl, T., Prchal-Murphy, M., Bastl, K., Pisa, H., Knab, V. M., Brandstoetter, T., Doma, E., Sperr, W. R., Lagger, S., Farlik, M., Moriggl, R., Valent, P., Halbritter, F., Kollmann, K., Heller, G., Maurer, B., & Sexl, V. (2021). A STAT5B–CD9 axis determines self-renewal in hematopoietic and leukemic stem cells. *Blood*, *138*(23), 2347–2359. https://doi.org/10.1182/blood.2021010980

Kovacic, B., Stoiber, D., Moriggl, R., Weisz, E., Ott, R. G., Kreibich, R., Levy, D. E., Beug, H., Freissmuth, M., & Sexl, V. (2006). STAT1 acts as a tumor promoter for leukemia development. *Cancer Cell*, *10*(1), 77–87. https://doi.org/10.1016/j.ccr.2006.05.025

Kralovics, R., Passamonti, F., Buser, A. S., Teo, S., Tiedt, R., Passweg, J. R., Tichelli, A., Cazzola, M., & Skoda, R. C. (2005). A Gain-of-Function mutation ofJAK2in myeloproliferative disorders. *New England Journal of Medicine*, *352*(17), 1779–1790. https://doi.org/10.1056/nejmoa051113

Kralovics, R., Stockton, D. W., & Prchal, J. T. (2003). Clonal hematopoiesis in familial polycythemia vera suggests the involvement of multiple mutational events in the early pathogenesis of the disease. *Blood*, *102*(10), 3793–3796. https://doi.org/10.1182/blood-2003-03-0885

Langabeer, S. E. (2016). Chasing down the triple-negative myeloproliferative neoplasms: Implications for molecular diagnostics. *JAK-STAT*, *5*(2–4), e1248011. https://doi.org/10.1080/21623996.2016.1248011

Lee, J. Y., & Hong, S. (2019). Hematopoietic stem cells and their roles in tissue regeneration. *International Journal of Stem Cells*, *13*(1), 1–12. https://doi.org/10.15283/ijsc19127

Levine, R. L., Wadleigh, M., Cools, J., Ebert, B. L., Wernig, G., Huntly, B. J., Boggon, T. J., Wlodarska, I., Clark, J. J., Moore, S., Adelsperger, J., Koo, S., Lee, J. C., Gabriel, S., Mercher, T., D'Andrea, A., Fröhling, S., Döhner, K., Marynen, P., . . . Gilliland, D. G. (2005). Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell*, *7*(4), 387–397. https://doi.org/10.1016/j.ccr.2005.03.023

Liu, X., Robinson, G. W., Wagner, K. U., Garrett, L., Wynshaw-Boris, A., & Hennighausen, L. (1997). Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes & Development*, *11*(2), 179–186. https://doi.org/10.1101/gad.11.2.179

Maurer, B., Kollmann, S., Pickem, J., Hoelbl-Kovacic, A., & Sexl, V. (2019). STAT5A and STAT5B—Twins with Different Personalities in Hematopoiesis and Leukemia. *Cancers*, *11*(11), 1726. https://doi.org/10.3390/cancers11111726

Maurer, B., Nivarthi, H., Wingelhofer, B., Pham, H. T. T., Schlederer, M., Suske, T., Grausenburger, R., Schiefer, A., Prchal-Murphy, M., Chen, D., Winkler, S., Merkel, O., Kornauth, C., Hofbauer, M., Hochgatterer, B., Hoermann, G., Hoelbl-Kovacic, A., Prochazkova, J., Lobello, C., . . . Moriggl, R. (2019). High activation of STAT5A drives peripheral T-cell lymphoma and leukemia. *Haematologica*, *105*(2), 435–447. https://doi.org/10.3324/haematol.2019.216986

Moriggl, R., Sexl, V., Kenner, L., Duntsch, C., Stangl, K., Gingras, S., Hoffmeyer, A., Bauer, A., Piekorz, R., Wang, D., Bunting, K. D., Wagner, E. F., Sonneck, K., Valent, P., Ihle, J. N., & Beug, H. (2005). Stat5 tetramer formation is associated with leukemogenesis. *Cancer Cell*, *7*(1), 87–99. https://doi.org/10.1016/j.ccr.2004.12.010

Nangalia, J., & Green, A. R. (2017). Myeloproliferative neoplasms: from origins to outcomes. *Blood*, *130*(23), 2475–2483. https://doi.org/10.1182/blood-2017-06-782037

Orlova, A., Wagner, C., De Araujo, E. D., Bajusz, D., Neubauer, H. A., Herling, M., Gunning, P. T., Keserű, G. M., & Moriggl, R. (2019). Direct targeting options for STAT3 and STAT5 in cancer. *Cancers*, *11*(12), 1930. https://doi.org/10.3390/cancers11121930

O'Shea, J. J., Schwartz, D. M., Villarino, A. V., Gadina, M., McInnes, I. B., & Laurence, A. (2015). The JAK-STAT Pathway: Impact on human disease and therapeutic intervention. *Annual Review of Medicine*, *66*(1), 311–328. https://doi.org/10.1146/annurev-med-051113-024537

Park, H. J., Li, J., Hannah, R., Biddie, S., Leal-Cervantes, A. I., Kirschner, K., Cruz, D. F. S., Sexl, V., Göttgens, B., & Green, A. R. (2015). Cytokine-induced megakaryocytic differentiation is regulated by genome-wide loss of a uSTAT transcriptional program. *The EMBO Journal*, *35*(6), 580–594. https://doi.org/10.15252/embj.201592383

Pham, H. T. T., Maurer, B., Prchal-Murphy, M., Grausenburger, R., Grundschober, E., Javaheri, T., Nivarthi, H., Boersma, A., Kolbe, T., Elabd, M., Halbritter, F., Pencik, J., Kazemi, Z., Grebien, F., Hengstschläger, M., Kenner, L., Kubicek, S., Farlik, M., Bock, C., . . . Moriggl, R. (2017). STAT5BN642H is a driver mutation for T cell neoplasia. *Journal of Clinical Investigation*, *128*(1), 387–401. https://doi.org/10.1172/jci94509

Ploderer Marie Master's ThesisThe Role of STAT5B in JAK2V617F-Positive Myeloproliferative Neoplasms 2020 Available at: www.utheses.univie.ac.at

Rampal, R., Al-Shahrour, F., Abdel-Wahab, O., Patel, J. P., Brunel, J., Mermel, C. H., Bass, A. J., Pretz, J., Ahn, J., Hricik, T., Kilpivaara, O., Wadleigh, M., Busque, L., Gilliland, D. G., Golub, T. R., Ebert, B. L., & Levine, R. L. (2014). Integrated genomic analysis illustrates the central role of JAK-STAT pathway activation in myeloproliferative neoplasm pathogenesis. *Blood*, *123*(22), e123–e133. https://doi.org/10.1182/blood-2014-02-554634

Saleiro, D., Wen, J. Q., Kosciuczuk, E. M., Eckerdt, F., Beauchamp, E. M., Oku, C. V., Blyth, G. T., Fischietti, M., Ilut, L., Colamonici, M., Palivos, W., Atsaves, P. A., Tan, D.,

Kocherginsky, M., Weinberg, R. S., Fish, E. N., Crispino, J. D., Hoffman, R., & Platanias, L. C. (2022a). Discovery of a signaling feedback circuit that defines interferon responses in myeloproliferative neoplasms. *Nature Communications*, *13*(1). https://doi.org/10.1038/s41467-022-29381-7

Schaller-Schönitz, M., Barzan, D., Williamson, A. J. K., Griffiths, J. R., Dallmann, I., Battmer, K., Ganser, A., Whetton, A. D., Scherr, M., & Eder, M. (2014). BCR-ABL affects STAT5A and STAT5B differentially. *PLoS ONE*, *9*(5), e97243. https://doi.org/10.1371/journal.pone.0097243

Schreiberhuber Lena Isabel Master's Thesis The Function of STAT5 in JAK2V617F-Positive Myeloproliferative Neoplasms and Megakaryocyte Lineage Regulation 2021 Available at: www.utheses.univie.ac.at

Schuringa, J. J., Chung, K. Y., Morrone, G., & Moore, M. A. (2004). Constitutive activation of STAT5A promotes human hematopoietic stem cell Self-Renewal and erythroid differentiation. *The Journal of Experimental Medicine*, *200*(5), 623–635. https://doi.org/10.1084/jem.20041024

Shammo, J. M., & Stein, B. L. (2016). Mutations in MPNs: prognostic implications, window to biology, and impact on treatment decisions. *Hematology*, *2016*(1), 552–560. https://doi.org/10.1182/asheducation.v2016.1.552.088349

Szybinski, J., Sasca, D., Heidelberger, J., Klumb, K., Shah, V., Dolnik, A., Theobald, M., Bullinger, L., Beli, P., & Kindler, T. (2019). Abstract 5223: Epigenetic silencing mediated by non-phosphorylated STAT5B prevents differentiation in acute myeloid leukemia. *Cancer Research*, *79*(13_Supplement), 5223. https://doi.org/10.1158/1538-7445.am2019-5223

Tabilio, A., Herrera, A., D'agay, M., Vainchenker, W., Allard, C., Clauvel, J., & Breton-Gorius, J. (1984). Therapy-related leukemia associated with myelofibrosis. Blast cell

characterization in six cases. *Cancer*, *54*(7), 1382–1391. https://doi.org/10.1002/1097-0142(19841001)54:7

Tefferi, A., & Barbui, T. (2020). Polycythemia vera and essential thrombocythemia: 2021 update on diagnosis, risk-stratification and management. *American Journal of Hematology*, *95*(12), 1599–1613. https://doi.org/10.1002/ajh.26008

Udy, G. B., Towers, R. P., Snell, R. G., Wilkins, R. J., Park, S., Ram, P. A., Waxman, D. J., & Davey, H. W. (1997). Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. *Proceedings of the National Academy of Sciences*, *94*(14), 7239–7244. https://doi.org/10.1073/pnas.94.14.7239

Wang, W., McDonald, M. C. L., Kim, C., Ma, M., Pan, Z., Kaufmann, C., & Frank, D. A. (2023). The complementary roles of STAT3 and STAT1 in cancer biology: insights into tumor pathogenesis and therapeutic strategies. *Frontiers in Immunology*, *14*. https://doi.org/10.3389/fimmu.2023.1265818

Wang, Z., & Bunting, K. D. (2013). STAT5 in hematopoietic stem cell biology and transplantation. *JAK-STAT*, *2*(4), e27159. https://doi.org/10.4161/jkst.27159

Xue, C., Yao, Q., Gu, X., Shi, Q., Yuan, X., Chu, Q., Bao, Z., Lu, J., & Li, L. (2023). Evolving cognition of the JAK-STAT signaling pathway: autoimmune disorders and cancer. *Signal Transduction and Targeted Therapy*, *8*(1). https://doi.org/10.1038/s41392-023-01468-7

Yan, D., Hutchison, R. E., & Mohi, G. (2011). Critical requirement for Stat5 in a mouse model of polycythemia vera. *Blood*, *119*(15), 3539–3549. https://doi.org/10.1182/blood-2011-03-345215

Zhang, M., Meng, Y., Ying, Y., Zhou, P., Zhang, S., Fang, Y., Yao, Y., & Li, D. (2023). Selective activation of STAT3 and STAT5 dictates the fate of myeloid progenitor cells. *Cell Death Discovery*, *9*(1). https://doi.org/10.1038/s41420-023-01575-y

Zou, S., Tong, Q., Liu, B., Huang, W., Tian, Y., & Fu, X. (2020). Targeting STAT3 in cancer immunotherapy. *Molecular Cancer*, *19*(1). https://doi.org/10.1186/s12943-020-01258-7

6. Appendix

6.1. Abstract

Myeloproliferative neoplasms (MPNs) are blood malignancies that develop due to the over proliferation of mutated hematopoietic stem cells (HSC) in the bone marrow, followed by the subsequent malignant expansion of mature cells of the myeloid lineage. The three classical MPN subtypes are polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), all of which are frequently driven by a mutation in the *JAK2* gene. This mutation, $JAK2^{V617F}$, results in a constitutively active JAK2 kinase and therefore increased activation of the proliferation and survival genes targeted by the downstream transcription factor STAT5. STAT5 comprises two different gene products, STAT5A and STAT5B, whose differential roles in human disease remain under investigation. Previous work in our lab has shown the proliferative advantage of MPN cell lines (HEL, SET-2) positive for the $JAK2^{V617F}$ mutation upon increased levels of STAT5B over those with increased levels of STAT5A. This suggests that STAT5B may be the main downstream effector of $JAK2^{V617F}$ and leads us to hypothesize that STAT5B is the more oncogenic sibling in this context. The aim of this project was to expand our understanding of the functions of the STAT5 variants in MPNs by investigating whether the two variants have different roles in the disease. For this we used both human MPN and murine hematopoietic cell lines.

We first assessed the proliferation of a human MPN cell line (HEL), homozygous for the $JAK2^{V617F}$ mutation, upon knock-out or knock-down of either STAT5A or STAT5B, using an MTS assay. We hypothesized that reduced STAT5B levels would reduce HEL cell proliferation to a higher extent than the reduction of STAT5A protein. We observed some degree of variability between the repeats of our experiments. In the first two assays the absence of STAT5B did decrease the proliferation of cells to a higher degree, compared to the absence of STAT5A. However, this effect was not consistent over time. We proceeded to optimize different methods of measuring cell

proliferation and different assay conditions, however due to further inconsistency of the results, we were not able to conclude on the effect of a lack of STAT5A or STAT5B on the proliferation of HEL cells. We also studied the effect of the absence of either STAT5 variant on the expression and activation levels of two other transcription factors of the STAT family, STAT1 and STAT3. These are, in addition to STAT5, known to be activated by $JAK2^{V617F}$ in hematopoietic cells and could serve as compensation mechanisms upon STAT5A/B loss. We observed no effect of the absence of either STAT5 variant on STAT1 and STAT3 expression and activation levels. Additionally, we were interested in investigating the effect of STAT5A or STAT5B on apoptosis. For this we used HEL cells with increased STAT5A or STAT5B levels and induced apoptosis with interferon-alpha (IFNα). Unfortunately, only a very weak effect of IFNα on cell viability was observed even after some optimization of the assay. We did however observe a trend that STAT5B over-expressing cells had decreased levels of apoptosis compared to STAT5A over-expressing cells or the empty vector control. This effect however was small and needs to be confirmed by further experiments and optimization of the assay. Previous data also showed that $JAK2^{V617F}$ positive MPN patients have increased levels of STAT5B mRNA expression (Rampal *et al.,* 2014). To investigate whether this is a direct consequence of the $JAK2^{V617F}$ mutation itself, we used a murine hematopoietic precursor cell line (HPC-7) which transgenically expresses the $JAK2^{V617F}$ mutant and examined the STAT5A and STAT5B levels. Surprisingly, we observed decreased STAT5A and STAT5B both at the mRNA and protein level in cells with $JAK2^{V617F}$ compared to wildtype cells.

To summarize, our results indicate that knocking out STAT5B in a human MPN cell line positive for the $JAK2^{V617F}$ mutation may decrease cell proliferation, and STAT5B over-expression may reduce cell apoptosis, but our results were variable and further experiments and optimization are required to make clear conclusions. The absence of either STAT5 variant had no effect on the protein levels nor the activation levels of STAT1 and STAT3. Lastly, our experiments showed an unexpected decrease in STAT5A/B mRNA and protein levels in a mouse progenitor cell line transgenically expressing the $JAK2^{V617F}$ mutation. In conclusion, to better understand the pathogenesis of MPNs, studying the differential roles of STAT5A/B proteins downstream of the $JAK2^{V617F}$ in these diseases remains a topic of interest and serves as an important step towards development of novel and improvement of currently available targeted therapies.

6.2. Zusammenfassung

Myeloproliferative Neoplasien (MPN) sind maligne Bluterkrankungen, die sich aufgrund der übermäßigen Vermehrung mutierter hämatopoetischer Stammzellen (HSC) im Knochenmark entwickeln, gefolgt von der anschließenden bösartigen Ausbreitung reifer Zellen der myeloiden Linie. Die drei klassischen MPN-Subtypen sind Polycythaemia vera (PV), essentielle Thrombozythämie (ET) und primäre Myelofibrose (PMF), die alle häufig durch dieselbe Mutation im JAK2-Gen verursacht werden. Diese Mutation, die $JAK2^{V617F}$-Mutation, führt zu einer konstitutiv aktiven JAK2-Kinase und damit zu einer erhöhten Aktivierung der Proliferations- und Überlebensgene, die vom nachgeschalteten Transkriptionsfaktor STAT5 angesteuert werden. STAT5 besteht aus zwei verschiedenen Genprodukten, STAT5A und STAT5B, deren unterschiedliche Rollen bei menschlichen Erkrankungen noch untersucht werden. Frühere Untersuchungen in unserem Labor haben gezeigt, dass MPN-Zelllinien (HEL, SET-2), die positiv für die $JAK2^{V617F}$-Mutation sind, bei erhöhten STAT5B-Genexpression einen proliferativen Vorteil gegenüber solchen mit erhöhten STAT5A-Genexpression haben. Diese Beobachtungen lassen darauf schließen, dass STAT5B der wichtigste nachgeschalteter Effektor von $JAK2^{V617F}$ sein könnte, und führt uns zu der Hypothese, dass STAT5B in diesem Zusammenhang das stärker onkogene Geschwister sein könnte. Ziel dieses Projekts war es, unser Verständnis der Funktionen der STAT5-Varianten in MPNs zu erweitern und zu untersuchen, ob und wie die beiden Varianten unterschiedliche Rollen auf die Krankheitsentwicklung spielen. Dazu haben wir sowohl menschliche MPN- als auch murine hämatopoetische Zelllinien verwendet.

Wir untersuchten zuerst die Proliferation einer humanen MPN-Zelllinie (HEL), die homozygot für die $JAK2^{V617F}$-Mutation ist, nach Knock-out oder Knock-down von entweder STAT5A oder STAT5B Variante mithilfe eines MTS-Assays. Wir stellten die Hypothese auf, dass reduzierte STAT5B die Proliferation von HEL-Zellen stärker reduzieren würde als die Reduzierung des STAT5A Proteins. Wir beobachteten eine gewisse Variabilität zwischen den Wiederholungen unserer Experimente. In den ersten beiden Assays verringerte die Abwesenheit von STAT5B die Zellproliferation stärker als die Abwesenheit von STAT5A. Dieser Effekt war über die Zeit nicht konsistent. Dann haben wir verschiedene Methoden zur Messung der Zellproliferation verwendet und unterschiedliche Testbedingungen optimiert. Aufgrund weiterer Dateninkonsistenzen zwischen den Wiederholungen der Experimente konnten wir keine Schlussfolgerungen auf die Auswirkungen des Mangels an STAT5A oder an STAT5B auf die Proliferation von HEL-Zellen

ziehen. Wir untersuchten auch die Auswirkungen des Fehlens einer der beiden STAT5-Varianten auf die Expressions- und Aktivierungsniveaus zweier anderer Transkriptionsfaktoren der STAT-Familie, STAT1 und STAT3. Es ist bekannt, dass diese zusätzlich zu STAT5, in hämatopoetischen Zellen durch JAK2^{V617F} aktiviert werden und als Kompensationsmechanismen bei STAT5A/B-Verlust dienen könnten. Wir beobachteten keine Auswirkung des Fehlens einer der beiden STAT5-Varianten auf die Expressions- und Aktivierungsniveaus von STAT1 und STAT3. Darüber hinaus waren wir daran interessiert, die Wirkung von STAT5A oder STAT5B auf die Apoptose zu untersuchen. Dazu verwendeten wir HEL-Zellen mit erhöhten STAT5A- oder STAT5B-Genexpression und induzierten Apoptose mit Interferon-Alpha (IFNα). Leider wurde selbst nach einer gewissen Optimierung des Assays nur eine sehr schwache Wirkung von IFNα auf die Zelllebensfähigkeit beobachtet. Wir beobachteten jedoch einen Trend, dass STAT5B-überexprimierende Zellen im Vergleich zu STAT5A-überexprimierenden Zellen oder der leeren Vektorkontrolle geringere Apoptosewerte aufwiesen. Dieser Effekt war jedoch gering und muss durch weitere Experimente und Optimierung des Assays bestätigt werden. Frühere Daten zeigten auch, dass JAK2^{V617F}-positive MPN-Patienten ein erhöhtes Maß an STAT5B-mRNA-Expression aufweisen (Rampal *et al.,* 2014). Um zu untersuchen, ob dies eine direkte Folge der JAK2^{V617F}-Mutation selbst ist, verwendeten wir eine murine hämatopoetische Vorläuferzelllinie (HPC-7), die die JAK2^{V617F}-Mutation transgen exprimiert, und bestimmten ihre STAT5A- und STAT5B-Expressionswerte. Überraschenderweise beobachteten wir in Zellen mit JAK2^{V617F} im Vergleich zu Wildtyp-Zellen einen Rückgang von STAT5A und STAT5B sowohl auf mRNA- als auch auf Proteinebene. Zusammenfassend deuten unsere Ergebnisse darauf hin, dass das Ausschalten von STAT5B in einer für die JAK2^{V617F}-Mutation positiven menschlichen MPN-Zelllinie die Zellproliferation verringern kann und eine Überexpression von STAT5B die Zellapoptose verringern kann. Unsere Ergebnisse waren jedoch unterschiedlich und weitere Experimente und Optimierungen sind für die klare Schlussfolgerungen erforderlich. Das Fehlen einer der STAT5-Varianten hatte keinen Einfluss auf die Proteinebene oder die Aktivierungsebene von STAT1 und STAT3. Schließlich zeigten unsere Experimente einen unerwarteten Rückgang der STAT5A/B-mRNA- und Proteinebene in einer Maus-Vorläuferzelllinie, die die JAK2^{V617F}-Mutation transgen exprimierte. Um die Pathogenese von MPNs besser zu verstehen, bleibt die Untersuchung der unterschiedlichen Rollen von STAT5A/B-Proteinen unten des JAK2^{V617F} bei diesen Krankheiten ein interessantes Thema und dient als wichtiger Schritt zur Entwicklung neuer und Verbesserung der derzeit verfügbaren zielgerichteten Therapien.