Arab American University

Faculty of Graduate Studies

Potential Role of WW Domain Containing Oxidoreductase Gene in Myeloproliferative Neoplasms

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This thesis was submitted in partial fulfillment of the requirements for the Master`s degree in molecular genetics and genetic toxicology

10/2023

Thesis Approval

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I certify that the work provided in this thesis unless otherwise referenced, is the researcher's work and has not been submitted for a higher degree to any other university or institution.

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Dedication

I would like to dedicate this work and effort to my great parents.

Kholoud Saleh Abo-Saleh

Acknowledgement

I would like to begin by expressing my heartfelt gratitude to Allah, whose guidance and blessings have been the cornerstone of my journey in completing this master's thesis. My family's unwavering support and encouragement have been my driving force, and I am deeply thankful for their presence in my life. I am truly indebted to Prof. Zaidoun Salah for his exceptional supervision, invaluable guidance, and unwavering commitment to my academic growth. My heartfelt thanks also extend to my colleagues, Nadeen Balqis and Khaled Herzallah, for their great support.

Abstract

Myeloproliferative Neoplasms (MPN) represent a group of clonal hematologic disorders characterized by aberrant proliferation of myeloid cells in the bone marrow. One class of MPNs is classical Philadelphia-negative MPN, which is caused by several driver mutations, with the most common one being JAK2V617F mutation, leading to constitutive activation of the JAK-STAT pathway. The tumor suppressor WW domain-containing oxidoreductase (*WWOX*), located in one of the most active fragile sites in the human genome (FRA16D), is known to play a significant role in cancer suppression. Alterations in *WWOX*, especially null mutations resulting in the loss of the protein, along with epigenetic modifications involving the methylation of the *WWOX* gene's CpG island, have been identified in various types of cancer. However, *WWOX* alteration and its direct contribution to MPN development and progression remain largely unknown. This study aims to investigate the role of the *WWOX* gene in JAK2V617F mutation and Philadelphia-negative myeloproliferative neoplasms. Our findings reveal diverse WWOX protein expression patterns across MPN cell lines, indicating potential subtype-specific alterations, with detectable WWOX expression in UKE1 and HEL cell lines exhibiting varying levels, while notably absent in the SET2 cell line. This heterogeneity in WWOX expression underscores the complexity of MPN subtypes. To investigate the role of WWOX in this MPN, we manipulated its expression in SET2 and UKE1 cell lines using both overexpression and CRISPR-knockout techniques respectively. We then employed a combination of Sanger sequencing, RT-PCR, western blotting, and proliferation assays to show our findings. Surprisingly, *WWOX* overexpression led to elevated levels of phospho-ERK and phospho-STAT3 (Y705), along with a reduction in cell viability and an increase in the expression of BCL2 proteins. Conversely, *WWOX* knockout resulted in reduced levels of phospho-ERK, challenging our initial expectations regarding phospho-ERK and phospho-STAT3

(Y705). These findings suggest a potential tumor suppressor role for both WWOX, STAT3 and ERK in MPNs. Further research is needed to elucidate the underlying molecular mechanisms of these tumor suppressor functions. Importantly, understanding the complex interplay between WWOX, JAK-STAT signaling, and MPN development could have significant implications for the diagnosis and treatment of these disorders.

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List of Abbreviations

MPN	Myeloproliferative Neoplasms
WWOX	WW domain-containing oxidoreductase
HSC	Hematopoietic Stem Cell
CML	Chronic Myeloid Leukemia
PV	Polycythemia Vera
ET	Essential Thrombocythemia
PMF	Primary Myelofibrosis
CNL	Chronic Neutrophilic Leukemia
CEL	Chronic Eosinophilic Leukemia
MPN-U	Unclassifiable Myeloproliferative Neoplasms
JAK2	Janus Kinase 2
V617F	Valine to Phenylalanine substitution at position 617
CALR	Calreticulin
MPL	myeloproliferative leukemia virus
WHO	World Health Organization
AML	Acute Myeloid Leukemia
ER	endoplasmic reticulum
TPOR	thrombopoietin receptor
EPOR	erythropoietin receptor
G-CSFR	granulocyte colony-stimulating factor receptor

STAT	signal transducer and activator of transcription protein
SH2	Src homology 2 domains
MAPK	mitogen-activated protein kinase
PI3K	phosphoinositide 3-kinase
SOCS	suppressors of cytokine signaling
CFS	chromosomal fragile site
CNVs	copy number variants
Mbp	million base pairs
ORF	open reading frame
NLS	nuclear localization signal
SDR	short-chain alcohol dehydrogenase/reductase
JNK	c-Jun N-terminal kinase
Zfra	Zinc finger-like protein that regulates apoptosis
CREB	cAMP response element-binding protein
TNBC	triple-negative breast cancer
HCC	hepatocellular carcinoma
CRISPR	clusters of regularly interspaced short palindromic repeats
OE	Overexpression
KO	Knockout
cDNA	Complementary DNA
PCR	Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate

FBS	Fetal Bovine Serum
ND	N-terminal domain
CC	coiled-coil domain
DBD	DNA-binding domain
LK	helical linker domain
SH2	Src homology 2
TAD	transactivation domain
ERK	Extracellular signal-regulated kinase 1/2
MAPKAPK	MAPK-activated protein kinases
BAD	BCL2 associated agonist of cell death
BAX	BCL2 associated X
LIF	Leukemia Inhibitory Factor

Chapter 1: Introduction

1.1. Myeloproliferative Neoplasms

Hematological cancers, also known as hematologic malignancies or blood cancers, are a group of cancers that originate in the cells of blood-forming tissues, primarily the bone marrow and the lymphatic system. These cancers affect the production and function of blood cells, including red blood cells, white blood cells, and platelets. One of these hematological malignancies is Myeloproliferative Neoplasms (MPN). MPNs include a heterogeneous group of clonal disorders arising from hematopoietic progenitors. These chronic and rare blood disorders involve abnormal overproduction of various blood cell types within the bone marrow, leading to the accumulation of mature myeloid cells in the bone marrow and peripheral blood. MPNs originate within the hematopoietic stem cell (HSC) compartment when a single HSC undergoes somatic mutations, including the most common mutation JAK2V617F. These mutations confer a specific advantage to the altered HSC over regular HSCs, driving the development of myeloid cells and resulting in a myeloproliferative phenotype. [1–3] A recent classification, introduced by WHO in 2016, divides MPN into four distinct sub-groups:

(i) Chronic Myeloid Leukemia (CML) with a positive BCR-ABL1 mutation; (ii) Classical Philadelphia-negative MPN, which include Polycythemia Vera (PV), Essential Thrombocythemia (ET), and Primary Myelofibrosis (PMF); (iii) Non-classical Philadelphia-negative MPN, including Chronic Neutrophilic Leukemia (CNL) and Chronic Eosinophilic Leukemia (CEL); and finally, (iv) Unclassifiable MPN (MPN-U).[4,5]

PV, a complicated disorder, displays distinct hematological hallmarks driven by JAK2 gain-of-function gene mutations. These genetic alterations lead to erythrocytosis, granulopoiesis, and

thrombopoiesis, often ending in thrombotic complications.[1] The classic PV presentation involves a rise in red blood cell mass, hematocrit, and leukocyte count, frequently accompanied by splenomegaly and sometimes myelofibrosis. Diagnosis typically hinges on the presence of splenomegaly, leukocytosis, and thrombocytosis, with the JAK2V617F mutation detected in around 95% of PV cases; exon 12 JAK2 mutations also present in some JAK2V617F negative cases. Clinically, PV stands out for its heightened susceptibility to early-phase thrombotic events alongside myeloproliferative neoplasms. The disease's instability adds complexity, as it can progress toward myelofibrosis or even leukemia. [6–9]

ET arises from isolated thrombocytosis. While it is typically observed in individuals over 60 years, it can also manifest in younger individuals, predominantly females, constituting around 20% of cases below 40 years.[10–12] Patients with ET, characterized by persistent thrombocytosis, may experience arterial or venous thrombosis similar to PV, occasionally accompanied by splenomegaly and leukocytosis, though less frequently than in PV.[13,14] Diagnosis depends on elevated platelet counts, supported by marrow biopsies displaying amplified megakaryocytic hematopoiesis. The majority of ET cases are driven by mutations in the JAK2 transduction pathway (JAK2V617F), calreticulin (CALR), or myeloproliferative leukemia virus (MPL), all resulting in constitutive activation of JAK2 signaling pathways. However, approximately 10% of patients progress to myelofibrosis (MF). Additionally, there is a possibility of secondary transformations to Acute Myeloid Leukemia (AML), which carries an unfavorable prognosis.[1]

PMF is characterized by bone marrow fibrosis, splenomegaly, and inflammatory cytokine production, attributed to mutations in JAK2V617F, CALR, or MPL. Notably, a significant proportion of patients, around 50–60%, carry JAK2V617F mutations, which underpin the pathogenesis of PMF. The progression potential of PMF to acute leukemia aligns it with related

conditions in terms of mutational origins and disruptions in hematopoiesis. Unlike PV or ET, PMF significantly shortens life expectancy.[1] The marrow landscape of PMF reveals erythroid-megakaryocytic hyperplasia, neo-angiogenesis, and osteosclerosis. Typically diagnosed after the age of 60, PMF exhibits a male bias, paralleling PV more than ET.[15,16] Clinical indicators include an array of symptoms including anemia, fever, and cachexia and bone pain, pruritus, and splenomegaly. Blood analysis unveils distinctive features like immature granulocytes, nucleated red cells, and abnormal teardrop-shaped erythrocytes. Marrow examination shows magakaryocytosis accompanied by reticulin and/or collagen fibrosis, typically of grade 2 or 3.[17] Around 20% of PMF cases progress to AML within a few years, while other case experience various complications including cardiovascular problems accompanied by consequences of cytopenias like bleeding or inflammation.[18]

1.2. Molecular basis of Classical Philadelphia-negative MPN (PV, ET, and PMF)

The molecular basis of Classical Philadelphia-negative MPN involves a complex interplay of somatic driver mutations, epigenetic alterations, and genetic predisposition factors that collectively contribute to the pathogenesis, diagnosis, and potential treatment of these disorders. The clinical characteristics of these conditions (PV, ET, and PMF) exhibit a degree of overlapping in clinical features where one MPN type is able to transform into another. This suggests a common pathogenesis in these MPNs.

Somatic driver mutations play a pivotal role in the sustained activation of the JAK2-STAT signaling pathway within MPN. A clear example is the JAK2 V617F mutation, unveiled in 2005, which is predominant in the majority of MPN patients.[19–21] This mutation is present in approximately 95% of PV cases and 50-60% of ET and PMF cases. A G to T transition in exon 14

of JAK2 gene induces a valine-to-phenylalanine alteration at codon 617 of the JAK2 protein. Consequently, the inhibitory effect of the JAK2 pseudokinase domain on the kinase domain is abolished and JAK2 remains constitutively activated, driving continuous signaling via the transcription factors STAT1, STAT3, and STAT5, as well as the PI3K/AKT and MAPK pathways. This activation fuels the persistence of myeloid progenitor cells, influencing their proliferation, differentiation, and survival, thereby contributing significantly to MPN progression.[22,23] JAK2 V617F arises in hematopoietic stem cells and corresponds to the elevated levels of red blood cells, platelets, or white blood cells observed in MPN patients.[1] In addition to the V617F mutation, other mutations are reported in JAK2 exon 12 in MPN patients including several small insertions, deletions, or missense mutations. These exon 12 mutations are specific to PV, comprising about 2% to 3% of PV cases. Patients with JAK2 exon 12 mutations often exhibit marked erythrocytosis without concurrent thrombocytosis or leukocytosis.[24,25] Beyond JAK2 V617F, other driver mutations also shape the MPN landscape. One such mutation occurs in the MPL gene, specifically at position 515, affecting the thrombopoietin receptor MPL. This mutation is found in about 4% of patients with ET and in 5% to 9% of patients with PMF, but not in patients with PV. The most common variation of this mutation is W515L, although W515K and few other variants have also been observed. These mutations lead to continuous activation of the JAK2 signaling pathway, contributing to the development of MPNs.[26,27] In addition to JAK2V617F and MPL driver mutations, another significant somatic driver mutation occurs in the CALR gene. While CALR protein is primarily recognized for its function as an endoplasmic reticulum (ER) chaperone involved in glycoprotein folding and calcium balance, it has drawn attention due to its unexpected implication in cancer development. CALR mutations are mainly located in the C-terminal of exon 9, leading to frameshifts that result in the formation of novel C-terminal sequences. The two most

prevalent CALR mutations, CALRdel52 (type 1) and CALRins5 (type 2), interfere with the ER retention signal and activate the JAK2-STAT signaling pathway. These mutations perturb CALR function, causing altered localization and activation of JAK2 signaling. Distinct clinical features are linked with various categories of CALR mutations.[28,29]

Alongside the aforementioned three driver mutations, other genes are commonly mutated in MPN, particularly in advanced stages. Epigenetic modifiers like DNMT3A and TET2 are affected, impacting DNA methylation and gene expression.[30,31] Splicing factor mutations, such as SRSF2 and SF3B1, contribute to the disease by causing mRNA mis-splicing.[32,33] Signaling molecules like RAS isoforms are also involved, further shaping the disease phenotype.[34]

Familial clustering of MPN suggests a genetic predisposition, with about 5% of MPN patients having affected family members, indicating a hereditary link.[35] The 46/1 or GGCC haplotype, particularly in the JAK2 gene, increases the risk of sporadic or familial MPN by three to four times.[36,37] Other genes like TERT and MECOM also contribute to germline susceptibility.[38] Rarer variants, such as those in the RBBP6 involved in p53 function, are more specific to familial cases.[39]

1.3. JAK2-STAT pathway in MPN

Janus kinases (JAKs) are a family of non-receptor tyrosine kinases that exert significant control over immune responses, hematopoiesis, and various physiological processes. The JAK family comprises four members: JAK1, JAK2, JAK3, and Tyk2.[40] These kinases are integral components of cytokine receptors, such as those for interleukins, interferons, and other immune-modulating molecules. Structurally, JAKs consist of multiple domains, including the FERM domain, SH2-like domain, pseudokinase domain, and the active kinase domain. The FERM domain participates in receptor recognition and binding, while the SH2-like domain contributes to

stabilizing interactions with cytokine receptors. The pseudokinase domain, although lacking key catalytic residues, plays a crucial role in regulating JAK activity by modulating kinase domain function.[41,42]

JAK activation is initiated when cytokines or growth factors bind to their respective receptors, such as thrombopoietin receptor (TPOR or c-MPL), erythropoietin receptor (EPOR), and granulocyte colony-stimulating factor receptor (G-CSFR), leading to receptor dimerization and juxtaposition of JAKs. This spatial arrangement enables one JAK to phosphorylate tyrosine residues on the other JAK molecule, resulting in their activation. These activated JAKs phosphorylate tyrosine residues on the receptor's cytoplasmic tail, creating docking sites for signaling molecules with Src homology 2 (SH2) domains, such as signal transducer and activator of transcription proteins (STATs). STAT proteins are latent transcription factors that, upon tyrosine phosphorylation by JAKs, dimerize, translocate to the nucleus, and modulate gene expression (Fig.1).[43] Additionally, JAKs also activate other downstream signaling pathways, such as the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways, contributing to the diverse cellular responses triggered by cytokine signaling.[44] To ensure the effective termination of signaling, multiple proteins play a role in attenuating cytokine signaling at various stages of the pathway. Notably, a family of suppressors of cytokine signaling (SOCS) functions as inhibitors in the signaling cascade, providing negative feedback.[43] Aberrant JAK-STAT signaling is implicated in autoimmune disorders like rheumatoid arthritis and inflammatory bowel disease, as well as in certain malignancies like myeloproliferative neoplasms.[3,45] Consequently, JAK inhibitors have emerged as promising therapeutic agents to alleviate these diseases by specifically targeting dysregulated JAK activity and downstream signaling events.

JAK2, in particular, has garnered significant attention due to its involvement in various physiological and pathological processes. [3]

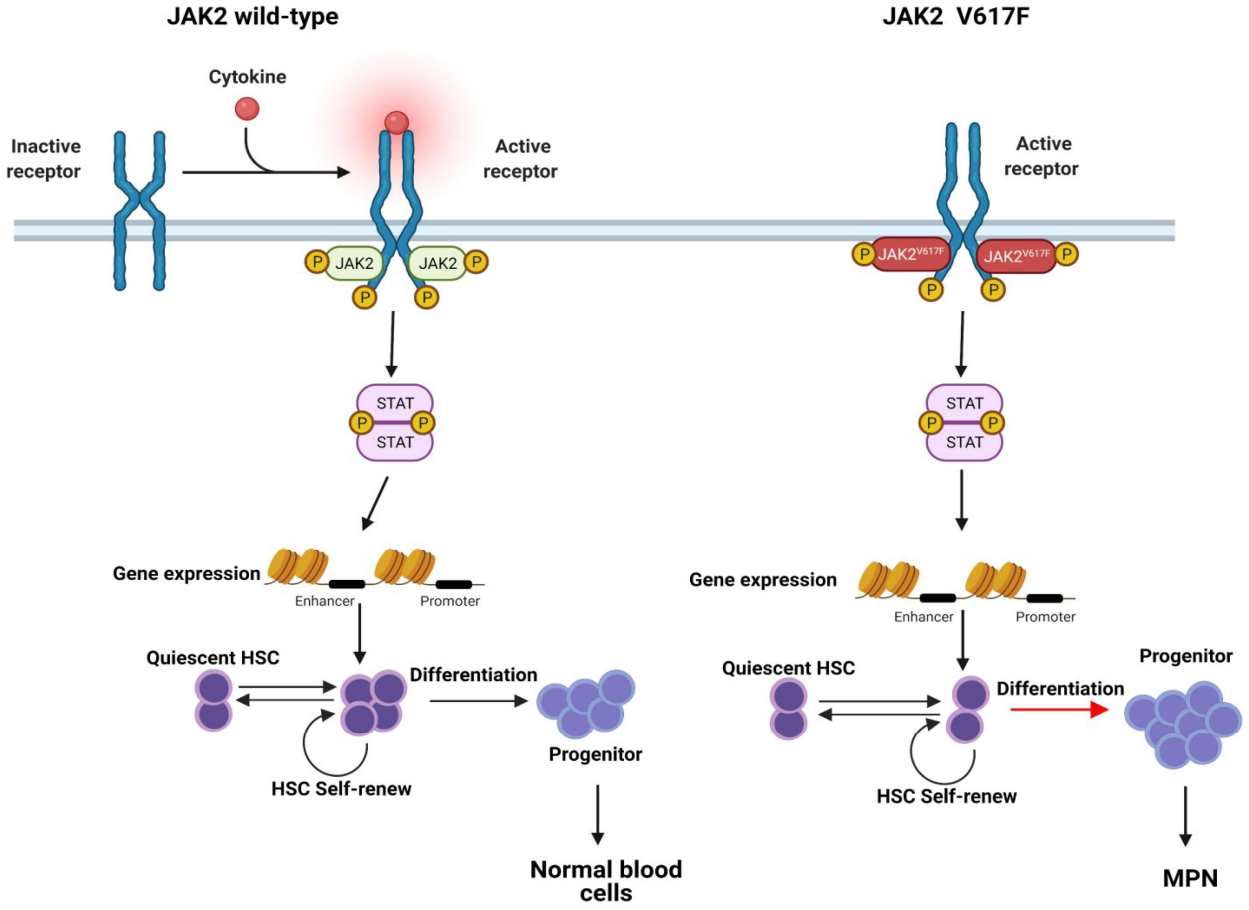


Figure 1: Dysregulation of JAK2-STAT Signaling in JAK2V617F. In the left panel (Wild-Type JAK2), normal signaling maintains cell balance. Cytokines and growth factors like EPO and TPO regulate cell fate for controlled blood cell formation. The right panel shows JAK2V617F mutation leading to constitutive JAK-STAT activation. This alters cell fate, promoting more progenitor cell development and MPN progression.[3]

1.4. The Dual Role of STAT3 in Cancer

The Signal Transducer and Activator of Transcription (STAT) protein family involves intracellular transcription factors that Coordinate numerous critical cellular processes, including immunity, proliferation, apoptosis, and differentiation. These proteins are predominantly activated via Janus kinases in association with membrane receptors.[46] Dysregulation of this pathway is a

prevalent occurrence in primary tumors, promoting enhanced angiogenesis that supports tumor survival and promotes immunosuppression. Within this protein family, seven distinct STAT proteins (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6) exist.

Each STAT protein has six functional domains: a helical N-terminal domain (ND) facilitating protein interactions, a coiled-coil (CC) domain governing regulatory interactions, a DNA-binding domain (DBD) for recognizing target genes, a helical linker (LK) domain for nuclear export and DNA binding, a Src homology 2 (SH2) domain for receptor binding and dimerization, and a C-terminal transactivation domain (TAD) responsible for transcriptional activation.[47,48]

Among the STAT proteins, STAT3 emerges as a particularly intricate transcriptional regulator with a pivotal role in cell proliferation, maturation, and survival. Activation of STAT3 occurs through a variety of cytokines. Aberrant STAT3 activation has been closely associated with malignant transformations across various cancer types. However, recent research has shed light on the dual function of STAT3 in cancer, where it can act as either a tumor promoter or tumor suppressor, depending on specific conditions.[49] The multifaceted role of STAT3, a key signaling protein, is profoundly influenced by various factors, particularly its integration into diverse signaling pathways within distinct oncogenic microenvironments. Notably, STAT3 function exhibits remarkable context-dependent variations, determined by its interactions with different signaling molecules. Additionally, different STAT3 isoforms contribute to this multifaceted role, with four primary isoforms - STAT3α, STAT3β, STAT3γ, and STAT3δ - possessing unique structural and functional characteristics that impact STAT3 diverse roles in cancer biology.[50,51] STAT3α, the full-length isoform, primarily drives the expression of genes associated with cancer hallmarks, making it a significant player in tumorigenesis.[52] In contrast, STAT3β acts as a repressor of STAT3 activity, inhibiting cancer progression by interfering with the activation of

specific genes and enhancing apoptosis in tumor cells, potentially overcoming chemoresistance.[53,54] For instance, in glioma cells with intact PTEN function, STAT3 exhibits tumor-suppressive activity by regulating genes associated with cell proliferation, migration, and survival. However, in the context of EGFRvIII-expressing gliomas and glioblastomas, STAT3 takes on an oncogenic role, promoting cancer progression.[55,56] In prostate cancer, STAT3 indirectly suppresses tumor progression by participating in the ARF-p53 pathway.[57] In colorectal cancer, STAT3 serves as a tumor suppressor by inhibiting the expression of pro-proliferative genes.[58] Moreover, STAT3 role in thyroid cancer is complex, as it can induce aerobic glycolysis or suppress tumor growth depending on the cellular context.[59]

In the context of MPN characterized by the JAK2V617F mutation, STAT3 plays a significant role. This was demonstrated through experiments involving conditional STAT3 knockout (STAT3 floxed) and JAK2V617F knock-in mice. The findings revealed that the deletion of STAT3 led to an increase in neutrophil counts in peripheral blood and promoted the expansion of hematopoietic progenitor cells and myeloid precursor cells within the bone marrow and spleens of JAK2V617F knock-in mice. Additionally, STAT3 deletion had a marked negative impact on the survival rates in a mouse model of MPN driven by the JAK2V617F mutation. Furthermore, the elimination of STAT3 in hematopoietic cells resulted in elevated JAK2-V617F-induced thrombocytosis, possibly due to increased expression and activation of STAT1.[60,61] So, STAT3 role in cancer is complicated and context-dependent, spanning from tumor promotion to tumor suppression, depending on specific genetic and environmental factors within different cancer types.

1.5. The Dual Role of ERK in Cancer

Extracellular signal-regulated kinase 1/2 (ERK) belongs to the mitogen-activated protein kinase (MAPK) family, responsible for transmitting signals from the extracellular environment to intracellular targets. This signaling cascade comprises several layers of protein kinases, including MAP4K, MAP3K, MAPKK, MAPK, and MAPK-activated protein kinases (MAPKAPK). The first three layers form the core unit of this intricate pathway.[62] In its default state within normal, unstimulated cells, ERK1/2 resides in the cytoplasm. Upon activation, ERK1/2 translocate to the nucleus, where it plays a crucial role in regulating the activity of transcription factors through phosphorylation. This nuclear activity, triggered by extracellular stimuli, influences gene expression, impacting cell metabolism and function.[63,64] Simultaneously, in the cytoplasm, ERK phosphorylates various cytoskeletal proteins, exerting influence over cellular processes like cell movement, trafficking, metabolism, adhesion, and the regulation of other signaling pathways.[65] ERK signaling is a central player in tumorigenesis. Elevated ERK expression has been detected in numerous human tumors, including ovarian, gastric, and breast cancer. Activation of the ERK/MAPK pathway is closely associated with tumor cell proliferation, dedifferentiation, and resistance to apoptosis. It promotes cell survival and proliferation while inhibiting apoptosis, a critical factor in uncontrolled cell growth characteristic of tumors. Furthermore, this pathway plays a pivotal role in tumor invasion and metastasis by regulating adhesion, degradation, and migration of tumor cells. It can also promote angiogenesis by upregulating factors like VEGF.[66–69]

The MAPK/ERK pathway has dual role in cancer, acting as both an oncogene and a tumor suppressor. Its impact on cancer development varies based on the specific tissue microenvironment. In some contexts, it promotes cellular proliferation and invasion, while in others, it induces cellular apoptosis and counters pro-oncogenic signals from other pathways.[70]

One mechanism through which the ERK/MAPK pathway can exhibit tumor-suppressive activity is by inducing cellular senescence. This process involves ERK-mediated proteasome-dependent degradation of proteins crucial for cell cycle progression, mitochondrial functions, cell migration, RNA metabolism, and cell signaling. Inhibition of ERK/MAP kinase signaling prevents this senescence mechanism and allows oncogenic transformation, particularly in the presence of constitutively activated growth factor signaling pathways.[71] Within the context of breast cancer, ERK1 and ERK2 exhibit distinct roles. ERK1 functions as a negative regulator of breast cancer progression by suppressing the YAP1 signaling pathway. Silencing ERK1 leads to an increase in YAP1 expression and TEAD activity, promoting the proliferation of breast cancer cells. Interestingly, higher expression of ERK1 is associated with a more favorable prognosis in breast cancer patients, whereas higher expression of ERK2 predicts poorer prognosis.[72] The balance between MEK1 and MEK2 activity significantly influences cell cycle progression within the context of the ERK/MAPK pathway. MEK1 primarily serves as a promoter of cellular proliferation, driving cells through the cell cycle. In contrast, MEK2 plays a distinct role by triggering growth arrest or senescence, essentially acting as a checkpoint mechanism to halt cell division and induces growth arrest at the G1/S boundary. Intriguingly, when MEK1 or MEK2 is individually knocked down, it leads to divergent outcomes. MEK1 deficiency results in the sustained activation of ERK, which, paradoxically, leads to cell growth arrest. Conversely, MEK2 depletion delays the onset of mitosis and initiates a cascade of cellular changes. These findings underscore the unique and complementary roles of MEK1 and MEK2 in fine-tuning ERK activity and modulating cell cycle progression.[73,74]

In the context of myeloproliferative neoplasms (MPNs), ERK2 has been found to play a dual role in JAK2V617F-driven MPNs, manifesting these dual roles through its substrate-binding domains.

Specifically, the ERK2-docking (ERK2-D) domain has emerged as a potential therapeutic target that promotes cancer, showing promise in suppressing MPN development. In contrast, the ERK2-DEF–binding pocket (ERK2-DBP) domain hinders disease progression in a mouse model, opposing the pathogenesis of MPN and promoting JAK2-mediated oncogene-induced senescence by promoting the physical interaction of ERK2 with the transcription factor Egr1.[75]

1.6. WWOX gene

The WW domain-containing oxidoreductase (*WWOX*) gene is located on chromosome 16 at the genomic locus 16q23.3-24.1.[76] This genetic locus is of particular interest due to its fragile site, FRA16D, which is known as the second most prevalent chromosomal fragile site (CFS) within the human genome. This region, prone to genomic instability, exhibits susceptibility to chromosomal breaks, thereby contributing to the emergence of germline and somatic copy number variants (CNVs). *WWOX* is recognized as one of the largest human genes, spanning an extensive region of 1.1 million base pairs (Mbp), consisting of nine exons with an open reading frame (ORF) of 1245 base pairs long, and producing a spliced 2.2 kb mRNA. [76–78]

WWOX gene encodes the human WW domain-containing oxidoreductase, also referred to as *WWOX* or *FOR*, as well as its murine counterpart *WOX1*.[76,78,79] The *WWOX* gene encodes a tumor suppressor protein consisting of 414 amino acids. The WWOX protein consists of distinct functional domains: two N-terminal WW domains (contain two conserved tryptophan residues), a nuclear localization signal between the WW domains, a C-terminal SDR domain, and a proapoptotic C-terminal tail termed D3 (Fig.2). The N-terminal WW domains are crucial for mediating various protein-protein interactions, specifically for binding to proteins containing proline-rich motifs (PPxY) and others, which is essential for its function. Meanwhile, the C-

terminal SDR domain is involved in catalyzing oxidation and reduction reactions, regulating processes related to various molecules such as lipid hormones, alcohols, sugars, and retinoids. The interplay between these domains contributes to WWOX protein diverse functions, including tumor suppression and apoptotic signaling.[76,78,80,81]

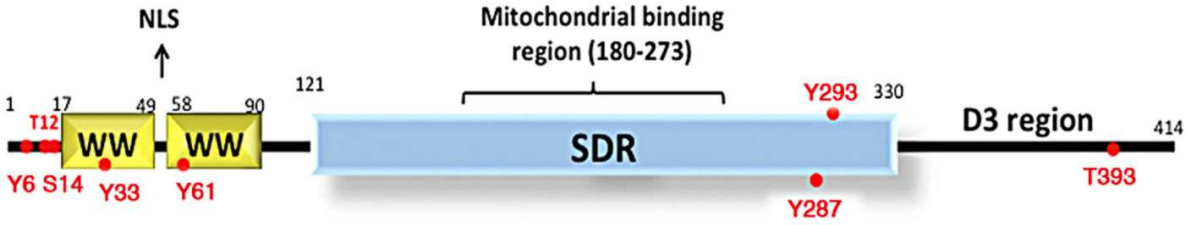


Figure 2: Schematic representation of the WWOX protein. WWOX is composed of two N-terminal WW domains, a nuclear localization signal (NLS), a C-terminal domain with short-chain alcohol dehydrogenase/reductase (SDR) activity, and a D3 region. Amino acids indicated in red dots are potential phosphorylation sites in the WWOX protein. [82]

WWOX is involved in various cellular processes, such as cell differentiation, proliferation inhibition, apoptosis induction, and suppression of tumor growth. [83–86] These functions are achieved through interactions with different proteins.[81,87] So far, more than 600 interactors of WWOX have been identified (https://thebiogrid.org/119707/summary/homo-sapiens/wwox.html, accessed date: 01 September 2023). The tumor suppressor activity of WWOX in various cell types relies on the presence of specific protein partners, including P73, AP-2γ, ErbB4, p53, c-Jun N-terminal kinase (JNK), Zinc finger-like protein that regulates apoptosis (Zfra), cAMP response element-binding protein (CREB), and others. WWOX exerts its ability to suppress protein activity by sequestering them in the cytoplasm, which consequently inhibits their transcriptional and oncogenic activities. Additionally, WWOX can also suppress protein activity by binding to their partners and competing for interaction with them.[84,88–94]

The *WWOX* gene plays a pivotal role in a range of conditions including cancer and neural diseases. Genomic loss plays a significant role in suppressing the expression of the *WWOX* gene.[95] Alterations in the *WWOX* gene, particularly null mutations that lead to the loss of protein expression, have been associated with various cancers such as breast[96–98], esophageal [99,100], ovarian [101], bladder [102], colon [103], liver[104], prostate[105], gastric carcinomas [106], and others. Epigenetic modifications involving methylation of the *WWOX* gene's CpG Island have also been observed in different types of cancer [107]. Additionally, as in prostate cancer, the WWOX protein can undergo degradation through polyubiquitination triggered by phosphorylation of tyrosine 287 by the Ack-1 tyrosine kinase.[108] Moreover, Loss of *WWOX* expression has been shown to contribute to the development of osteosarcomas, lung papillary carcinoma, and other malignancies in animal models.[109] In the context of cancer progression, the expression and activation of *WWOX* differ. While it is upregulated and activated in the early stages of certain cancers, its expression tends to decrease as cancer progresses toward more advanced and metastatic states.[110,111]

Interestingly, *WWOX* gene alterations are not limited to cancer but extend to neural diseases as well. Null mutations of *WWOX* have been linked to severe neural diseases like epilepsy, retinal degeneration, microcephaly, and ataxia, often leading to early death.[112–114] WWOX significance in neural health is emphasized by its role in neuronal survival. Studies suggest that under WWOX deficiency, protein conformation changes can occur, leading to protein aggregation associated with neurodegenerative processes.[115,116]

WWOX, an essential protein within cells, can undergo different forms of phosphorylation, which greatly impact its functions. When WWOX is phosphorylated at Tyr33 (pY33-WWOX), it exhibits a protective effect, suppressing cancer growth and supporting normal neuronal functions. This phosphorylation state is associated with inhibiting tumor growth and maintaining cellular

health. However, in Alzheimer's disease (AD) patients, there is a decrease in pY33-WWOX, potentially contributing to the development of AD-related brain changes. Conversely, when WWOX is phosphorylated at Ser14 (pS14-WWOX), it promotes cancer growth and AD progression. This differential phosphorylation state appears to have dual roles, both in supporting disease progression and, intriguingly, in facilitating the maturation of T cell leukemia.[117]

WWOX protein has been found to play a significant role in controlling oncogenic pathways, particularly the TGFβ1/SMAD [118], Wnt/β-catenin [119,120], and JAK2/STAT3 pathways.[121] Within the JAK2/STAT3 pathway, which is linked to various cancers and poor prognosis, WWOX was found to interact with both JAK2 and STAT3 through its WW1 domain. This interaction prevents the phosphorylation of JAK2 and STAT3, consequently inhibiting the transcriptional activity of STAT3. In the context of triple-negative breast cancer (TNBC), a notable observation is the inverse correlation between WWOX expression and phosphorylated STAT3 levels. Overexpression of WWOX into TNBC cell lines inhibits migration, tumor growth, and metastasis. Current data strongly indicates that the absence of WWOX contributes to TNBC metastasis through the JAK2/STAT3 axis (Fig.3).[121] Similarly, similar outcomes were identified in hepatocellular carcinoma (HCC). Studies have revealed that TSN (toosendanin) exerts its WWOX-dependent anti-HCC effects, inhibiting proliferation and metastasis. The mechanisms underlying TSN-mediated inhibition of proliferation and metastasis in HCC cells, as induced by WWOX, are likely based on the significant suppression of aberrant JAK2/STAT3 and Wnt/β-catenin activation.[122]

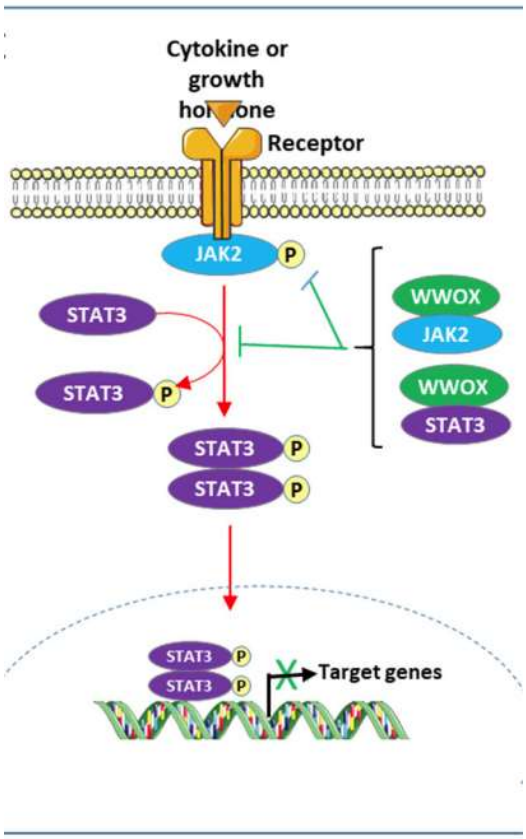


Figure 3: Schematic representation of inhibiting the transcriptional activity of JAK2/STAT3 pathways by WWOX in triple negative breast cancer.[130]

1.7. Study Problem, Aim, and Objectives

Myeloproliferative neoplasms (MPN) are hematological malignancies characterized by the constitutively active JAK-STAT pathway, which plays a pivotal role in driving disease progression. The *WWOX* gene has been identified as a tumor suppressor in various solid tumors and has been shown to interact with and inhibit JAK2 in triple negative breast cancer. However, the role of WWOX in MPN and its interaction with the JAK2-STAT pathway remain largely unexplored.

Aim: This study aims to investigate the potential tumor-suppressive role of WWOX in MPN through its interaction with the JAK-STAT signaling pathway or other signaling pathways.

The **objectives** of this study include:

1. Characterizing *WWOX* expression levels in MPN cell lines.
2. Investigating the influence of WWOX on the constitutively active JAK2-STAT pathway.
3. Manipulating *WWOX* expression using lentivirus vectors for overexpression and employing a cluster of regularly interspaced short palindromic repeats (CRISPR) approach for *WWOX* knockout to assess alterations in MPN cell phenotypes, including proliferation and cell death.
4. Analyzing changes in JAK2 downstream signaling molecules (STAT3 and ERK) phosphorylation levels in response to *WWOX* manipulation.
5. Investigating the impact of WWOX on the proliferation rate of MPN cell lines.
6. Examining the expression of downstream genes (LIF and BCL2 family).

By addressing these objectives, this study shed light on the potential tumor-suppressive functions of WWOX in MPN, providing valuable insights into the molecular mechanisms underlying MPN

development and progression. These findings could potentially pave the way for novel therapeutic strategies targeting the WWOX-JAK2 interaction.

Chapter 2: Materials and Methods

2.1. Cell lines

2.1.1 HEK293T Cell Line

Description: HEK293T cell line is a genetically modified version of the HEK293 cell line, derived from human embryonic kidney cells. It has been engineered to stably express the SV40 large T-antigen. HEK293T is a widely used model system for molecular and cell biology studies.

2.1.2 SET-2 Cell Line

Description: The SET-2 cell line was established from the peripheral blood of a 71-year-old woman with essential thrombocythemia at megakaryoblastic leukemic transformation in 1995. These cells carry the JAK2 V617F mutation and the DNMT3A R882H mutation. (https://www.dsmz.de/collection/catalogue/details/culture/ACC-608)

2.1.3 UKE1 Cell Line

Description: UKE1 cell line was established from a 59-year-old female patient with essential thrombocytosis that transformed into acute leukemia. These cells carry the JAK2 V617F mutation. (https://www.cellosaurus.org/CVCL_0104)

2.1.4 HEL Cell Line

Description: The HEL cell line was established from the peripheral blood of a 30-year-old man with erythroleukemia (AML M6) in relapse after treatment for Hodgkin lymphoma in 1980. These cells are known for their ability to spontaneously and induced globin synthesis and carry the JAK2 V617F mutation. (https://www.dsmz.de/collection/catalogue/details/culture/ACC-11)

2.1.5 KG-1 Cell Line

Description: The KG-1 cell line was established from the bone marrow of a 59-year-old man with erythroleukemia that developed into acute myeloid leukemia (AML) at relapse in 1977. (https://www.dsmz.de/collection/catalogue/details/culture/ACC-14)

2.1.6 NB-4 Cell Line

Description: The NB-4 cell line was established from the bone marrow of a 23-year-old woman with acute promyelocytic leukemia (APL = AML FAB M3) in the second relapse in 1989. These cells carry the t(15;17) PML-RARA fusion gene.
(https://www.dsmz.de/collection/catalogue/details/culture/ACC-207)

2.1.7 HL-60 Cell Line

Description: The HL-60 cell line was established from the peripheral blood of a 35-year-old woman with acute myeloid leukemia (AML FAB M2) in 1976.
(https://www.dsmz.de/collection/catalogue/details/culture/ACC-3)

2.2. Cell culture

HEK293T, HEL, KG1, NB4, and HL60 cells were grown in RPMI 1640 (Biological industries, Cat# 01-100-1A), supplemented with 10% fetal bovine serum (Gibco, REF:10270-106), 1% L-glutamine (Biological industries, REF:03-020-1A) , and 1% penicillin streptomycin (Gibco, Cat# 15140-122). UKE1 cells were grown in RPMI 1640 media supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin streptomycin, and 1 µmol/L hydrocortisone. SET2 cells

were grown in RPMI 1640 media supplemented with 20% fetal bovine serum, 1% L-glutamine, and 1% penicillin streptomycin.

2.3. Plasmid construction

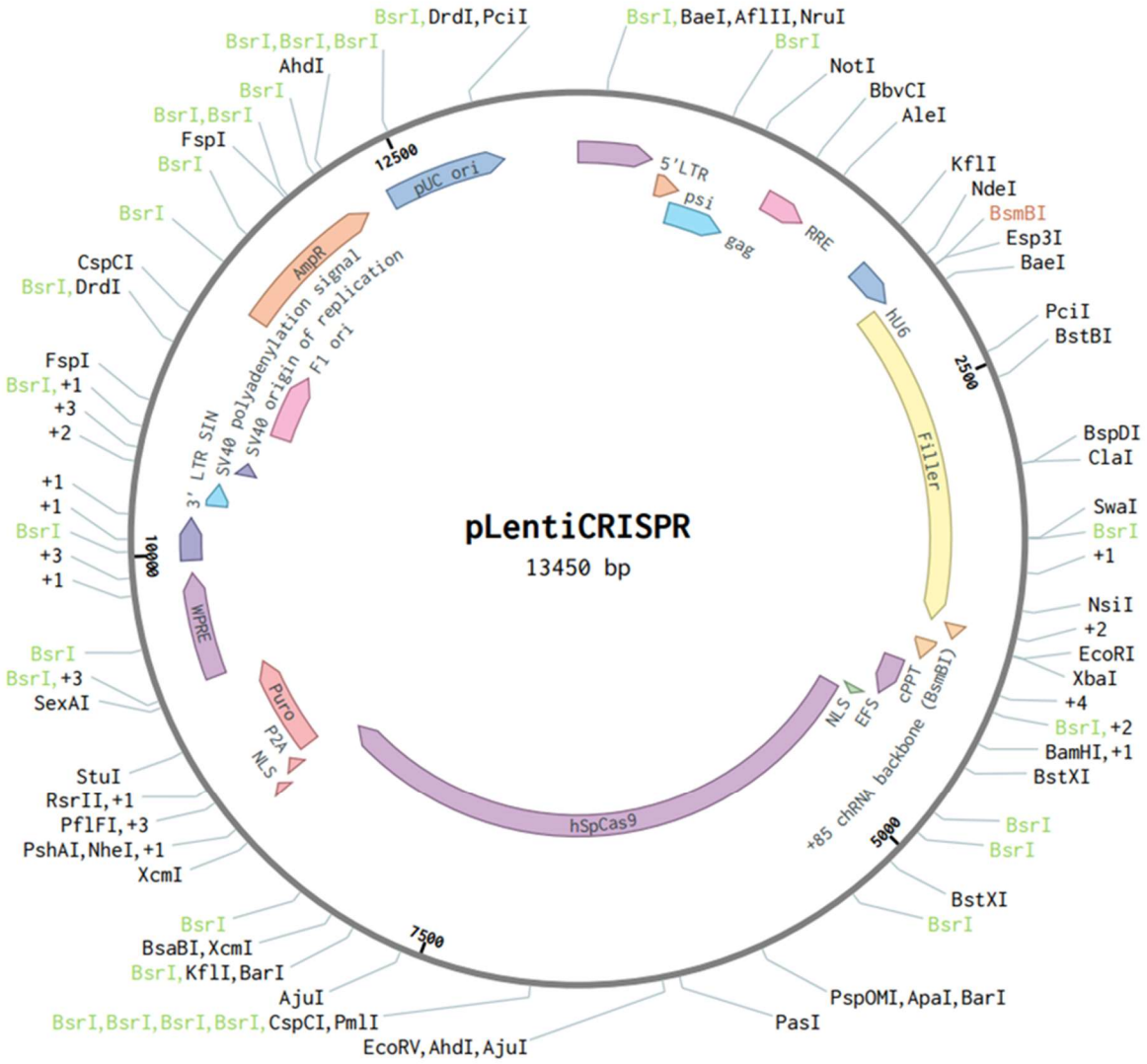
gRNA of WWOX (Forward: 5'-CACCGTGGTCACTGGAGCTAATTC, Reverse: 5'-AAACGAATTAGCTCCAGTGACCAC) was cloned into a pLenti CRISPR vector using standard protocols. In brief, the plasmid was digested by incubating 5µg of plasmid with 5µl BsmBI (Thermo Scientific, Cat# ER0452) and 10µl of 10X Tango buffer in a final volume of 100µl for 60 minutes at 55ºC. Next, the plasmid was dephosphorylated by adding 12.2µl of 10X phosphatase buffer and 10µl of Shrimp Alkaline phosphatase (TaKaRa, Cat#2660A), followed by incubation at 37ºC for 30 minutes. The resulting product was gel-purified, targeting the 11 kb band using the GenJET Gel Extraction Kit (Thermo Scientific, #K0691 #K0692).

The guides were then annealed and phosphorylated by incubating 100µM of oligo1, 100µM of oligo2, 1µl of 10X T4 Ligase buffer, and 0.5µl of T4 PNK (New England Biolabs, Cat# M0201S) in a total volume of 10µl for 30 minutes at 37 ºC, followed by a 5-minute incubation at 95 ºC and ramp down to 25 ºC at 5 ºC/min. This mixture was then diluted 1:200 with DW.

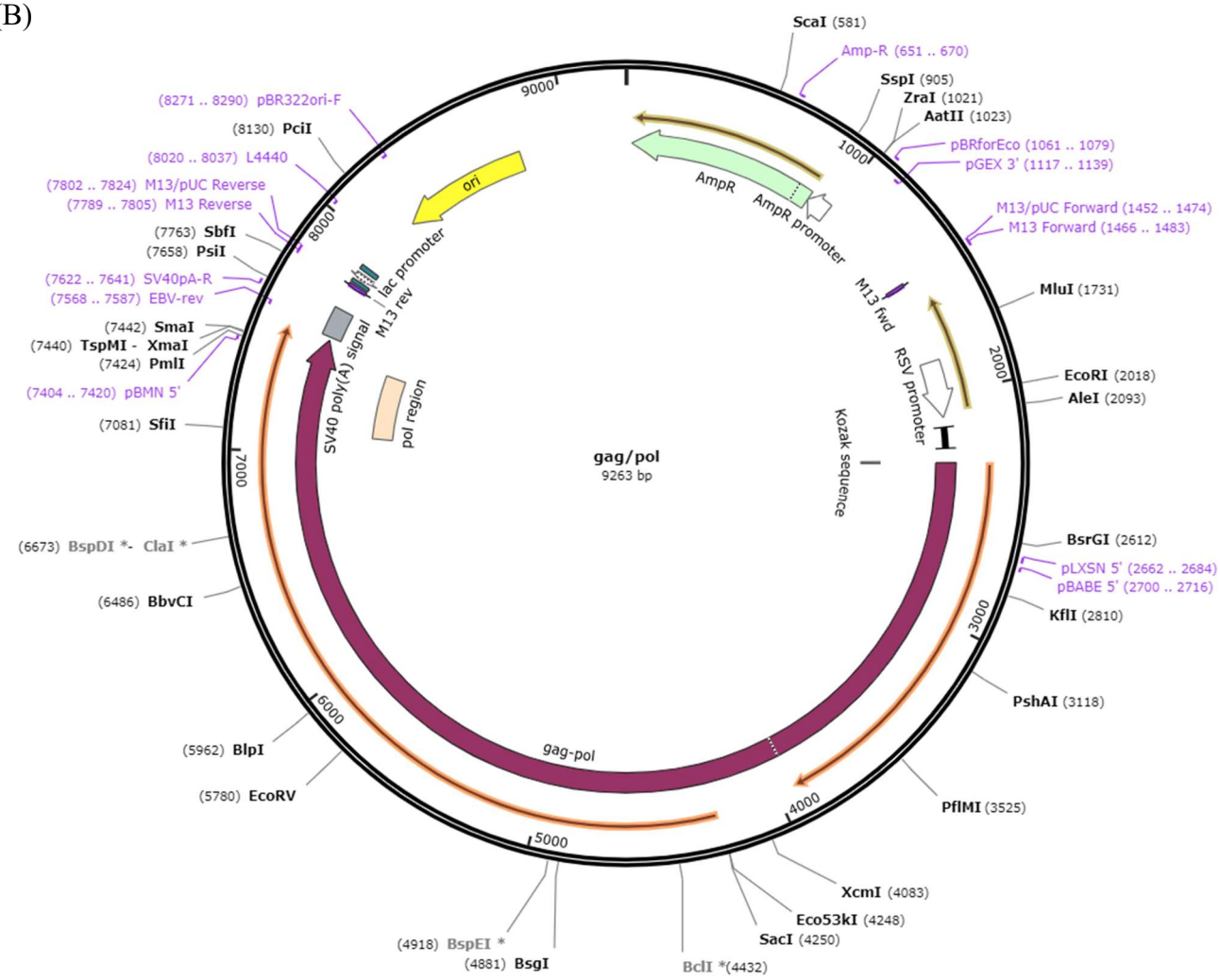
Subsequently, 50 ng of the prepared cut plasmid was incubated with 1 µl of the diluted oligo, 10µl of 2X ligase buffer, and 1µl of T4 ligase (New England Biolabs, Cat# M2200S) in a total volume of 20µl for 20 minutes at room temperature. The ligation product was transformed into DH5α competent cells by adding 10µl of the ligation reaction to 50µl of competent cells on ice for 30 minutes, followed by a heat shock for 45 seconds at 42 ºC and a 3-minute incubation on ice. The transformed cells were then spread on ampicillin LB plates and incubated at 37 ºC overnight. Finally, a Maxi-Prep was prepared using PureLink™ HiPure Plasmid Maxiprep Kit (Invitrogen,

Cat# K210007). For the Lenti-Att-neo WWOX, VSVG, and GAG-POL plasmids, they were provided by Dr. Zaidoun Salah. (Fig.4)

(A)



(B)


ScaI (581)
Amp-R (651 .. 670)
SspI (905)
ZraI (1021)
AatII (1023)
pBRforEco (1061 .. 1079)
pGEX 3' (1117 .. 1139)
M13/pUC Forward (1452 .. 1474)
M13 Forward (1466 .. 1483)
MluI (1731)
EcoRI (2018)
AleI (2093)
BsrGI (2612)
pLXSN 5' (2662 .. 2684)
pBABE 5' (2700 .. 2716)
KflI (2810)
PshAI (3118)
PflMI (3525)
XcmI (4083)
Eco53kI (4248)
SacI (4250)
BclI *(4432)
(4881) BsgI
(4918) BspEI *
(5780) EcoRV
(5962) BlpI
(6486) BbvCI
(6673) BspDI *- ClaI *
(7081) SfiI
(7424) PmlI
(7440) TspMI - XmaI
(7442) SmaI
(7404 .. 7420) pBMN 5'
(7568 .. 7587) EBV-rev
(7622 .. 7641) SV40pA-R
(7658) PsiI
(7763) SbfI
(7789 .. 7805) M13 Reverse
(7802 .. 7824) M13/pUC Reverse
(8020 .. 8037) L4440
(8130) PciI
(8271 .. 8290) pBR322ori-F
1000
2000
3000
4000
5000
6000
7000
8000
9000
ori
AmpR
AmpR promoter
M13 fwd
RSV promoter
Kozak sequence
gag/pol
9263 bp
gag-pol
pol region
SV40 poly(A) signal
M13 rev
lac promoter

(C)

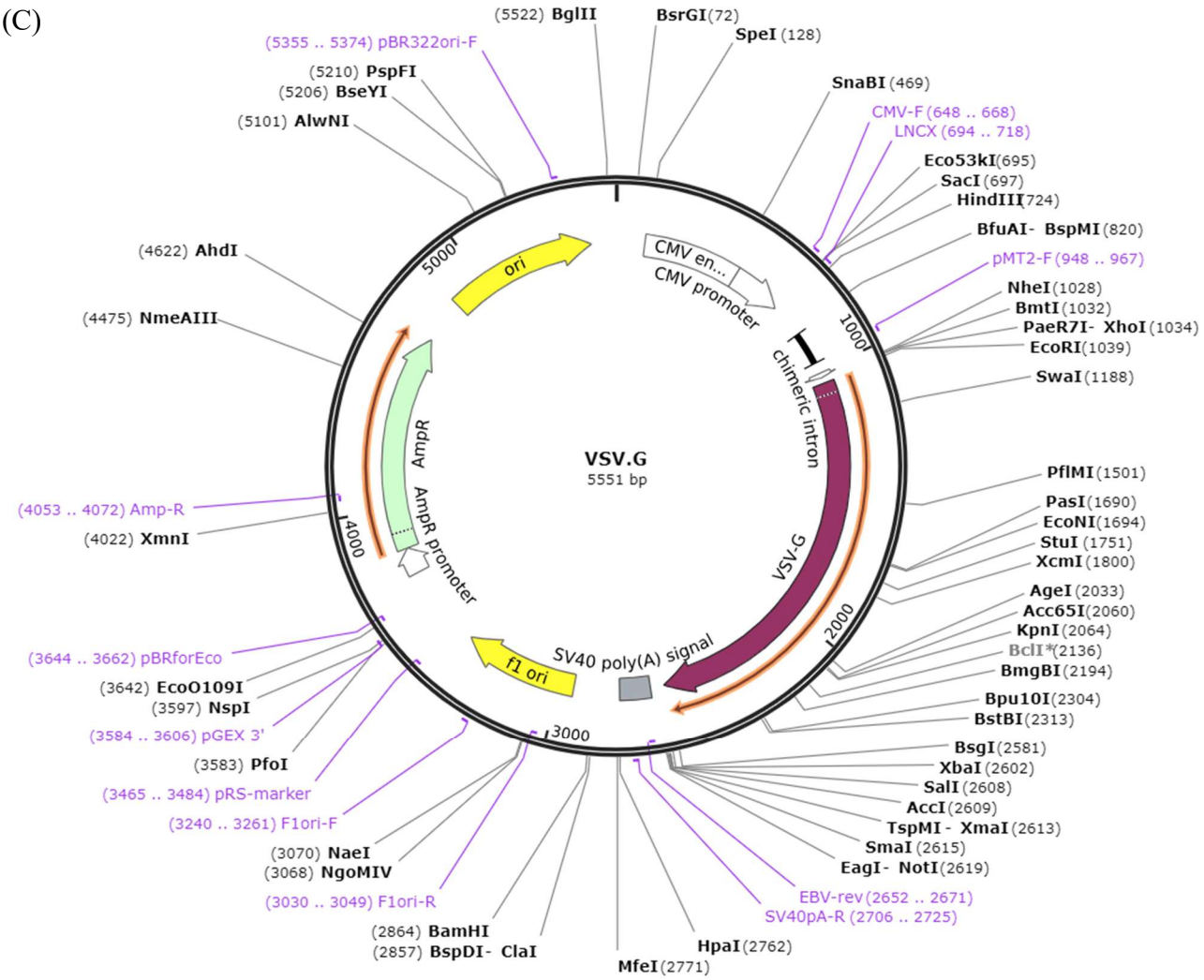


Figure 4: Plasmid Constructs Used in Experiments - CRISPR and Viral Packaging Genes

(A) PLenti CRISPR plasmid from Benchling accessed in 19-10-2023

(file:///C:/Users/user/Downloads/pLentiCRISPR-sequence%20(4).pdf)

(B) GAG/POL plasmid from Addgene website accessed in 19-10-2023

(https://www.addgene.org/browse/sequence/366616/)

(C) VSV-G plasmid from Addgene website accessed in 19-10-2023

(https://www.addgene.org/browse/sequence/221993/)

2.4. Preparation of lentivirus

Lentivirus particles were prepared by a three-plasmid expression system, in which HEK293T cells were co-transfected with the following three vectors: GAG-POL, VSV-G, and the transfer vector. One day before transfection, HEK293T cells were plated to 60% confluency. Next day, cells were fed with fresh medium and transfected with the three plasmids using TransIT®-LT1 (Mirus, Cat#MIR2300) transfection reagent. Medium was changed 24 hrs after transfection. On days 2 and 3 after transfection, medium was collected to recover viral particles. The collected medium was filtered and kept at-80°C.

2.5. RNA extraction and reverse transcription-PCR and Real Time PCR

Total RNA from UKE1 and SET2 cell lines and their clones was prepared using the TRIzol® Reagent (Thermo Scientific, Cat#15596026) as described by the manufacturer. One microgram of RNA was used for cDNA synthesis using First-Strand cDNA Synthesis kit (Thermo Scientific, Cat# K1642). Real-time PCR was performed to evaluate the expression of WWOX, LIF, BAD, and BAX using the PowerTrack™ SYBR Green Master Mix (Applied Biosystems, Cat# A46109). All measurements were conducted in triplicate and standardized to the levels of UBC expression, employing the primers listed in Table 1.

Table 1: Primers of WWOX, UBC, LIF, BAD, and BAX used for RT-PCR:

Gene	Primer type	5′-3′ primer sequence
WWOX	Forward	5′-TGAAGCAGTGTCACGCATTT-3'
	Reverse	5′-TGGTGAGACTCCAGGGTAGA-3'
UBC	Forward	5′-GTCGCAGTTCTTGTTTGTGG-3'
	Reverse	5′-GATGGTGTCACTGGGCTCAA-3'
LIF	Forward	5′-AACCAGATCAGGAGCCAACT -3'
	Reverse	5′-GTTGGGGCCACATAGCTTG -3'
BAD	Forward	5′-CTCCTTTAAGAAGGGACTTC -3'
	Reverse	5′-GATGTGGAGCGAAGGTCA -3'
BAX	Forward	5′-GGTTGTCGCCCTTTTCTACT -3'
	Reverse	5′-AAGTCCAATGTCCAGCCCAT -3'

2.6. DNA extraction

DNA was prepared from UKE1 clones using the Wizard® genomic DNA purification kit (Promega USA, Cat# A1120) as described by the manufacturer.

2.7. PCR

Amplification of the indicated DNA fragments was carried out in 20 µl reaction mix containing 10 µl of GoTaq® PCR Master Mix (Promega, Cat# M712B), 50 ng of DNA, and 1 µl from a 10 µmol primer mix (forward and reverse). The PCR was then run on a Thermal cycler (Biometra Tone, Analytikjena) using specific PCR conditions (Table 2 and Table 3).

Table 2: PCR protocol for amplification of WWOX (Exon 4):

PCR Conditions		
Initial denaturation	94º C , 3 minutes	
Second denaturation	94º C , 20seconds	33 cycle
Annealing	58º C, 30seconds	
Extension	72º C, 45seconds	
Final extension	72º C, 5 minutes	

Table 3: Primers used for WWOX and CRISPR plasmid:

Gene	Primer type	5′-3′ primer sequence
WWOX	Forward	5′-GTAAAACGACGGCCAGTCTGTGTTCATTGCTGTGGGT-3'
	Reverse	5′-AACAGCTATGACCATGCACAGGCTTCCATGACAACA-3'
Plenti CRISPR plasmid	Forward	5′- TTTCTTGGGTAGTTTGCAGTTTT -3'
	Reverse	5′- GTTGATAACGGACTAGCCTT -3'

2.8. Sanger sequencing

Sanger sequencing was performed as follows: PCR products were cleaned using the EPPIC Fast kit (A&A Biotechnology, Cat# 1021-500F). First, 1 µL of the EPPIC Fast enzymatic solution was added to 5 µL of the PCR product. The mixture was then placed in a thermal cycler at 37°C for 10 minutes, followed by 1 minute at 80°C. Subsequently, Sanger sequencing was conducted using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Cat# 4336774), following the manufacturer's instructions.

2.9. Western Blot

Total protein from all cell lines and clones was lysed by using lysis buffer containing 50 mM Tris (pH7.5), 150 mM NaCl, 10% glycerol, 0.5% Nonidet P-40 (NP-40), with addition of protease and phosphatase inhibitors. The lysates were subjected to SDS-PAGE under standard conditions. The antibodies used were polyclonal WWOX (ThrmoFisher; PA5-98916) (1:1000), monoclonal pERK1/2 (Thr202/Tyr204) (Cell Signaling; 4370) (1:2000), monoclonal ERK (Cell Signaling;

4695) (1:1000), monoclonal pSTAT3(y705) (abcam; ab76315) (1:2000), monoclonal STAT3 (abcam; ab68153) (1:1000), and polyclonal β-actin (abcam; ab8227) (1:10000).

2.10. XTT Test

Triplicate of 1.5 x 10^3 cells/well were seeded in a 96-well plate and incubated at 37°C with 5% CO2 for 24, 48, 72, 96, and 120 hours. Cell proliferation was assessed using a cell proliferation Kit (XTT-based) according to the manufacturer's instructions (cat# 20-300-1000). The absorbance of the samples was measured at 490 nanometers using an ELISA reader and compared against control blank.

2.11. Statistical analysis

Results were expressed as mean ± standard deviation (SD). The Student t-test was employed to compare values between the test and control samples, with statistical significance defined as $P < 0.05$. For real-time PCR analysis, we utilized the delta-delta-CT (ΔΔCT) method to quantify the fold change in gene expression.

Chapter 3: Results

3.1. WWOX Protein Expression Patterns in Philadelphia-Negative MPN Cell Lines with JAK2V617F Mutation

Alterations in the WWOX gene, particularly loss of its expression, have been associated with various cancers. We hypothesized that WWOX might also be altered in MPN. To investigate WWOX protein expression in Philadelphia-negative MPN cell lines with JAK2V617F mutation, we chose different MPN cell lines, including UKE1, SET2, and HEL. Additionally, we tested leukemic cell lines with wild-type JAK2, including NB4, KG1, and HL60. Cell lysates from each cell line were subjected to immunoblotting with WWOX antibody. The results revealed detectable WWOX expression in UKE1, HEL, KG1, NB4, and HL60 cell lines with varying levels. However, WWOX expression was notably absent in the SET2 cell line. These results suggest the potential involvement of WWOX alterations in cell lines associated with JAK2V617F MPN and emphasize the complexity of WWOX's role in various cancers (Fig.5).

3.2 Experimental Manipulation of WWOX Expression in JAK2V617F MPN Cell Lines

Building upon the results indicating the potential involvement of WWOX alterations in different cell lines associated with JAK2V617F MPN, we carried out further experiments to explore the functional implications of WWOX in these contexts. We selected the UKE1 and SET2 cell lines for our upcoming experiments because they both carry the JAK2V617F mutation. Furthermore, the SET2 cell line lacks WWOX protein expression, while the UKE1 cell line exhibits elevated levels of WWOX protein. Specifically, we employed two distinct strategies to modulate WWOX expression levels in different cell lines: overexpression (OE) in the SET2 cell

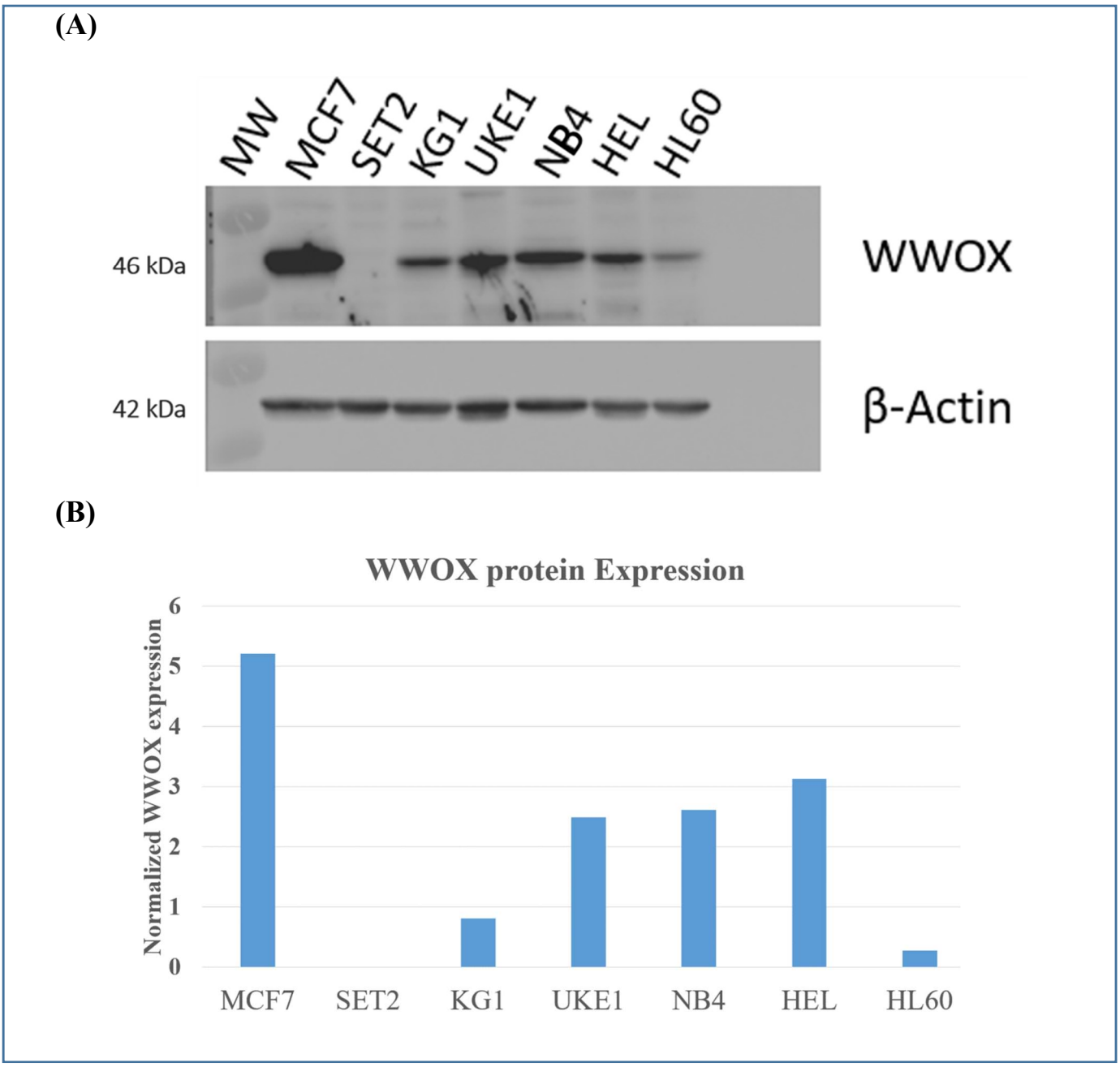


Figure 5: WWOX protein expression in MPN cell lines. (A) Western blot analysis of WWOX protein expression in various cell lines including SET2, KG1, UKE1, NB4, HEL, and HL60. MCF7 cells were utilized as the control for WWOX antibody, while β-actin served as the loading control. (B) Quantification of WWOX protein expression using ImageJ software, revealing relative expression levels across the analyzed cell lines.

line (which exhibited absent WWOX expression) and knockout (KO) in the UKE1 cell line (which showed high WWOX expression).

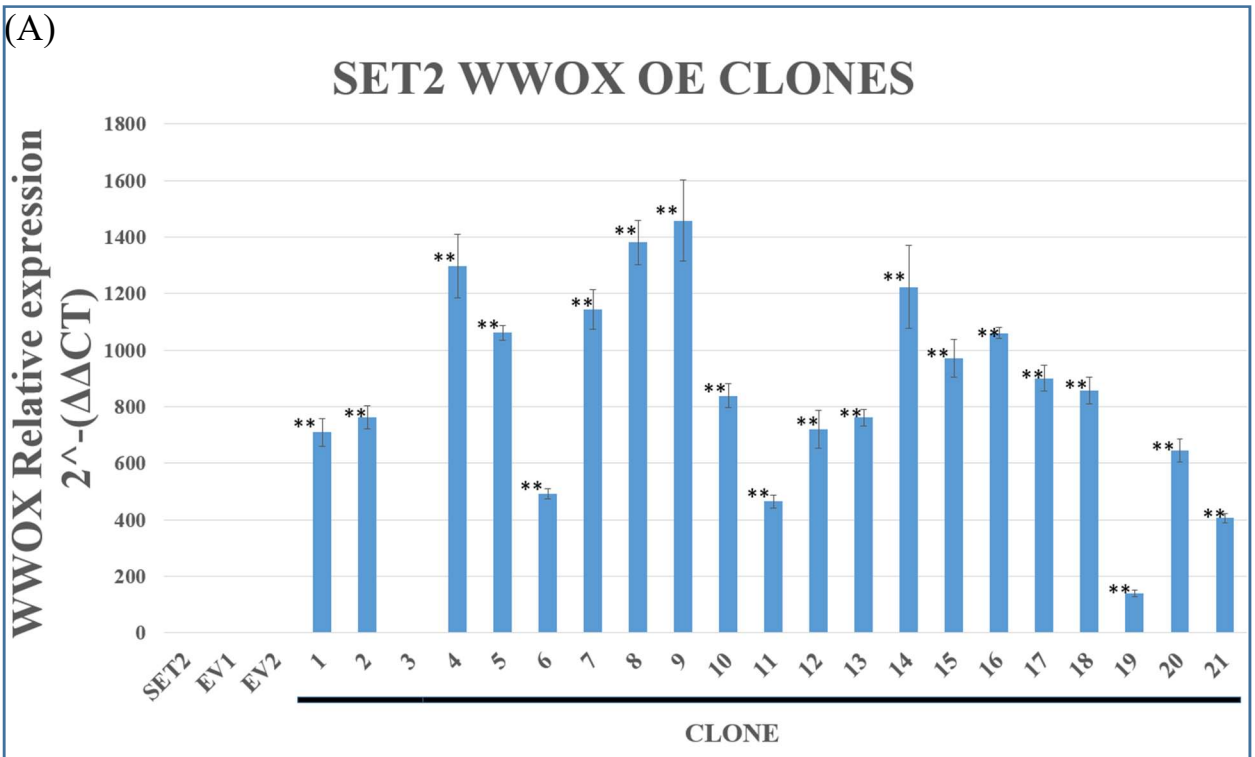
To enhance WWOX expression in the SET2 cell line, we employed a targeted overexpression approach. The SET2 cells were infected with a lentivirus carrying the WWOX gene (using the Lenti-Attneo WWOX plasmid). As a result, WWOX was successfully expressed in SET2 cell line clones. RT-PCR and WB confirmed the effective overexpression of WWOX in these SET2 clones (Fig.6-A, Fig.9-A). In contrast, to investigate the impact of WWOX knockout in UKE1 cell line, we employed a CRISPR based knockout technique (Fig.7). The UKE1 cells were infected with a lentivirus carrying the pLenti-CRISPR-WWOX plasmid. This resulted in the generation of UKE1 clones with suppressed WWOX expression due to gene knockout. The successful knockout of WWOX in these UKE1 clones was confirmed through a combination of Sanger sequencing, RT-PCR, and WB analyses (Fig.6-B, Fig.8, and Fig.9-B).

3.3 WWOX Expression Modulates ERK and STAT3 Activation in JAK2V617F MPN

After successfully manipulating the cell lines, we proceeded to assess the impact of these WWOX manipulations on the SET2 and UKE1 cell line phenotypes. The ERK signaling pathway and the JAK-STAT pathways are considered to play pivotal roles in cellular functions and have implications in MPN associated with the JAK2V617F mutation. Therefore, we decided to investigate the impact of these manipulations on these pathways.

Given the known involvement of the ERK signaling pathway in various cellular processes such as regulating cell proliferation, differentiation, and survival, we initially investigated the expression levels of ERK and Phospho-ERK in both manipulated cell lines using WB analysis.

(A)


SET2 WWOX OE CLONES
WWOX Relative expression
2^-(ΔΔCT)
1800
1600
1400
1200
1000
800
600
400
200
0
SET2
EV1
EV2
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
CLONE

(B)

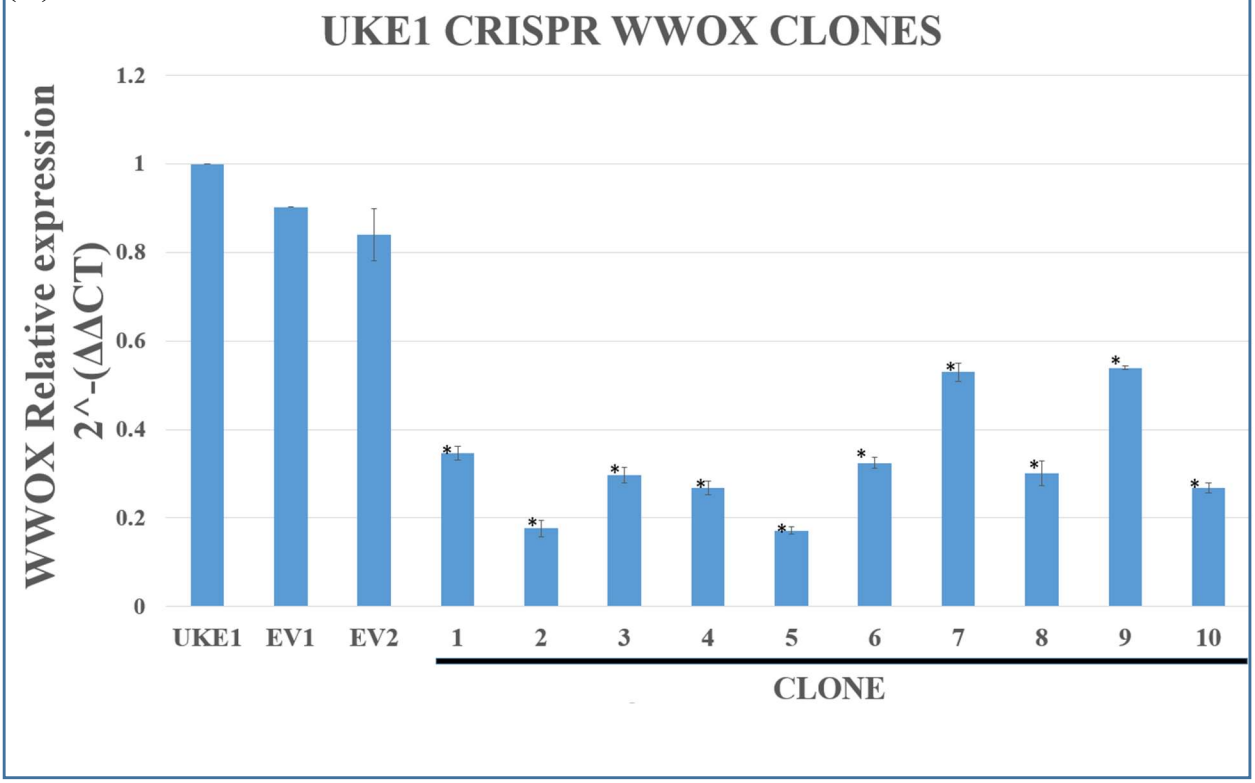

UKE1 CRISPR WWOX CLONES
WWOX Relative expression
2^-(ΔΔCT)
1.2
1
0.8
0.6
0.4
0.2
0
UKE1
EV1
EV2
1
2
3
4
5
6
7
8
9
10
CLONE

Figure 6: Validation of WWOX Overexpression and Knockout in manipulated cells(A) RT-PCR validates successful WWOX overexpression in SET2 clones through Lenti-Attneo-WWOX plasmid.SET2 cells were infected with Lenti-Attneo-WWOX virus. After 48 hours, cells were selected with 0.5 mg/ml G418 antibiotic twice during 6 days. Then clones were grown. DNA, RNA, and lysate were prepared for all of them. cDNA was prepared from RNA, RT-PCR for WWOX relative expression levels were normalized to UBC house keeping gene for all clones. The Final relative expression index for each SET2 WWOX OE clones were correlated relative to its EV control (B) RT-PCR validates successful WWOX Knockout in UKE1 clones through pLenti CRISPR WWOX plasmid. UKE1 cells were infected with pLenti CRISPR WWOX virus. After 48 hours, cells were selected with 1µg/ml puromycin antibiotic twice during 6 days. Then clones were grown. DNA, RNA, and lysate were prepared for all of them. cDNA were prepared from RNA, RT-PCR for WWOX relative expression levels were normalized to UBC house keeping gene for all clones. The Final relative expression index for each UKE1 WWOX KO clones were correlated relative to its EV control. Bars indicate standard error mean (SEM) of three replicates. The statistical significance of the results was determined by measuring the p value. (* indicates p-value<0.005,** indicates p-value<0.001)

The results demonstrated significant effects on Phospho-ERK1/2 (Thr202/Tyr204) in both cell clones. In SET2 clones that overexpressed WWOX, Phospho-ERK levels were notably elevated. In contrast, UKE1 clones with WWOX knockout exhibited a significant reduction in Phospho-ERK levels (Fig.9-A, B). These results suggest a potential regulatory role of WWOX in ERK signaling pathway.

Building upon the ERK pathway results, we turned our attention to the JAK-STAT pathway. Given the central role of this pathway in MPN driven by the JAK2V617F mutation, we investigated the activation levels of STAT3 in our manipulated cell clones. The results demonstrated significant effects on Phospho-STAT3 (Y705) mainly in SET2 clones. SET2 clones overexpressing WWOX exhibited a significant elevation in Phospho-STAT3 levels at Y705. However, in UKE1 clones with WWOX knockout, it was difficult to determine if the phospho-STAT3 had changed or not. This is because the level of phospho-STAT3 was already low in the

UKE1 parental and EV cells (Fig.9-C). These results suggest a potential crosstalk between WWOX and the JAK-STAT pathway through STAT3 or other protein that affect STAT3.

(A)

TTTCAGACCCACCTCCCAACCCCGAGGGGACCCAGAGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGAG
AGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAA
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GAGACGGTTGTAAATGAGCACACAAAATACACATGCTAAAATATTATATTCTATGACCTTTATAAAATCAACCAAAATCTTCTTTTTAATAACTTT
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(B)

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(C)

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Range 1: 4 to 160 Graphics ▼ Next Match ▲

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Figure 7: Validation of CRISPR-WWOX Cloning by Sanger Sequencing. (A) Original Plenti CRISPR Plasmid Sequence. This sequence displays a partial sequence of the Plenti CRISPR plasmid before cloning with the WWOX gRNA. The red nucleotide colors represent the "filler" region in the CRISPR plasmid, which will be excised during the cloning experiment. The black nucleotide colors represent the surrounding region of the target cloning site. The yellow nucleotide color represents the primer sequences flanking the cloning region. (B) Predicted Cloning Outcome. Illustration of the sequence expected after cloning. The yellow nucleotide colors represent the primer sequences flanking the cloning region. The black nucleotide colors represent the surrounding region of the target cloning site. The green nucleotide colors represent the WWOX gRNA that has been successfully cloned into the CRISPR plasmid. (C) Sanger Sequencing Results for CRISPR-WWOX Cloning. The sequencing results of the cloned Plenti CRISPR plasmid with WWOX gRNA are presented here, indicated in yellow. These results were obtained through the alignment of Sanger sequencing data from the cloned CRISPR-WWOX gRNA plasmid with the predicted sequence of the cloning experiment (as shown in “B”) using the NCBI BLAST website. The "sbjct" sequence represents the cloned CRISPR with the WWOX sequence. The "query" sequence represents the predicted sequence of our cloning experiment.

(A)

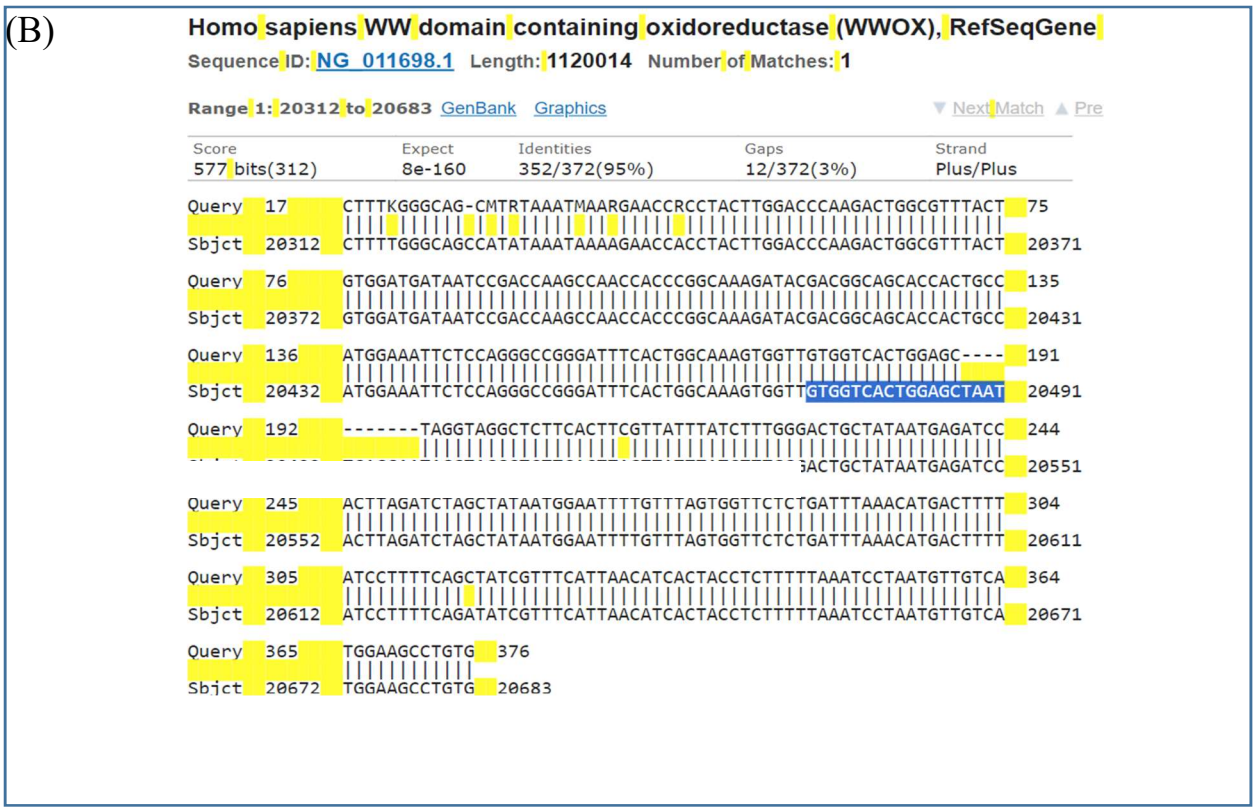
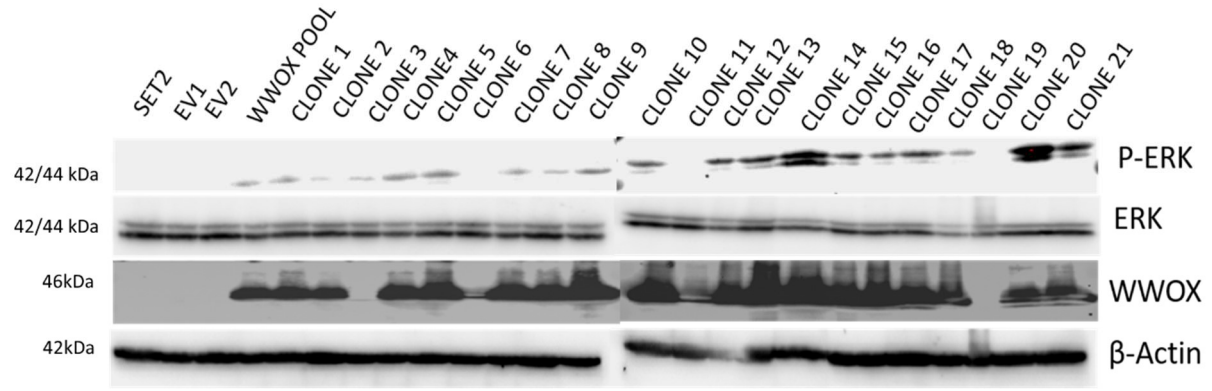


Figure 8: Validation of CRISPR-WWOX Knockout by Sanger Sequencing. (A): Sequencing Result of Representative UKE1 Plenti CRISPR EV. The result was obtained through alignment of Sanger sequencing data of UKE1 CRISPR EV with the WWOX gene (Sequence ID: NG_011698.1) using the NCBI BLAST site. The blue-highlighted sequence represents the gRNA used in the CRISPR cloning. The sbjct sequence represents the reference WWOX gene sequence (EXON 4 and its surrounding region) (Sequence ID: NG_011698.1), while the query sequence represents the WWOX gene (EXON 4 and its surrounding region) sequence for UKE1 Plenti CRISPR EV. (B): Sequencing Result of Representative UKE1 Plenti CRISPR WWOX Clone Number 4 . The result was obtained through alignment of Sanger sequencing data of UKE1 CRISPR WWOX clone number 4 with the WWOX gene (Sequence ID: NG_011698.1) using the NCBI BLAST site. The blue-highlighted sequence represents the gRNA used in the CRISPR cloning. The yellow-highlighted mismatches around the gRNA sequence confirm the occurrence of a frameshift deletion. The sbjct sequence represents the reference WWOX gene sequence (EXON 4 and its surrounding region) (Sequence ID: NG_011698.1), while the query sequence represents the WWOX gene (EXON 4 and its surrounding region) sequence for UKE1 Plenti CRISPR WWOX clone number 4.

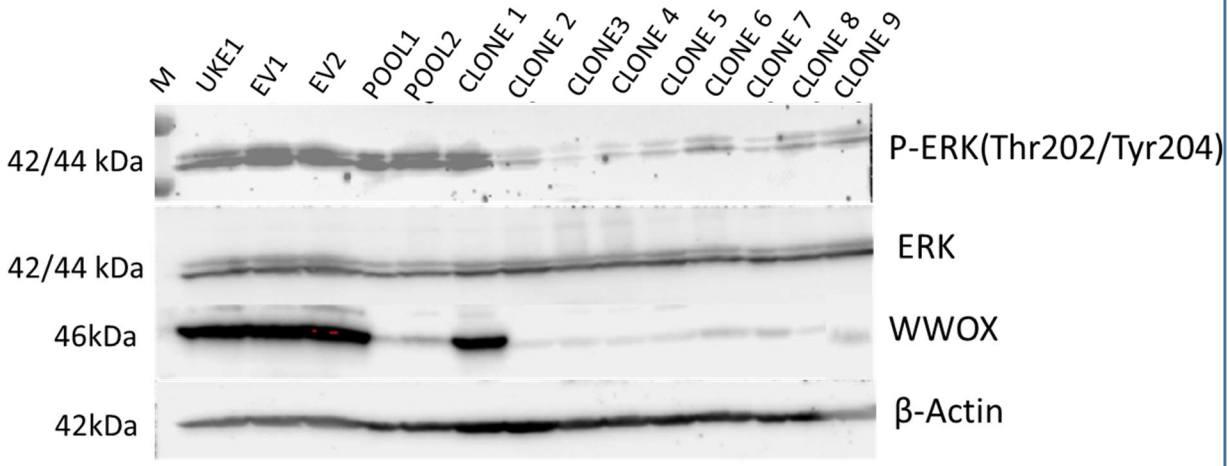
(A)

Elevated ERK Activation in SET2 WWOX OE Clones



(B)

Decrease ERK Activation in UKE1 WWOX KO Clones



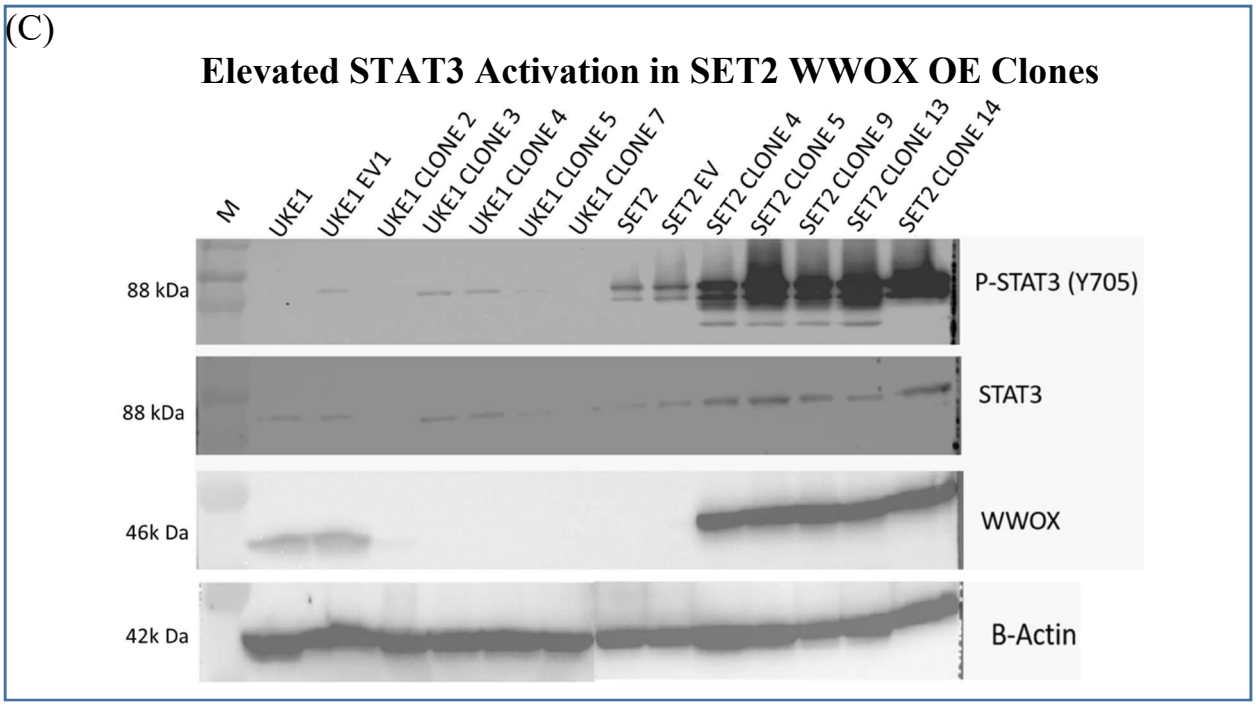


Figure 9: WWOX Expression Modulates ERK and STAT3 Activation in JAK2V617F MPN. (A) WWOX Overexpression Increases ERK activation in SET2. WB analysis was conducted using lysates from various cell samples, including SET2, SET2 Lenti Attneo EV1, SET2 Lenti Attneo EV2, SET2 Lenti Attneo WWOX POOL, and SET2 Lenti Attneo WWOX clone 1-21. Antibodies against WWOX, ERK, Phospho-ERK (Thr202/Tyr204), and β-actin were utilized for detection. Clones #3 and #19 were excluded from analysis. (B) WWOX Knockout Inhibits ERK activation in UKE1. Similar WB analysis was performed using lysates from different cell samples, including UKE1, UKE1 pLenti-CRISPR EV1, UKE1 pLenti-CRISPR EV2, UKE1 pLenti-CRISPR WWOX POOL1, UKE1 pLenti-CRISPR WWOX POOL2, and UKE1 pLenti-CRISPR WWOX clone 1-9. Antibodies against WWOX, ERK, Phospho-ERK (Thr202/Tyr204), and β-actin were used for detection. Clone #1 was excluded. (C) WWOX Expression Modulates STAT3 Activation in JAK2V617F MPN. Further WB analysis was carried out using lysates from UKE1 and SET2 cells. For UKE1, lysates from UKE1, UKE1 pLenti-CRISPR EV1, and UKE1 pLenti-CRISPR WWOX clones 2, 3, 4, 5, and 7 were analyzed. For SET2, lysates from SET2, SET2 lenti-att-neo EV1, and SET2 lenti-att-neo clones 4, 5, 9, 13, and 14 were used. Antibodies against WWOX, STAT3, Phospho-STAT3 (Y705), and β-actin were used for detection. β-actin serving as the loading control in all experiments.

3.4. WWOX Overexpression Suppresses Proliferation and Modulates Apoptosis-Related Gene Expression in MPN

After observing an elevation in phospho-STAT3 and phospho-ERK levels upon WWOX overexpression, both of which are known to influence cell proliferation, we proceeded to assess the proliferation status of the WWOX-overexpressed clones after a 120-hour interval. We chose

SET2 WWOX clone #4 as the representative WWOX-overexpressing clone. The results were intriguing: SET2 WWOX clone #4 exhibited 1.48-fold reduction in relative cell viability compared to SET2 EV, indicating a significant impact on proliferation. (Fig.10)

After obtaining significant results that demonstrated WWOX's ability to reduce proliferation in the SET2 cell line, we proceeded to investigate the expression of target genes associated with apoptosis and proliferation in these cells. Given that Leukemia Inhibitory Factor (LIF) expression has been linked to key cancer hallmarks such as proliferation, metastasis, and chemoresistance across various cancer types[123,124], and considering the previously observed high level of LIF in SET2 cell lines[125], we conducted RT-PCR to assess its RNA levels following WWOX overexpression in the SET2 cell line. The results revealed a significant downregulation of LIF expression in SET2 cells overexpressing WWOX, with 3.8-fold reduction compared to SET2 EV. (Fig.11-A)

In addition to LIF, we determined the level of RNA expression of BCL-2 family members known to regulate programmed cell death and function as apoptotic activators, namely, BCL2 associated agonist of cell death (BAD) and BCL2 associated X (BAX)[126,127], in the same clone that overexpresses WWOX (SET2 clone 4). The results revealed a significant upregulation of both BAD and BAX expression in SET2 cells overexpressing WWOX, with a 1.28-fold and 1.45-fold increase, respectively, compared to SET2 EV (Fig. 11- B, C)

Collectively, these results show that WWOX plays a role in JAK2V617F MPN cells, possibly by regulating JAK downstream signaling pathways and, in turn, the expression of key transcription factors involved in these cells.

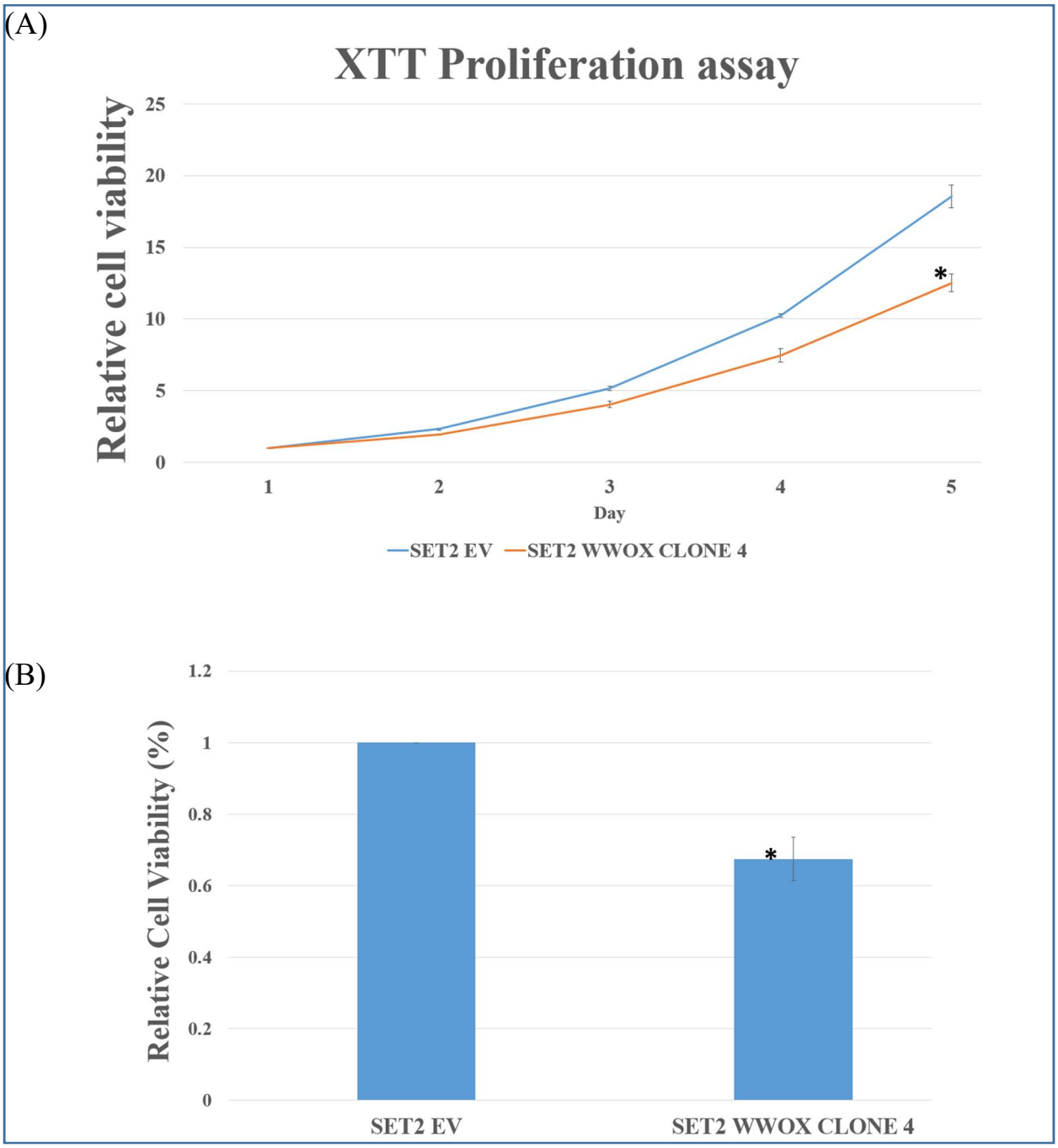
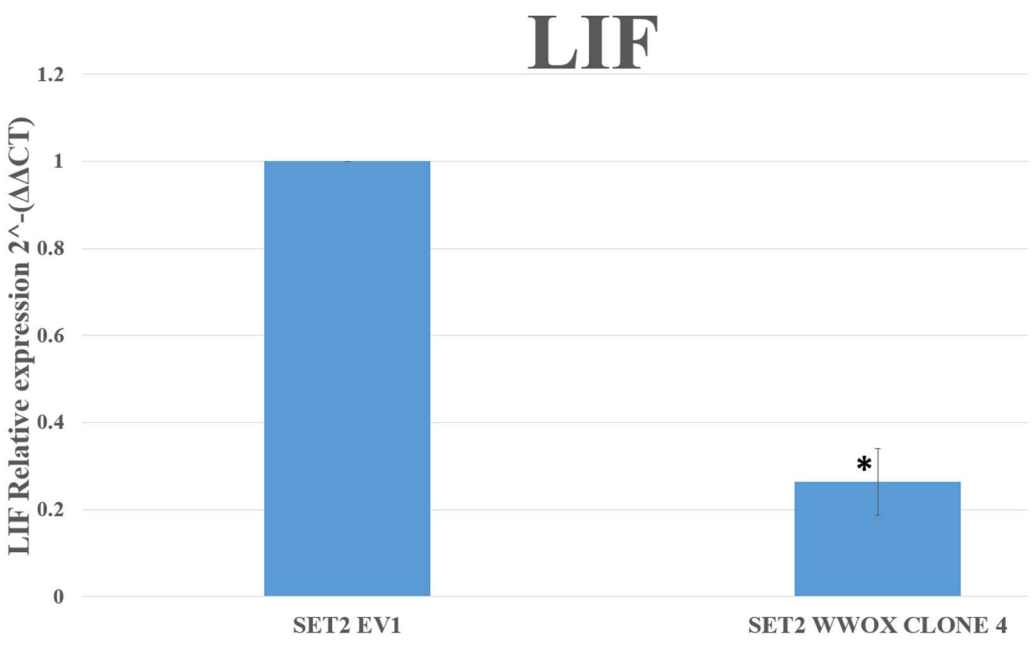
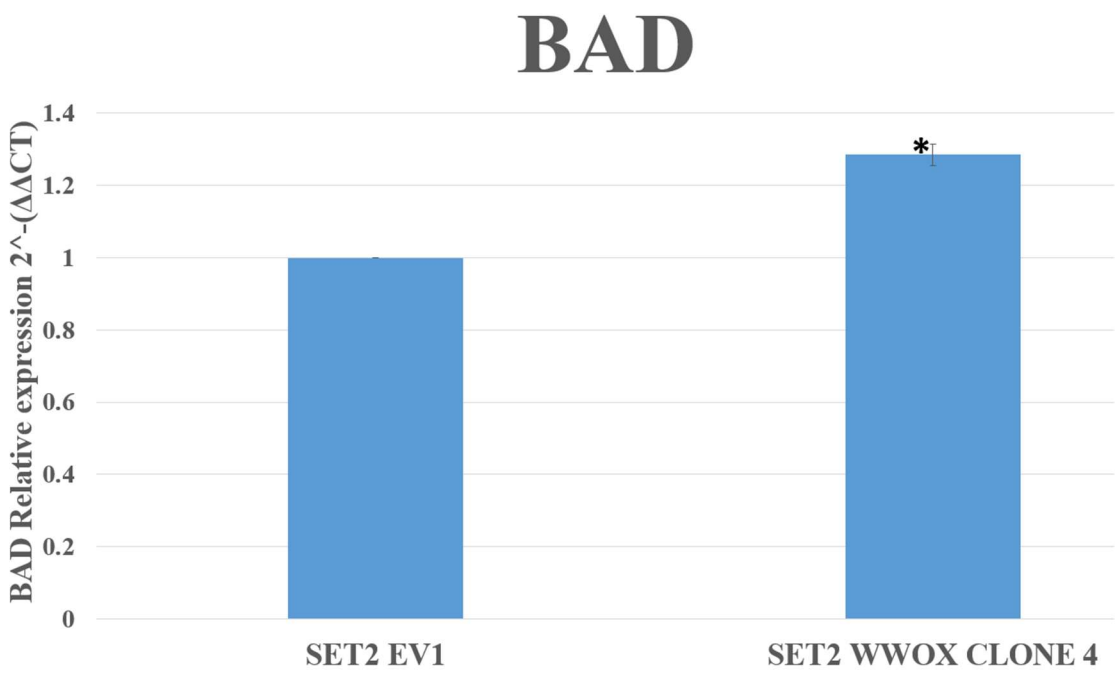


Figure 10: WWOX Overexpression Reduces JAK2V617F-Driven MPN Proliferation. (A) Relative Cell Viability Assessment. Relative cell viability was assessed for SET2 WWOX Clone 4 (representative of all SET2 clones) using the XTT assay. This graph demonstrates the impact of WWOX overexpression on SET2 cell proliferation. The relative proliferation rate index on day 5 was compared to day 1 for both Clone 4 and SET2 EV. (B) Quantification of Relative Cell Viability (%). The results presented in day5 in (A) were quantified to determine relative cell viability. The final relative proliferation index for SET2 WWOX Clone 4 was compared to its EV control. Error bars represent the standard error of the mean (SEM) for three replicates. Statistical significance was assessed by calculating the p-value (p-value < 0.001). (*p-value <0.001).

(A)


LIF
LIF Relative expression 2^-(ΔΔCT)
1.2
1
0.8
0.6
0.4
0.2
0
*
SET2 EV1
SET2 WWOX CLONE 4

(B)


BAD
BAD Relative expression 2^-(ΔΔCT)
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1.2
1
0.8
0.6
0.4
0.2
0
*
SET2 EV1
SET2 WWOX CLONE 4

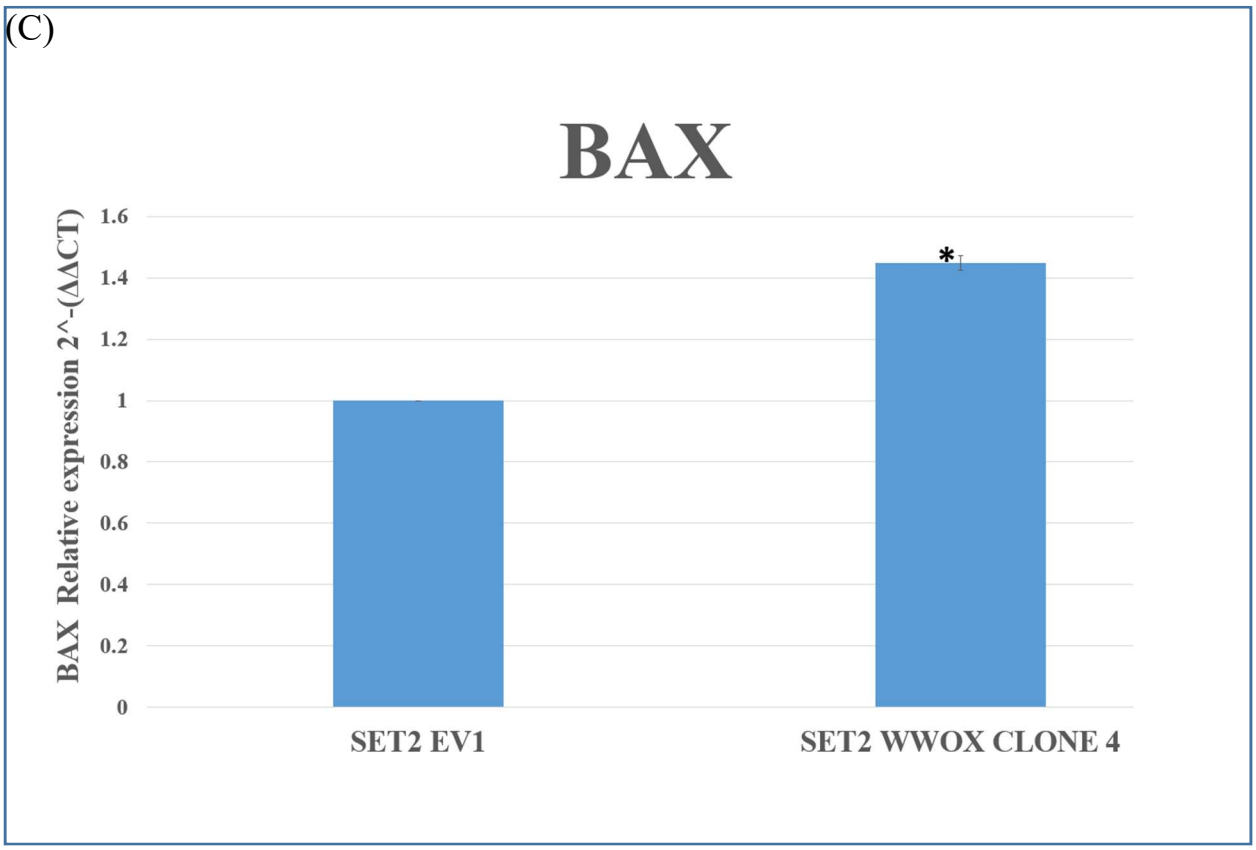

Figure 11: WWOX Modulates Expression of Apoptosis and Proliferation-Related Genes in MPN with JAK2V617F. Real-Time PCR Analysis of Gene Expression, (A) Relative expression levels of LIF. (B) Relative expression levels of BAD. (C) Relative expression levels of BAX. Gene expression levels were normalized to the UBC housekeeping gene. The final relative expression index for SET2 WWOX CLONE 4 were correlated relative to its EV control. Error bars represent the standard error of the mean (SEM) calculated from three replicates. Statistical significance was determined using p-values (* indicates p-value < 0.001)

Chapter 4: Discussion

In this study, we have shown results that provide valuable insights into the role of the WWOX gene in Philadelphia-negative myeloproliferative neoplasms (MPN) with JAK2V617F mutation. The alterations in WWOX expression observed in different cell lines and the subsequent functional implications suggest a complex interplay between WWOX and critical signaling pathways involved in MPN pathogenesis.

The initial investigation into WWOX protein expression patterns in Philadelphia-negative MPN cell lines with JAK2V617F mutations highlighted a diverse landscape. The detectable WWOX expression in UKE1, HEL, KG1, NB4, and HL60 cell lines, along with the absence of WWOX in the SET2 cell line, suggests that WWOX alterations may be linked to specific subtypes of MPN. This observation is consistent with previous findings linking WWOX alterations in various cancer types as a tumor suppressor gene, underlining the potential significance of WWOX in MPN pathogenesis. Furthermore, the presence of WWOX protein expression in leukemic cell lines with wild-type JAK2 emphasizes the complexity of WWOX role across different malignancies.

Our data unveiled unexpected outcomes concerning the impact of WWOX overexpression on critical signaling pathways in JAK2V617F MPN cells. Specifically, the elevated levels of phospho-ERK and phospho-STAT3(Y705) resulting from WWOX overexpression were surprising, given the established roles of these phospho-proteins in promoting cancer and proliferation, especially in the context of MPN with JAK2V617F mutations. Conversely, the reduction of Phospho-ERK level in UKE1 clones with WWOX knockout further supports this finding. This result contradicts our initial expectations, as constitutive activation of the JAK-STAT pathway is a hallmark of this cancer type.

Since WWOX was shown to act as a tumor suppressor gene in many cancers, we expected opposite results in regard to the JAK-STAT signaling pathways. We anticipate a downregulation of both activated ERK and STAT3 signaling molecules upon WWOX overexpression and the opposite scenario upon WWOX knockout. In previous studies, WWOX tumor suppressor activity was demonstrated in Triple-Negative Breast Cancer (TNBC) and Hepatocellular carcinoma (HCC) by downregulating the JAK-STAT pathway.[121,122] For example, in TNBC, lower WWOX expression correlated with higher STAT3 activation, and overexpressing WWOX inhibited BC cell proliferation and metastasis by suppressing STAT3 activation through interactions with JAK2 and STAT3. However in our current study we didn't check whether WWOX interacts with mutated JAK2 in the studied cells and thus, further investigations are needed in this regard.[121] Similarly, in HCC, WWOX was shown to inhibit HCC growth by influencing the JAK2/STAT3 and Wnt/β-catenin pathways. Overexpression of WWOX hindered the nuclear translocation of phosph-Stat3 (Y705) and β-catenin, leading to the inhibition of MMPs and C-MYC, thus suppressing HCC progression and metastasis.[122] However, our results are not consistent with these mechanisms. Since WWOX overexpression in JAK2V617F MPN cells caused elevation of phospho-ERK and phospho-STAT3 (Y705). In light of these findings, it is crucial to investigate deeper into the specific phosphorylation status of WWOX in these cell lines in future studies. Determining whether pS14-WWOX, which has been associated with enhancing cancer progression and metastasis, or pTyr33-WWOX, which is considered to have an anticancer effect, could provide further insights into WWOX roles in MPN.[117]

The unexpected results of elevated levels of phospho-STAT3 (Y705) upon WWOX overexpression raises intriguing questions about the dual role that STAT3 can play in cancer. STAT3 transcription factor, exhibits a dual nature, functioning as either a tumor promoter or a

tumor suppressor depending on specific conditions.[49] This duality has been observed in various cancer types, including glioblastomas [56], thyroid cancer [59], prostate cancer [57], colorectal cancer [58], and other type of cancers. The complex involvement of STAT3 in cancer adds an intriguing layer of complexity to our observations. Elevated phospho-STAT3 (Y705) levels can be interpreted in two ways: as an anti-tumor signal, aligning with WWOX's tumor suppressor activity, or as a cancer-promoting signal, consistent with WWOX's potential oncogenic role. Both scenarios are plausible and underscore the intricate nature of WWOX's involvement in cancer, emphasizing the need for further research to uncover the precise molecular mechanisms and contextual factors governing these interactions.

In a related study by Yan et al., the impact of STAT3 knockout in JAK2V617F mutant mice with MPN was investigated. Conditional JAK2V617F knock-in and STAT3-deficient mice were used to examine STAT3 role in MPN development. Surprisingly, STAT3 ablation did not prevent MPN development but instead resulted in myeloid cell expansion and reduced survival in the Jak2V617F MPN mouse model. STAT3 deletion led to increased numbers of neutrophils, myeloid precursors, and HSC in the bone marrow and spleen, suggesting that STAT3 might normally restrain granulopoiesis and HSC proliferation. The study also identified potential molecular mechanisms involving STAT3, including its role in inducing SOCS3 and regulating Notch1 and C/EBP expression. These findings provided insight regarding the complex role of STAT3 in MPN pathogenesis. The study concluded that deletion of STAT3 worsens the severity of MPN caused by the JAK2V617F mutation, suggesting that STAT3 plays a negative regulatory role in this context.[60] This result is consistent with our finding showing increase phospho-STAT3 (Y705) upon WWOX tumor suppressor gene overexpression suggesting that WWOX play antitumor

activity in these MPN by inducing STAT3-Y705 phosphorylation, and this might be the real scenario.

Our findings demonstrating that elevated levels of WWOX is consistent with an increase in phospho-ERK is supported by other related findings. Previous studies investigated WWOX interaction with MEK2 and its subsequent activation of the ERK pathway. Notably, this activation has been associated with the induction of growth arrest, particularly at the G1/S boundary.[74] Furthermore, in a related study by Yong Zhang et al., it was found that ERK2 plays a dual role in JAK2V617F-driven MPNs through its substrate-binding domains. Specifically, the ERK2-docking (ERK2-D) domain has emerged as a potential therapeutic target that promotes cancer. In contrast, the ERK2-DEF–binding pocket (ERK2-DBP) domain opposes the pathogenesis of MPN and promotes JAK2-mediated oncogene-induced senescence.[75] Therefore, we can anticipate that WWOX, as a tumor suppressor, may bind to the ERK2-DBP domain, activating it and supporting its role in opposing the pathogenesis of MPN. On the other hand, WWOX may interact with the ERK2-D domain, blocking it from binding with other oncoproteins. Both scenarios are possible. Thus, our data showing an increase in phospho-ERK upon WWOX overexpression introduces an intriguing layer of complexity. It suggests that while increased WWOX level could potentially stimulate ERK-mediated cell proliferation, it may also favor a scenario where growth arrest is induced. These different outcomes underscore the multifaceted role of both WWOX and ERK in the regulation of cell behavior and emphasizes the need for further research to clarify the precise molecular mechanisms that determine the specific cellular responses controlled by WWOX and its interactions with MEK or ERK2 in the context of the ERK pathway.

Our results demonstrate a significant reduction in proliferation observed in SET2 JAK2V617F MPN cells in response to WWOX overexpression. Additionally, we observed a significant

elevation in RNA levels of BCL2 pro-apoptotic family members (BAX and BAD) and a reduction in LIF RNA expression. These findings strongly support our initial hypothesis that WWOX functions as a tumor suppressor in this specific subtype of MPN. In a previous study conducted by Elisa M. Salas et al., they identified LIF as a direct transcriptional target of STAT5 and found increased expression of LIF in various myeloid cell lines with active JAK2/STAT5 signaling, including the SET2 cell line[125]. Our results are consistent with these findings, suggesting that LIF plays a pivotal role in MPN pathogenesis. We propose a regulatory link between WWOX and LIF in controlling proliferation and apoptosis. This provides strong evidence for a potential interplay between WWOX and the JAK-STAT pathway. Furthermore, when they used a STAT5 inhibitor, they found that LIF RNA expression decreased by 4.2-fold in SET-2 cells. This result closely mirrors our findings, where WWOX overexpression in SET2 cells caused a 3.8-fold decrease in LIF levels. This suggests that WWOX might inhibit STAT5, as STAT5 is known to regulate the transcription of LIF in these cells. Such evidence supports the hypothesis of a potential interplay between WWOX and the JAK-STAT pathway in MPN with JAK2V617F mutation.

Altered WWOX expression has also been observed in other types of hematological malignancies, such as lymphomas, suggesting that WWOX may serve as a potential tumor suppressor in these conditions. Moreover, studies involving WWOX hypomorphic mice have demonstrated a higher incidence of B-cell lymphomas.[128] Additionally, deletion of WWOX in mouse B cells has been linked to genomic instability, neoplastic transformation, monoclonal gammopathies, and the development of B-cell neoplasms, including B-cell lymphomas and plasma cell neoplasias.[129]

In our future research plan, we aim to extend our understanding of the intricate mechanisms involving JAK2, WWOX, STAT3, STAT5, ERK, and other crucial proteins within the context of

MPN. We will conduct functional assays including proliferation assays in WWOX-knockout (KO) clones, which will allow us to assess the impact of WWOX manipulation on MPN cell growth and to further confirm our results in the context of WWOX overexpression. Additionally, we plan to determine the phosphorylation status at specific sites of key proteins, including JAK2, WWOX, STAT3, STAT5, ERK, MEK1/2, and others. Furthermore, we will investigate protein localization and abundance to gain valuable insights into the cellular dynamics of WWOX, STAT3, STAT5, ERK, and other proteins. Employing inhibitors for upstream kinases such as MEK1, MEK2, ERK, and JAK2 will be integral to precisely pinpointing the pathways contributing to their activation, thereby offering a more comprehensive understanding of the signaling cascade in the presence of WWOX protein. In parallel, we will assess the sensitivity of MPN to drugs, exploring potential therapeutic interventions. Additionally, we will utilize immunohistochemistry on patient samples to validate our findings in a clinical context. Finally, our research plan includes the utilization of an animal model with a conditional knockout of the WWOX gene to further investigate the *in vivo* consequences of altering these key proteins. These comprehensive approaches collectively constitute our future research roadmap, aimed at advancing our understanding the role of tumor suppressor WWOX on MPN and identifying potential avenues for therapeutic development.

Chapter 5: Conclusion

In conclusion, the collective findings of this study demonstrate the complicated role of WWOX in JAK2V617F MPN cells. The altered expression of WWOX in different cell lines associated with JAK2 mutations, combined with the functional implications revealed through experimental manipulation, strongly suggest that WWOX plays a regulatory role in critical signaling pathways driving MPN pathogenesis. These pathways include ERK and JAK-STAT, which are closely involved in cellular processes crucial for disease progression. The combination of our findings with previous studies suggests that WWOX exhibits tumor suppressor activity in this type of MPN, in conjunction with the tumor suppressor activity of STAT3 and ERK as shown in Fig.12. Notably, our results indicate that WWOX overexpression in this MPN SET2 cells causes a significant reduction in cell proliferation, further supporting its role as a tumor suppressor in MPN. While this study advances our understanding of WWOX involvement in MPN, future research should investigate more deeply into the molecular mechanisms underpinning these interactions, potentially opening the way for novel therapeutic strategies targeting the WWOX-associated pathways in MPN treatment.

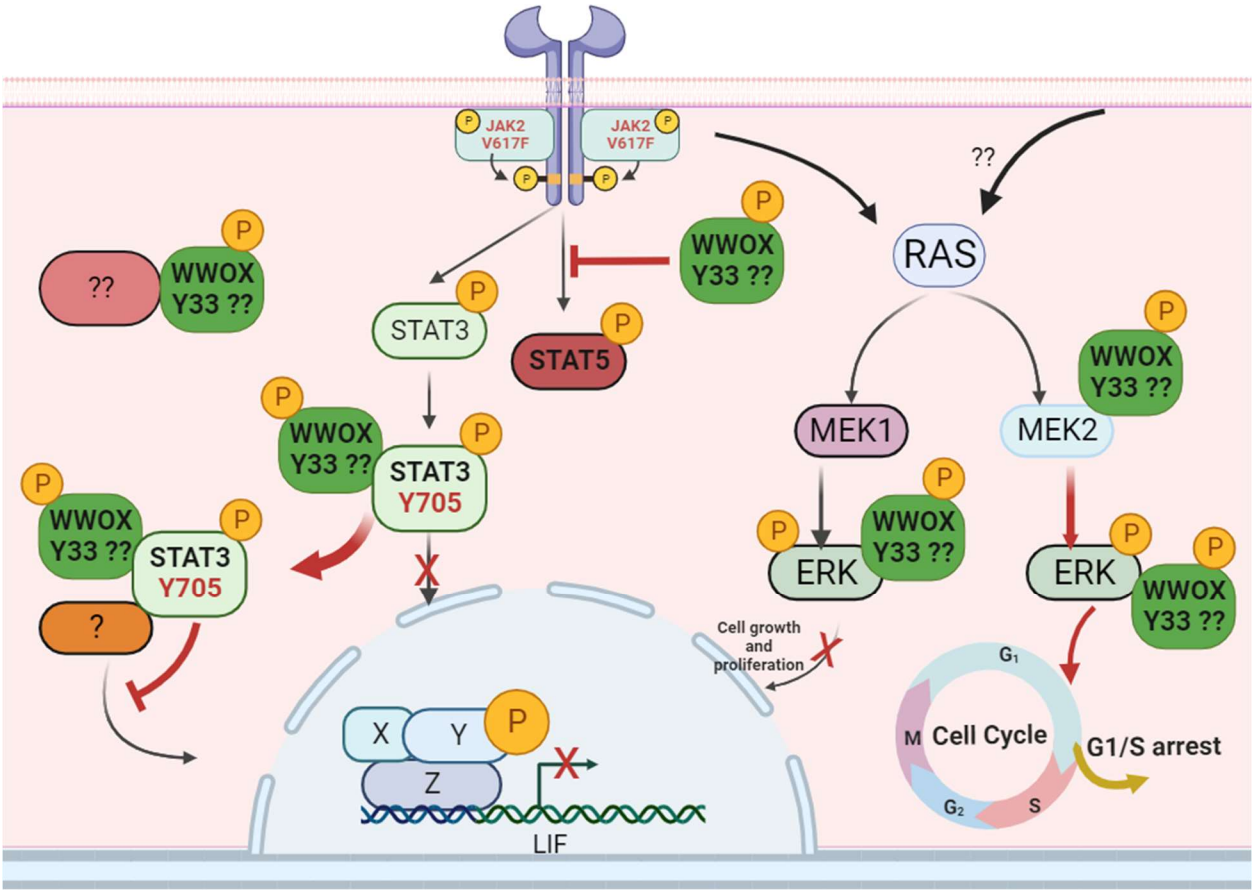

Figure 12: Illustration of a potential scenario involving WWOX overexpression in MPN with the JAK2V617F mutation suggests that WWOX may interact with Phospho-STAT3, resulting in the trans-phosphorylation of STAT3 at Y705. Both WWOX and Phospho-STAT3 are suggested to function as tumor suppressor proteins in this context, leading to the inhibition of their nuclear translocation and sequestration of STAT3 in the cytoplasm. This sequestration may also involve other proteins known to enhance cancer phenotypes by binding to this complex. Additionally, WWOX is suggested to inhibit STAT5 activation and its downstream target genes. Furthermore, WWOX is assumed to activate the MEK2/ERK pathway, which, in turn, induces cell cycle arrest in the G1/S phase. Moreover, WWOX may interact with ERK2 in a specific substrate binding domains. There is also a possibility that WWOX interacts with other proteins.

6. References:

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بسم الله الرحمن الرحيم

الملخص

تمثل الأورام التكاثرية النقوية (MPN) مجموعة من الاضطرابات الدموية النسيلية التي تتميز بانتشار غير طبيعي للخلايا النخاعية في نخاع العظم. إحدى فئات MPN هي classical Philadelphia-negative MPN ، والتي تنتج عن العديد من الطفرات المحركه، وأكثرها شيوعًا هي طفرة JAK2V617F، مما يؤدي إلى التنشيط المستمر لمسار JAK-STAT. من المعروف أن إنزيم التأكسد المحتوي على نطاقWW (*WWOX*)، الموجود في أحد أكثر المواقع الهشة في الجينوم البشري (FRA16D)، يلعب دورًا مهمًا في الحماية من السرطان. تم التعرف على التغيرات في *WWOX* في العديد من أنواع السرطان. ومع ذلك، فإن تغيير *WWOX* ومساهمته المباشرة في تطوير MPN وتقدمه لا يزالان غير معروفين إلى حد كبير. تهدف هذه الدراسة إلى دراسة دور جين *WWOX* في الأورام التكاثرية النقوية السلبية لفيلادلفيا (MPN) مع طفرة JAK2V617F. تكشف النتائج التي توصلنا إليها عن أنماط متنوعة للتعبير عن بروتين WWOX عبر خلايا MPN المختلفه، مما يشير إلى التعديلات المحتملة الخاصة بالنوع الفرعي. لمزيد من استكشاف تأثير WWOX على MPN هذا، قمنا بتغيير تعبير *WWOX* في الخلايا هذه من خلال كل من أساليب overexpression وCRISPR-knockout. استخدمنا بعد ذلك مزيجًا من Sanger sequencing ، وRT-PCR، وال Western blotting،و Proliferation Assay .من المثير للدهشة أن زيادة بروتين WWOX أدى إلى مستويات مرتفعة منphospho-ERK phospho-STAT3 (Y705) مع تثبيط نمو الخلايا وزيادة بروتينات BCL2 ,بينما أدى نقصان بروتين WWOX الى نقصان مستوى phospho-ERK ، مما يمثل تحديًا لتوقعاتنا الأولية حول(Y705) phospho-STAT3 و phospho-ERK. تشير هذه النتائج غير المتوقعة إلى دور محتمل لقمع الورم لكل من WWOX و STAT3 في MPNs. هناك حاجة إلى مزيد من البحث لتوضيح الآليات الجزيئية الأساسية. إن فهم التفاعل المعقد بين WWOX وJAK-STAT وتطوير MPN يمكن أن يكون له آثار كبيرة على تشخيص هذه الاضطرابات وعلاجها.