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DOKUZ EYLUL UNIVERSITY
IZMIR INTERNATIONAL
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INSTITUTE

THE ROLE OF MALT1 GENE IN THE CROSSTALK OF TGF–β AND NF–κB SIGNALING PATHWAYS

FATMA AYBÜKE MAZI

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

MASTER THESIS

IZMIR-2022

Thesis Code: DEU.HSI.MSc number/201885002

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Advisor: Assoc. Prof. Şerif Şentürk

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Dokuz Eylül Üniversitesi İzmir Uluslararası Biyotıp ve Genom Enstitüsü Genom Bilimleri ve Moleküler Biyoteknoloji Anabilim Dalı, Moleküler Biyoloji ve Genetik Yüksek Lisans programı öğrencisi Fatma Aybüke Mazı TGF–β and NF–κB Sinyal Yolaklarının Çapraz Konuşmasında Malt1 Geninin Rolü konulu Yüksek Lisans tezini 02/06/2022 tarihinde başarılı olarak tamamlamıştır.

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Fatma Aybüke Mazı, who is a student at the Department of Molecular Biology and Genetic Master's program of the Dokuz Eylul University's Izmir International Biomedicine and Genome Institute, with the student ID 2018850022, succeeded her Master's thesis titled "The Role of Malt1 Gene in the Crosstalk of TGF–β and NF–κB Signaling Pathways" as a result of her thesis defense on 02/06/2022.

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ABBREVIATIONS

Malt1: Mucosa-associated lymphoid tissue lymphoma translocation protein 1

TGF–β: Transforming growth factor-beta

NF–κB: Nuclear factor kappa-B

TNF-α: Tumor necrosis factor-alpha

MAPK: Mitogen-activated protein kinase

JNK: c-Jun N-terminal kinase

JAK/STAT: Janus kinases, signal transducer and activator of transcription proteins and receptors

TGFBR1: Transforming growth factor-b receptor 1

IκB: Inhibitor of kappa B

IKK: Inhibitor of kappa B kinases

RelA: REL-associated protein A, p65

NEMO: NF-kappa-B essential modulator

CARMA/CARD: CARD-containing membrane-associated guanylate kinase (MAGUK) protein

BCL-10: B-cell lymphoma/leukemia 10

TRAF6: Tumor necrosis factor receptor-associated factor 6

TAK1: Transforming growth factor-b-activated kinase 1

JUNB: Transcription factor jun-B

RhoA: Ras homology family member A

Rac1: Ras-related C3 botulinum toxin substrate 1

Cdc42: Cell division control protein 42 homology

IL-1β: Interleukin-1 beta

shRNA: Short hairpin ribonucleic acid

CRISPR: Clustered regularly interspaced short palindromic repeats

gRNA: Guide RNA

SBE: Smad-binding element

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The Role of Malt1 Gene in the Crosstalk of TGF−β and NF−κB Signaling Pathways

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ABSTRACT

Transforming growth factor-β (TGF−β) is a cytokine superfamily expressed in a variety of cell types. Through contextual regulation of a large number of genes, TGF−β ligands serve critical functions in key cellular and molecular mechanisms, including cancer cell survival and proliferation. Notably, canonical (Smad-dependent) and non-canonical (Smad-independent) signaling pathways are involved in its activity. Furthermore, TGF−β signaling is known to interact with other intracellular pathways, including MAPK, JNK, JAK/STAT and NF−κB. Importantly, the nuclear factor kappa-B (NF−κB) pathway constitutes a small family of transcription factors found in virtually every cell type and is involved in multiple aspects of biological functions relevant to physiological and pathophysiological processes. The activity of NF−κB pathway is also modulated by both canonical and non-canonical signals. Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (Malt1), a paracaspase first identified in a subset of MALT lymphoma, is a critical component of NF−κB pathway in several biological contexts. Yet, the role of Malt1 in TGF−β and NF−κB interaction is unknown. Here we report that Malt1 protein potentiates the crosstalk between TGF−β and NF−κB signaling pathways. Mechanistically, through extensive molecular studies in A549 and Huh7 cancer cell lines using RNAi and CRISPR/Cas9 perturbations, we find that Malt1 is a Smad3-dependent target gene of canonical TGF−β signaling. More significantly, TGF−β-mediated Malt1 induction facilitates NF−κB activation. Collectively, our pioneering work provides mechanistic insights into how TGF−β/Smad signaling regulates Malt1 expression and reveals a novel mode of crosstalk between TGF−β and NF−κB signaling pathways.

Key Words: TGF−β, NF−κB, Malt1, Smad3, RNAi, CRISPR/Cas9

TGF−β ve NF−κB Sinyal Yolaklarının Çapraz Konuşmasında Malt1 Geninin Rolü

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ÖZET

Dönüştürücü büyüme faktörü-β (TGF−β), çeşitli hücre tiplerinde ifadelenen ve hücre tipine ve durumuna bağlı olarak çok sayıda geni düzenleyerek çeşitli hücresel aşamalarda ve hücre kaderinde rol oynayan bir sitokin süper ailesidir. Kanonik (SMAD bağlı) ve kanonik olmayan (SMAD bağımsız) sinyaller etkinliğinde çalışır. MAPK, JNK, JAK/STAT gibi sinyal yolaklarına ek olarak, hemen hemen her hücre tipinde bulunan küçük bir transkripsiyon faktörü ailesi olan NF−κB'nin içerisinde bulunduğu çeşitli sinyal yolaklarıyla etkileşime girerek hayatta kalma ve kanser ilerlemesinde önemli roller oynar. Aktivitesi hem kanonik hem de kanonik olmayan sinyaller tarafından düzenlenir. İlk olarak MALT lenfomada keşfedilen mukoza ile ilişkili lenfoid doku lenfoma translokasyon proteini 1 (Malt1), nükleer faktör kappa-B (NF−κB)'nin aktivasyonunda rol oynadığı bilinen bir parakaspazdır. TGF−β-NF−κB ve Malt1-NF−κB arasındaki etkileşimler farklı konseptlerde çalışılmış olmasına rağmen, bunların çeşitli kanser türlerinde Malt1 yoluyla çapraz konuşması tanımlanmamıştır. Bu çalışma, TGF−β ve NF−κB sinyal yollarının çapraz konuşmasında Malt1 proteininin rolünü farklı bir yöntemle göstermektedir. TGF−β'ya yanıt veren iki hücre hattında, Malt1'ın kanonik TGF−β yolağının hedefi olduğunu gösterdik. Malt1'ın bir Smad3 hedef geni olduğu TGF−β kanonik yolağının shRNA ve CRISPR/Cas9 yöntemleri kullanılarak baskılanması aracılığıyla gösterildi. Ayrıca, TGF−β ile indüklenen arttırılmış Malt1 ekspresyonunun NF−κB aktivasyonuna aracılık ettiği NF−κB raportör deneyleri, immünofloresan boyaması ve NF−κB hedef genleri için qRT-PCR testleri ile gösterilmiştir.

Anahtar Kelimeler: TGF−β, NF−κB, Malt1, Smad3, RNAi, CRISPR/Cas9

1.1. Aim of The Study

This study aimed to investigate the role of the MALT1 in the crosstalk of two important signaling pathways; TGF−β and NF−κB. To achieve this goal, we employed two different cancer lines: Huh7, a hepatocellular cancer cell line, and A549, a non-small cell lung cancer cell line. Experimentally, we silenced SMAD2/3/4 and TGFBR1 expression using the RNAi system in order to investigate the regulation of Malt1 through canonical TGF−β signaling cascade. Within this context, we also blocked the kinase activity of TGFBR1 using a potent and selective small molecule inhibitor. To explore whether Malt1 expression could be directly regulated by Smad3, we employed CRISPR/Cas9 technology to engineer Smad3 knockout clones. Luciferase assay experiments were performed using reporter vectors consisting of Malt1 promoter regions to investigate whether Malt1 is regulated transcriptionally through TGF−β cascade. More significantly, the role of Malt1 in the crosstalk between TGF−β and NF−κB pathways was demonstrated by performing immunofluorescent staining of p65 translocation into the nucleus, luciferase assay with NF−κB reporter cell lines, and qRT-PCR with NF−κB target genes.

2. INTRODUCTION

2.1. TGF−β Signaling Pathway

The transforming growth factor-β superfamily is a great collection of cytokines that govern a variety of cellular and physiological processes, including apoptosis, cellular senescence, epithelial-mesenchymal transition (EMT), inflammation, and tissue formation.(1) Consistent with its multiple functions in practically all cell types, there can be pathophysiological repercussions and consequences as a result of dysregulation of TGF−β signaling, such as cancer. (2) TGF−β pathway activation is triggered by three ligands, namely TGF−β1, TGF−β2, and TGF−β3, known as TGF−β in general, all of which are expressed by separate genes in the mammalian genome. TGF−β1 is the most common isoform, and it can be released by almost any cell type. Ligand isoforms share transmembrane serine/threonine kinase receptors

and transmit signaling via a single type II receptor (TGFBR2). (1–3) Although the family contains a large number of factors with cell type-specific and developmental stage-dependent biological activities, they all signal through conserved signaling pathways. Specifically, active TGF−β dimers bind to homodimeric TGFBR2, inducing the formation of a heterotetrameric TGF−β receptor complex. Then, the type I receptor TGFBR1 (ALK5) is recruited, and the transphosphorylated by constitutively active TGFBR2 to activate intracellular cascade.(1) The type III receptor (TGFBR3), a membrane-anchored proteoglycan coreceptor found in numerous cell types, is another member of TGF−β receptor proteins which facilitates ligand presentation to TGFBR2 while not being directly involved in signal transduction. TGFBR1 is the key transmitter of regulatory instructions to the cytosol after activation, which can operate both canonical (Smad-dependent) and non-canonical (Smad-independent) signaling branches.(2–5)

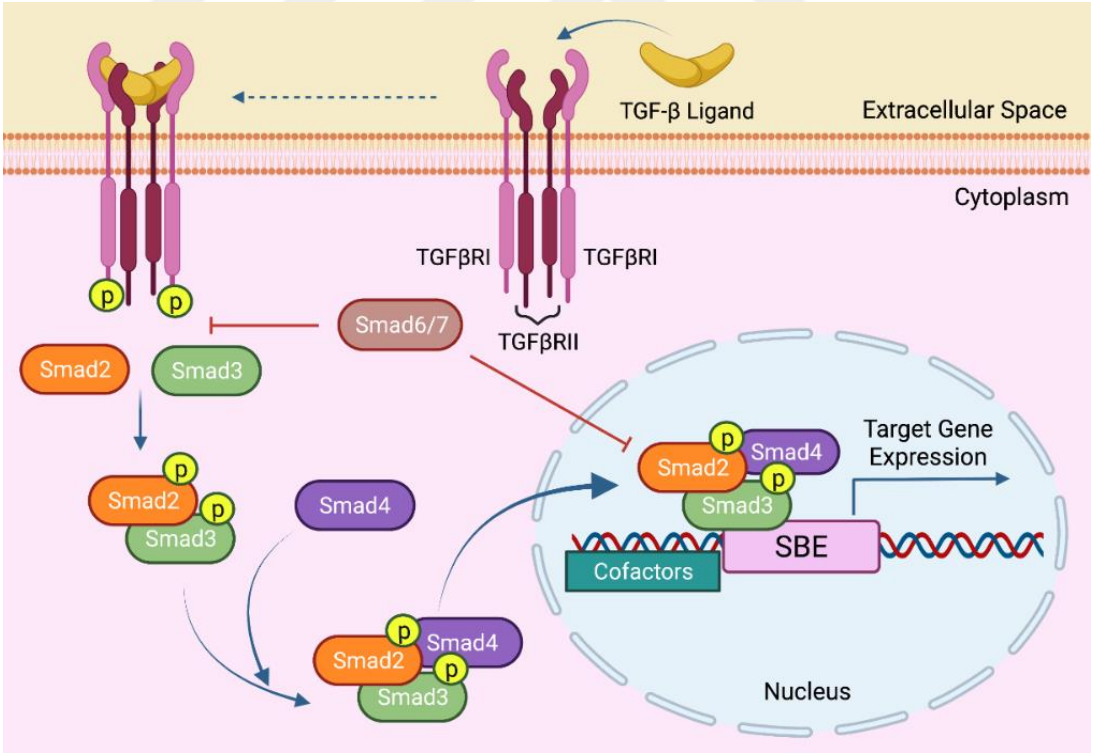


Figure 1. Canonical TGF−β signaling pathway. (6)

In canonical pathway activation, intracellular Smad molecules transmits the signals into the nucleus. Smad proteins have two conserved mad homology (MH) domains, N-terminal MH1 and C-terminal MH2 which are structurally connected by a proline-rich linker region. The MH1 domain is primarily important for DNA binding, while the MH2 domain regulates receptor Smad-TGFBR1, Smad-Smad, and Smad-coregulator interactions. (7) The linker region is commonly linked to crosstalking with other signaling pathways. (8)

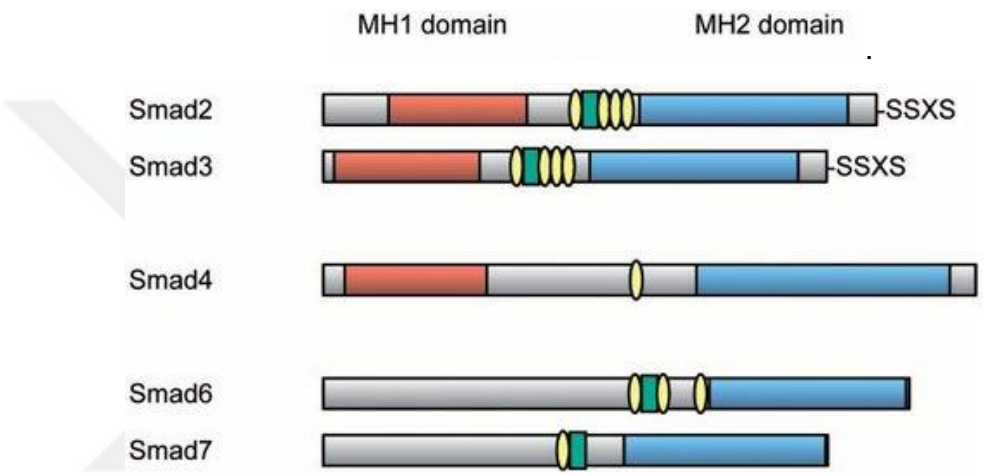


Figure 2. Smad proteins. Smad2 and Smad3 are R-Smads while Smad4 is co-Smad, Smad6 and Smad7 are I-Smads. The red region represents the MH1 domain while the blue region corresponds to MH2. (9)

TGF−β receptors undergo conformational changes when activated, allowing recruitment and phosphorylation of R-Smads, Smad2 and Smad3, at their c-terminal SSXS motif by TGFBR1. After phosphorylation, they detach from the receptor and form a heterodimeric complex with the common intermediator (co-Smad) Smad4, and this complex subsequently translocates into the nucleus to regulate transcription of over 500 genes together with coregulators. (4,10–12) The 8-bp palindromic sequence, namely Smad-binding element (SBE), (GTCTAGAC) is recognized by Smad3 and Smad4 while the most common variant of Smad2 is not able to bind DNA caused by additional 30 amino acids within the MH1 domain which interferes with binding to DNA. The regulation of TGF−β signaling is governed by inhibitory Smads (I-Smads), Smad6 and Smad7, by limiting R-Smad recruitment and phosphorylation

via interacting with TGFBR1 through their conserved MH2 domain similar to R-Smads and Smad4.(13,14)

The collective action of central pathway components such as receptors, ligands, Smads, and transcription factors interacting with Smads determines TGF−β receptors' ability to activate other signaling modules as well as the crosstalking with other signaling pathways besides Smads.(15,16)

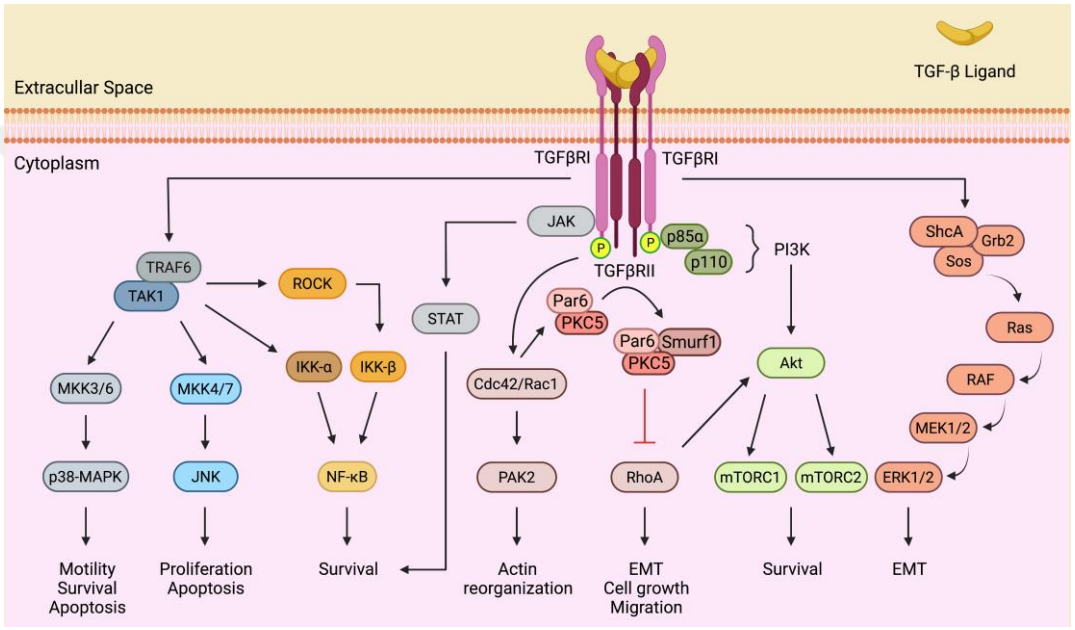


Figure 3. Non-canonical TGF−β signaling pathway. (6)

Non-canonical crosstalk with various signal transduction networks appears to be activated independently of Smads, via direct interaction of pathway factors with TGF−β receptors or phosphorylation. Furthermore, phosphorylation of the intrinsic tyrosine residues of TGF−β receptor kinase domains is thought to be a key factor in the intricate interaction between TGF−β and non-canonical signaling pathways including phosphatidylinositol-3-kinase (PI3K)/Akt, Janus kinases-signal transducer (JAK/STAT), MAPKs (extracellular signal-regulated kinases, c-Jun N-terminal kinases, and p38-MAPKs), Rho family GTPases like RhoA, Rac1, and Cdc42, and nuclear factor kappa B (NF−κB) which adds to the intricacy of TGF−β actions. (16,17)

2.2. NF–κB Signaling Pathway

The NF–κB pathway is a small family of structurally related transcription factors consisting of five DNA-binding heterodimer proteins such as NF–κB1 (p105 and p50), NF–κB2 (p100 and p52), c-Rel, RelA (p65), and RelB. NF–κB cascade controlling various gene expressions involved in proliferation, cell survival, apoptosis, and immune response. (18–21) As its dysregulation is common in human malignancies, NF–κB is considered to play a role in invasiveness and angiogenesis of tumor. Significantly, NF–κB and the signaling pathways that underlie its activation have appeared as interesting targets for new chemopreventive and chemotherapeutic medicines. (22)

The NF–κB pathway is also divided into two branches, namely canonical and non-canonical signaling. The common regulatory mechanism is the IκB family of inhibitory proteins, which are destroyed by the multi-subunit IκB kinase (IKK) complex.(19,20) The inactive NF–κB heterodimer complex, comprised of p65 and p50, is generally kept in the cytoplasm by the natural biological inhibitor IκB. Phosphorylation of IκB by IKK complex is then followed by ubiquitination resulting in its degradation. This allows the NF–κB subunits, p65 and p50, to be released and translocated to the nucleus, so that they can interact with DNA for gene expression regulation. (20,23)

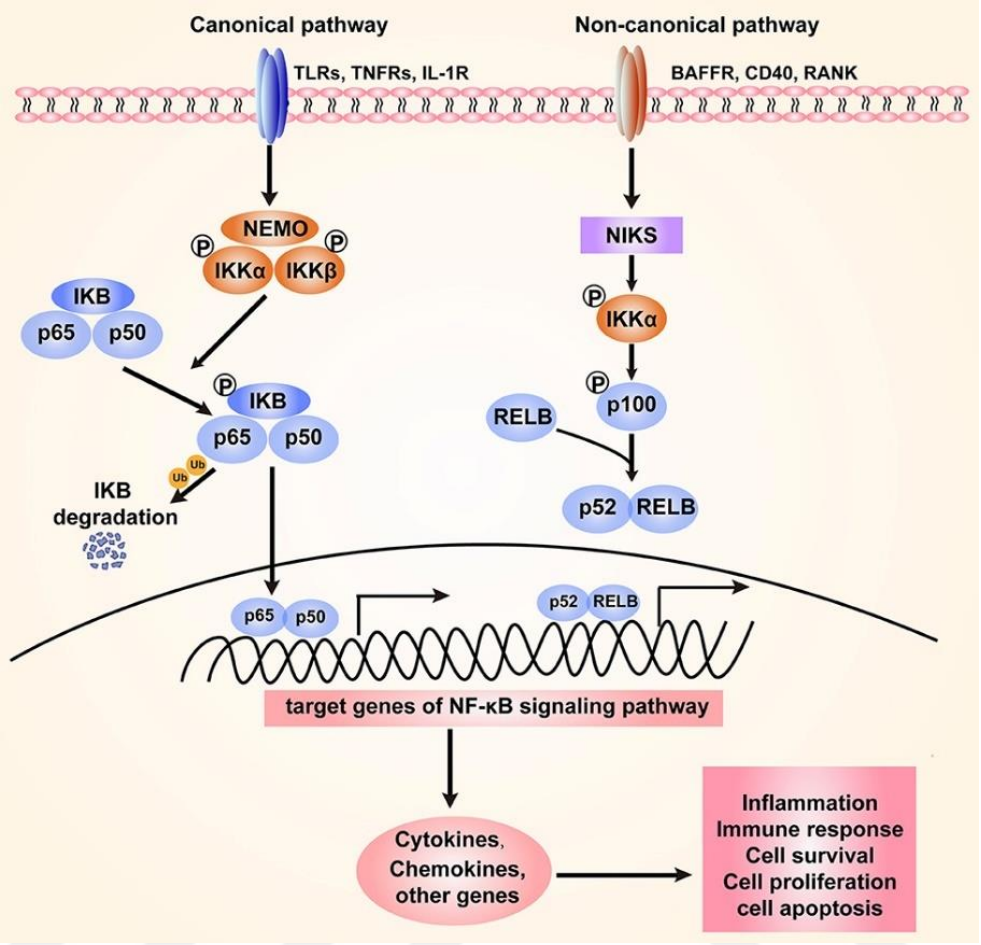


Figure 4. Canonical and non-canonical NF−κB signaling pathway.(24)

Tumor necrosis factor (TNF) and several immune system ligands and receptors are the common activating factors of NF−κB signaling. A variety of proinflammatory cytokines and cellular stressors can activate the canonical pathway such as TGF−β-activated kinase 1 (TAK1) or MAPK kinase kinase 3 (MEKK3). (25,26) Only a subset of cytokines from the TNF superfamily can also activate the non-canonical pathway.(18) In the non-canonical pathway activation, NF−κB inducing kinase (NIK) triggers IKK, which phosphorylates the precursor form p100 to be cleaved and create p52. When RelB and p52 form a dimer, the NF−κB complex translocates into the nucleus to regulate target gene expression. (25)

There is a variety of mechanisms to regulate NF−κB signaling. Mitogen and stress-activated kinase protein kinase 1/2 (MSK1/2), protein kinase A, PKCζ, IKKα/β, and TRAF-family-member associated (TANK)-binding kinase 1 (TBK1) have all been demonstrated to phosphorylate and hence activate p65. (20,27) Besides, histone

deacetylases, such as HDAC1/2/3, suppress transactivation by acetylating p65, resulting in NF−κB inactivation. In addition to these, one of the other known upstream regulators of the NF−κB pathway is the Malt1 protein (mucosa-associated lymphoid tissue lymphoma translocation protein 1). (28,29)

2.3. MALT1

Malt1 is a paracaspase discovered in a subset of MALT lymphomas (30,31) and has since been studied in immune cells, particularly T and B cells. Malt1 has a similar structure to caspase proteins containing a death domain at the N-terminus and a caspase-like domain at the C-terminus, both of which are followed by immunoglobulin-like domains. Besider, Malt1 protein constitutes a TRAF6 binding motif, which enhances activation of IKK complex. (30)

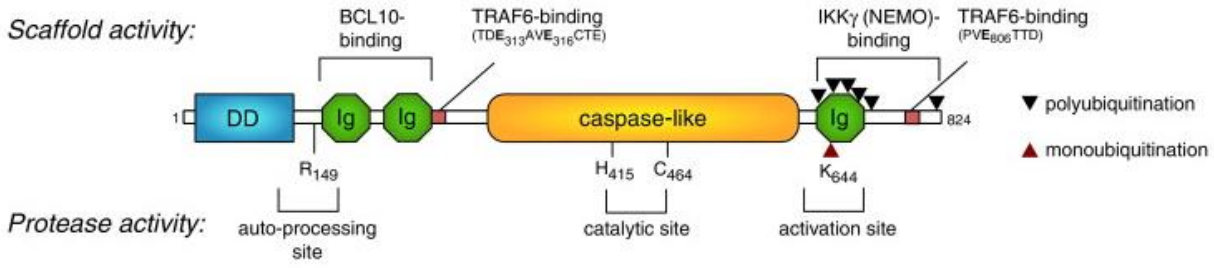


Figure 5. Malt1 protein structure. (32)

Compelling evidence suggest that Malt1 regulates downstream activation of NF−κB networks in response to surface receptor stimulation in a range of scenarios, including immunological settings like T- and B-cell maturation, as well as cancer cell proliferation and survival. (29,30) More importantly, Malt1 is a multifunctional protein in NF−κB signaling with its paracaspase and scaffold activities. (30,33) By its scaffolding function, Malt1 constructs the CBM complex with CARMA1/3, CARD9, and Bcl10, which then recruits TRAF6 and activates IKK complex, leading in NF−κB pathway activation. (31)

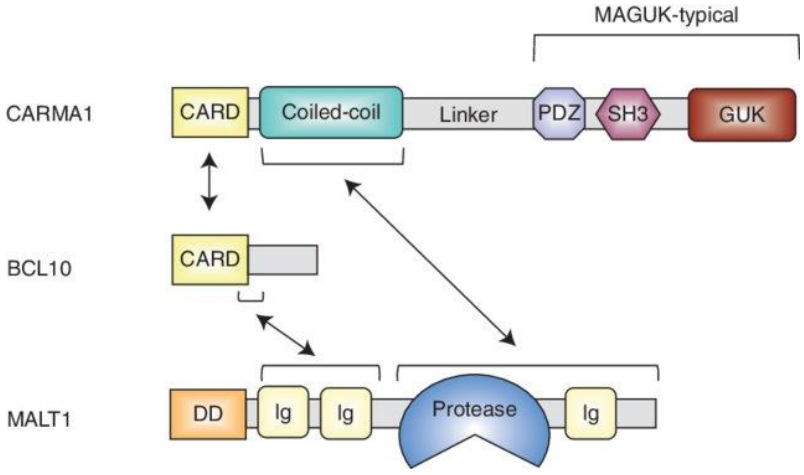


Figure 6. CBM complex formation. (34)

CBM complex formation is not specific to B and T cell receptors and can be triggered by different types of receptors such as epidermal growth factor receptors (EGFRs), G protein-coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs). (31,35,36)

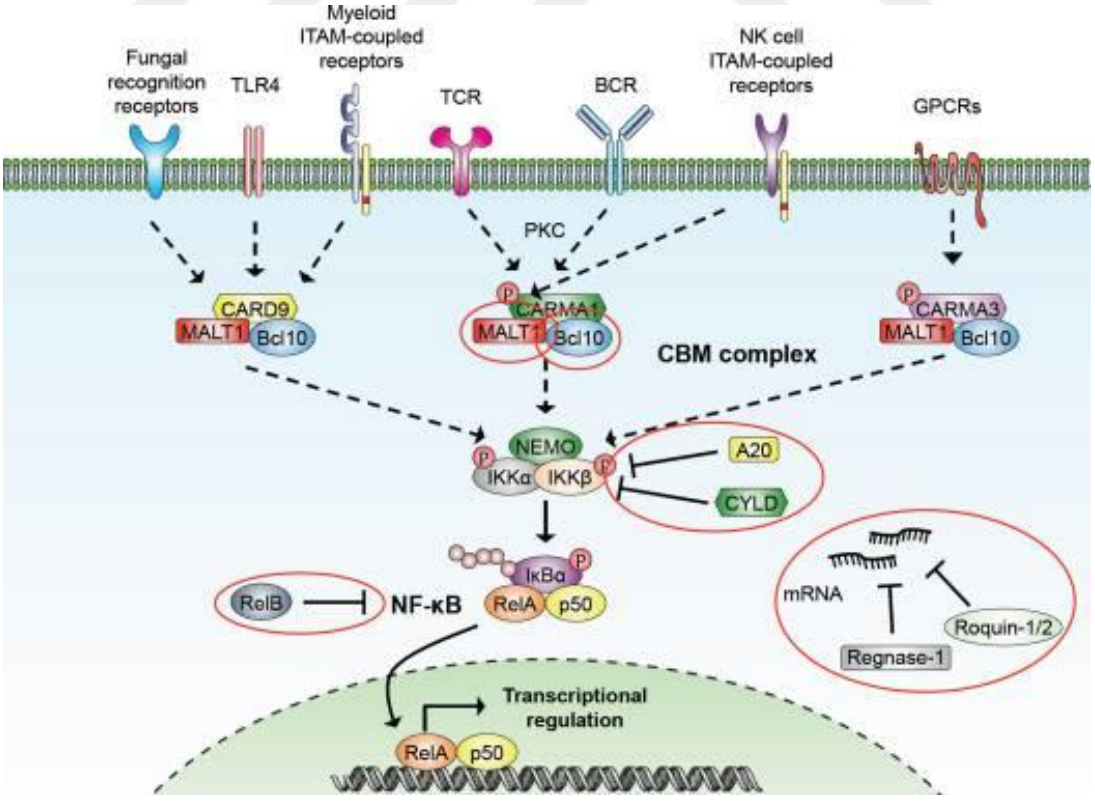


Figure 7. NF−κB pathway activation through CBM complex stimulation by a variety of receptor families. (30)

Besides its scaffolding function, Malt1 cleaves various proteolytic substrates through its protease function to stabilize NF–κB signaling. One of its targets is RelB which forms dimers with RelA and c-Rel to obscure their DNA-binding capabilities or competing with DNA binding to suppresses the NF–κB pathway. Malt1 cleaves RelB to prevent the inhibition of NF–κB pathway activation. (32) A20 and CYLD, deubiquitinating enzymes, are also Malt1 targets. They inhibit NF–κB signaling by detaching the polyubiquitin chains from important signaling factors such as MALT1, TRAF6, and NEMO. Their cleavage by Malt1 activates NF–κB, which increases cell survival. (37) As discovered in MALT lymphomas, MALT1 transfuses with the cIAP2 protein which enables for cleaving of NIK in the non-canonical NF–κB pathway, followed by cascade activation. Moreover, Malt1 cleaves roquin and RNase regnase-1 (MCPIP1), and indirectly governs posttranscriptional regulation of various genes like c-Rel, IL-2 and IL-6. Moreover, MALT1 cleaves itself as well as other targets, facilitated by its auto-processing site resulting in the NF–κB pathway activation in a TRAF6-dependent manner, but the exact mechanism is unknown. (35,37)

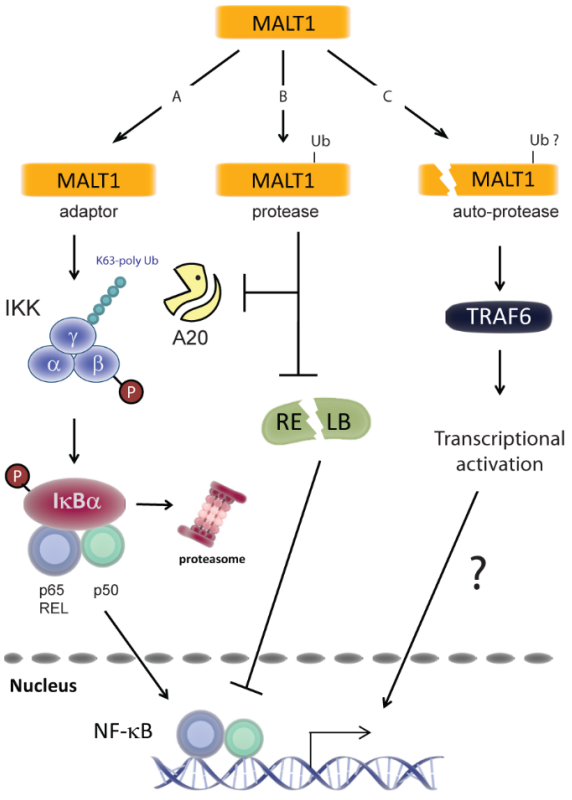


Figure 8. Malt1 in the regulation of NF–κB signaling. (38)

In the last few decades, scientific literature has witnessed a tremendous progress in the study of TGF−β and NF−κB crosstalk in distinct biological contexts. One of the molecules that is known to mediate this crosstalk is TAK1, which is activated through TGF−β signaling pathway resulting in IKK complex activation and p65 translocation to the nucleus in head and neck carcinoma.(39) Yet, the molecular mechanisms that link these pathways are not fully understood. Consistent with this statement, the role of Malt1 in this context has never been explored. As dysregulation of both pathways is common in various cancer cell types and Malt1 has been therapeutically targeted, defining its role in the crosstalk of TGF−β and NF−κB signaling pathways can be advantageous.(37)

3. TYPES OF RESEARCH

Type of research in this study is experimental.

4. TIME AND PLACE OF RESEARCH

The study was conducted in Senturk Functional Cancer Genomics Laboratory at Izmir Biomedicine and Genome Center between September 2019 and March 2022.

5. MATERIALS & METHODS

5.1. MATERIALS

5.1.1. Cell Culture Materials, Reagents & Cell Lines

Huh7, A549, and Hek293T cell lines were kindly provided by Prof. Dr. Mehmet Öztürk (Department of Medical Biology, Izmir Tinaztepe University), Prof. Dr. Ömer Faruk Bayrak (Department of Medical Biology, Yeditepe University), and Prof. Dr. Nuri Öztürk (Department of Molecular Biology and Genetics, Gebze Technical University) respectively. Cell culture plates, dishes, serological pipettes, cryogenic vials, sterile falcon, and microcentrifuge tubes were purchased from Sarstedt (Nümbrecht, Germany), SPL Life Sciences, Korea, or Cornomics Life Sciences. Common cell culture consumables are listed in Table 1.

Table 1. Cell culture materials

Name	Catalog Number	Vendor
DMEM High Glucose	41965039	ThermoFisher Scientific
FBS	10500064	ThermoFisher Scientific
Penicillin-Streptomycin	15140122	ThermoFisher Scientific
DMSO	472301	Sigma
PBS	70011036	ThermoFisher Scientific
Polybrene	H9268-5G	Sigma
PEI pH:6,9	23966	Polyscience
0,45µm PES membrane filter	AND2545	Gilson

5.1.2. Oligonucleotides

Oligonucleotides are purchased from Macrogen (Seoul, South Korea) and Eurofins Genomics (Ebersberg, Germany). Sequences are listed in Table 2. Sequences for shMalt1LT1 were retrieved from Sigma-Aldrich (https://www.sigmaaldrich.com/). Scrambled shRNA (shSCR) was used as a negative control for knock-down experiments Sequences for Smad3-Exon2 and Smad3-Exon4 gRNA constructions were obtained from a study published by Martufi, M. *et al*. (2019) (40) gRen was used as a negative control here since it targets the Renilla gene which does not exist in the human genome.

Table 2. List of the oligonucleotides used in this study.

Name	Sequence (5'→3')
shMalt1_F	CCGGCTACGATGATACCATTCCAATCTCGAGATTGGAATGG TATCATCGTAGTTTTTG
shMalt1_R	AATTCAAAAACTACGATGATACCATTCCAATCTCGAGATTGG AATGGTATCATCGTAG
U6_F	GAGGGCCTATTTCCCATGATT
gRen_F	CACCGGTAGCGCGGTGTATTATACC
gRen_R	AAACGGTATAATACACCGCGCTACC
Smad3 Exon2_F	CACCGGGCGAACTCACACAGCTCCA
Smad3 Exon2_R	AAACTGGAGCTGTGTGAGTTCGCCC
Smad3 Exon4_F	CACCGCCACCAGATGAACCACAGCA
Smad3 Exon4_R	AAACTGCTGTGGTTCATCTGGTGGC

5.1.3. PCR Reagents

Reagents used for cDNA conversion and PCR experiments, and nucleotide sequences of the primer pairs are shown in Table 3 and Table 4, respectively.

Table 3. PCR reagents

Name	Catalog	Vendor
iScript™ cDNA Synthesis Kit	1708891	Bio-Rad
BrightGreen 2X qPCR MasterMix-No Dye	MasterMix-S	abm
Blastaq™ Green 2X qPCR MasterMix	G892	abm
MicroAmp® Fast Optical 96-Well Reaction Plate	4346906	ThermoFisher Scientific
FastStart Essential DNA Green Mastermix	0640271201	Roche Life Sciences

Table 4. Primer list used in this study.

Name	Sequence (5'→3')
qSmad3_F	CAGAGTGCCTCAGTGACAGC
qSmad3-R	AGCGAACTCCTGGTTGTTGA
Smad3-Ex2_F	GCAAGCACAATCCACATTTCCCT
Smad3-Ex2_R	GCAGGCCAGGCAGCATACCTGGT
Smad3-Ex4_F	TACAGCCACGGATGCTAGCATCA
Smad3-Ex4_R	AGAGGAAGGGATGGAAGGGATGA
Malt1_F	GACCCATTCCATGGTGTTTACC
Malt1_R	AATAAATGCATCTGGAGTCCGG
TGFBR1_F	GGCCAAATATCCCAAACAGA
TGFBR1_R	TGATGCCTTCCTGTTGACTG
GAPDH_F	GGCTGAGAACGGGAAGCTTGTCAT
GAPDH_R	CAGCCTTCTCCATGGTGGTGAAGA
JUNB_F	CGATCTGCACAAGATGAACCACG
JUNB_R	CTGCTGAGGTTGGTGTAAACGG
ADAM19_F	CGAGAAGGTGAATGTGGCAGGA
ADAM19_R	AGCTCTGACACTGGATCTTCCC
IL-1β_F	GGCCACATTTGGTTCTAAGAAA
IL-1β_R	TAAATAGGGAAGCGGTTGCTC
CXCL1_F	AGCTTGCCTCAATCCTGCATCC
CXCL1_R	TCCTTCAGGAACAGCCACCAGT
Serpine1_F	CTCCAACCTCAGCCAGACAA
Serpine1_R	CCTCCGATGATACACGGCTG
PAI1_F	GCAGGACATCCGGGAGAGA
PAI1_R	CCAATAGCCTTGGCCTGAGA
LINC_F	CACACAACTTTCCGCTTCCC
LINC_R	CTCGCTCTGCACAAACAGAC
Target1_F	TGCCTGCTTTGATGACGGAG
Target1_R	AGCTGTGGCATATTTAGGGC
Target2_F	ACAAAACAGGAAGGCACCTGA
Target2_R	AACTCGGTAACTGGCACATCG
Target3_F	TCCAGACCTATGAGTGCTTTGA
Target3_R	GCCTTGGGAGTTCTTCCTCAT
NCT3_F	CATTTTAAGCAGACCCACGC
NCT3_R	GGCTCAATGACCTTCCGAAC

5.1.4. Plasmids

The plasmids used in this study are listed in Table 5.

Plasmids including truncated Malt1 promoter regions were kindly gifted by Takaomi Nozawa (Department of Biochemistry, The Nippon Dental University School of Life Dentistry at Tokyo). shLuc was used for Smad knock-down experiment as a negative control since it targets Luciferase mRNA which does not exist in the human genome.

Table 5. List of the plasmids used in this study.

Name	Source
pLKO.1-puro_empty	Addgene #8453
pLKO.1_shSCR	Addgene #17920
pLKO.1_shMALT1	TRCN0000073825
pLKO.1_shTGFBR1	TRCN0000221534
pMD2.G	Addgene #12259
psPAX2	Addgene #12260
pLP/VSVG	ThermoFisher Scientific #K497500
pCL-Eco	Gived from Scott Lowe Lab
pECPV	Senturk, S. *et al*., 2017
pSpCas9(BB)-2A-GFP (PX458)	Addgene #48138
Luc-pSUPER-Retro-GFP/Neo	Addgene #22133
pRetroSuper-GFP-Smad2	Addgene #15722
pRetroSuper-GFP-Smad3	Addgene #15723
pRetroSuper-GFP-Smad4	Addgene #15724
pGL4.10 [luc2]	Promega (E6651)
pRL-TK	Promega (E2241)
3TP-Lux	Addgene #11767
pHAGE NFkB TA-Luc LIBC GFP-W	Addgene #49343
pLV-EF1a-IRES-Puro	Addgene #85132
pcDNA3.1-eGFP	Addgene #13031
pGL4.10-Malt1PromoterRegion-2	Nozawa, T. *et al*., 2021
pGL4.10-Malt1PromoterRegion-4	Nozawa, T. *et al*., 2021
pGL4.10-Malt1PromoterRegion-5	Nozawa, T. *et al*., 2021
TTi-GFP-Smad3 WT	Senturk Lab
pLV-EF1a-Venus-IRES-Puro	Senturk Lab
pLV-EF1a-Smad3-IRES-Puro	Senturk Lab
pLV-EF1a-Smad3-3SA-IRES-Puro	Senturk Lab

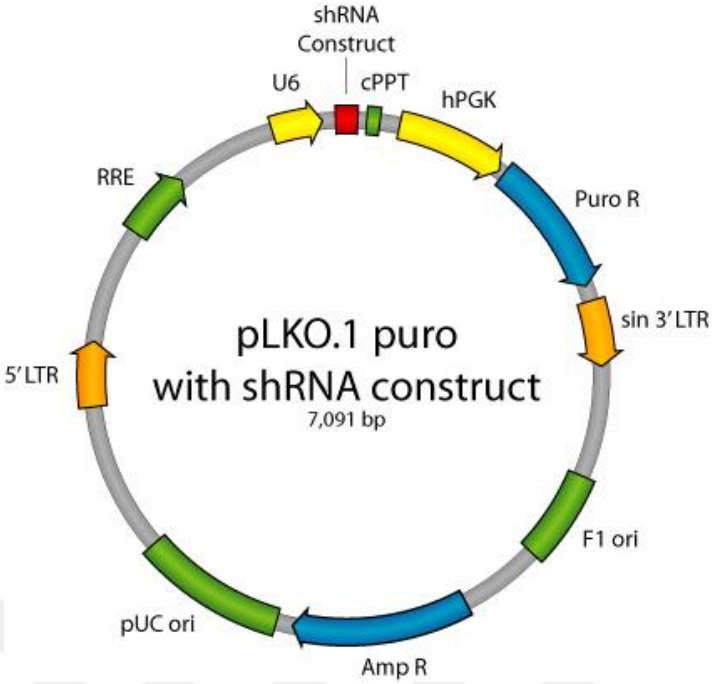


Figure 9. pLKO.1 vector map. (41)

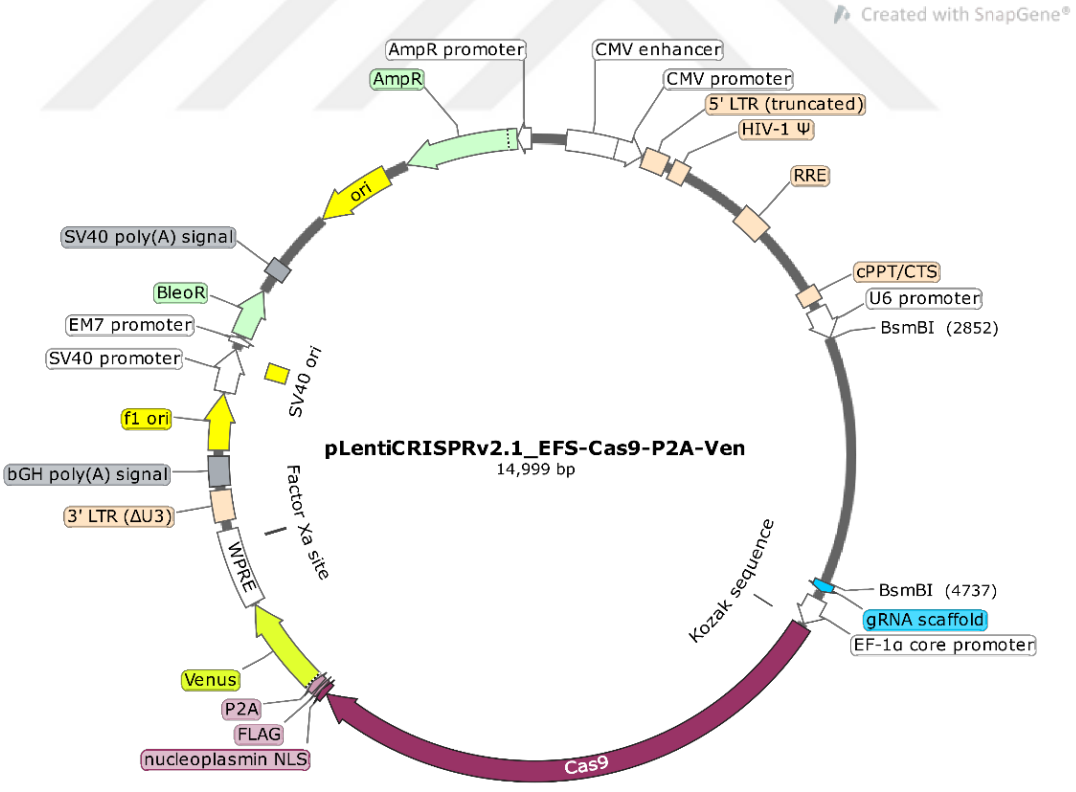


Figure 10. pECPV vector map.

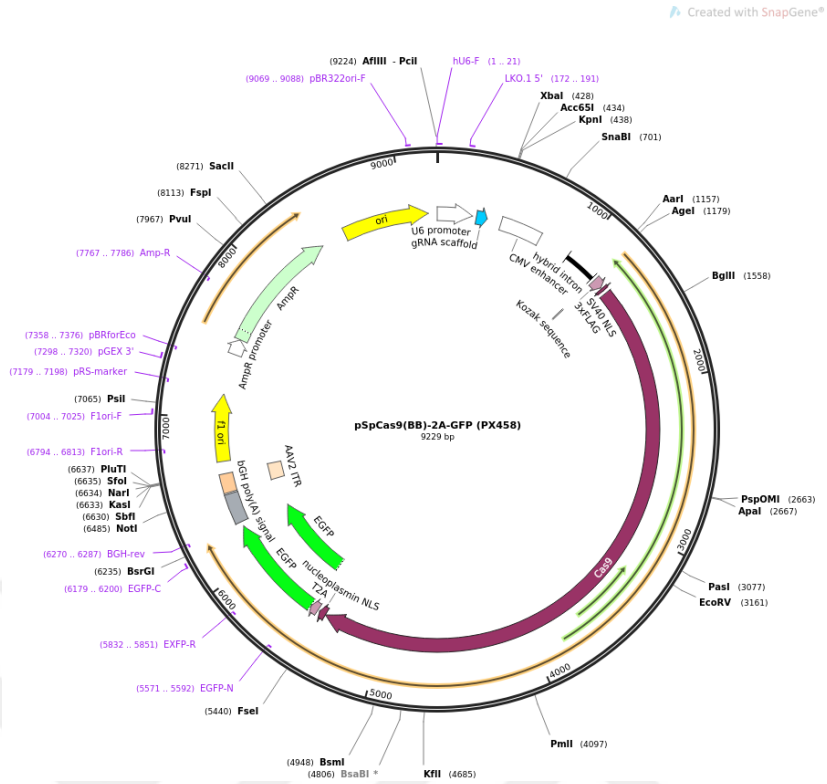


Figure 11. PX458 vector map with gRNA scaffold. (42)

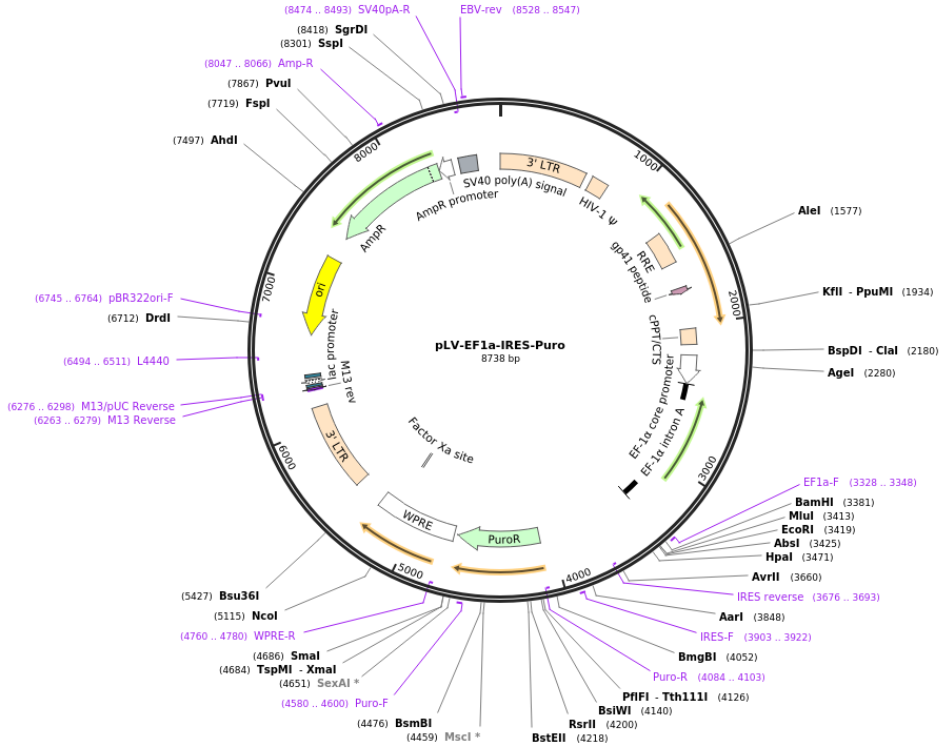


Figure 12. pLV-EF1α-IRES-Puro vector map. (43)

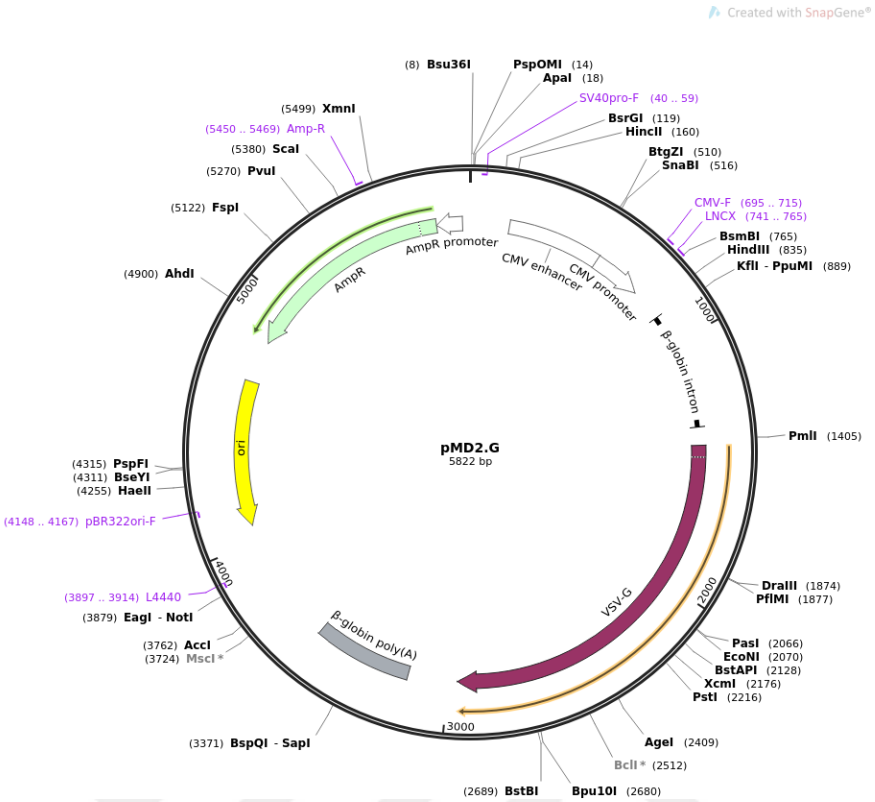


Figure 13. pMD2.G helper vector map. (44)

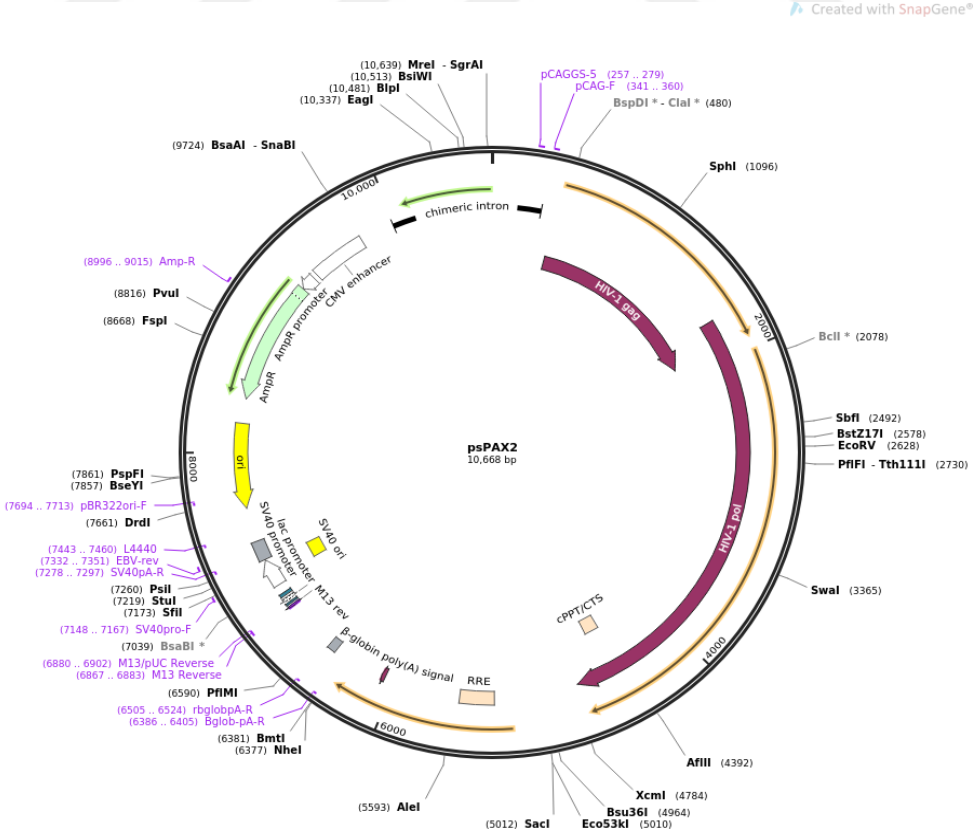


Figure 14. psPAX2 helper vector map. (45)

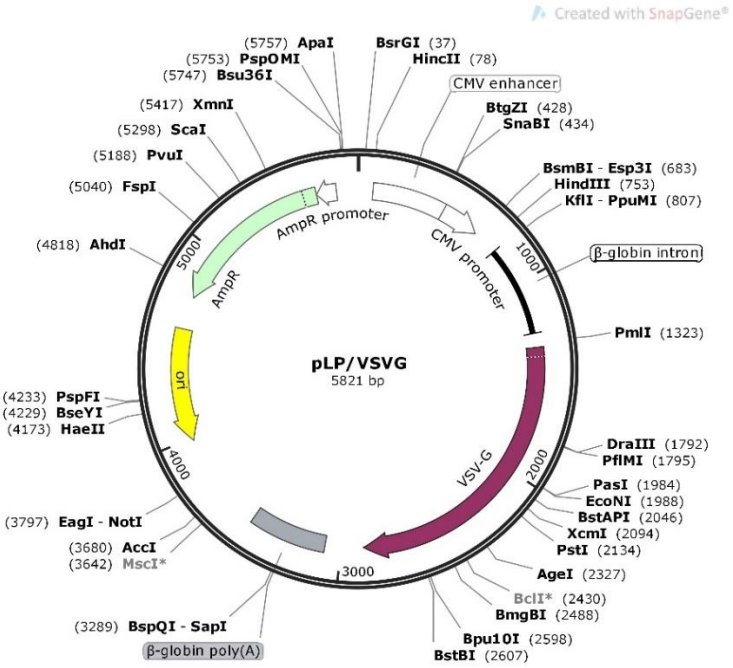


Figure 15. pLP/VSVG helper vector map.(46)

Figure 16. pCL-ECO helper vector map.

5.1.5. Bacterial Strains and Reagents for Bacterial Experiments

Bacterial strains used in the study are shown below.

Strain	Catalog	Vendor
10 Beta Competent Cells	C3019I	NEB
One Shot™ Stbl3™ chemically competent	C737303	ThermoFisher Scientific
E. Coli K12 (dam-) competent cells	E4109S	NEB

Chemical reagents used for bacterial experiments and their recipes were listed in Table 6 and Table 7, respectively.

Table 6. Chemical reagents used in bacterial experiments.

Name	Catalog	Vendor
Ampicillin	A0166	Sigma
Tryptone	1553.05	AppliChem
Yeast Extract	MB16401	Nzytech
LB Broth with Agar	L2897-1KG	Sigma
NaCl	M106404.1000	Merck

Table 7. Reagent recipes used in bacterial experiments.

Reagents	Recipe
Luria Bertani (LB) Media	– 10g NaCl – 5g yeast extract – 10g tryptone in 1 L ddH_2O – Autoclave
Ampicillin Stock (100mg/ml)	– Dissolve 1000mg ampicillin in 10ml ddH_2O – Filter, aliquot and store at -20°C.
LB Agar plates	– Dissolve 35 g (3.5%) LB broth with agar in 1L ddH_2O – Heat and stir – Autoclave to sterilize – Cool to 55°C – Add 1 ml of the 100mg/mL ampicillin stock – Pour into petri dishes

5.1.6. Molecular Cloning Experiments

Enzymes and buffers used in molecular cloning experiments are shown in Table 8.

Table 8. Enzymes & Buffers

Name	Catalog Number	Vendor
BamHI-HF	R3136S	New England Biolabs
BbSI	ER1011	ThermoFisher Scientific
BclI	R0160S	New England Biolabs
EcoRI	R3101S	New England Biolabs
KpnI-HF	R3142S	New England Biolabs
NheI-HF	R3131S	New England Biolabs
XbaI	R0145S	New England Biolabs
FastDigest (10X)	FD0454	ThermoFisher Scientific
FastAP Buffer (10X)	FD0454	ThermoFisher Scientific
FastAP	EF0651	ThermoFisher Scientific
CutSmart® Buffer	B7204S	New England Biolabs
NEB Buffer 2 (10X)	B7002S	New England Biolabs
T4 DNA Ligase	M0202S	New England Biolabs
T4 DNA Ligase Reaction Buffer	B0202S	New England Biolabs
T4 Polynucleotide Kinase	M0201S	New England Biolabs
Taq polymerase	EP1701	ThermoFisher Scientific

5.1.7. Western Blot Reagents & Antibodies

Mini-PROTEAN® Tetra Cell and Mini Trans-Blot® Module system (Bio-Rad, #1658029) were used for western blot experiments. Antibodies used in this study and reagents for western blot studies are listed in Table 9 and Table 10, respectively.

Table 9. Antibodies used in the western blot studies.

Name	Cat number	Vendor
MALT1	sc46677	Santa Cruz Biotechnology
B-actin	3700T	Cell Signaling Technology
Smad2 (D43B4 XP)	5339T	Cell Signaling Technology
phospho-Smad2 (Ser465/467) (138D4)	3108T	Cell Signaling Technology
Smad3 (C67H9)	9523T, 9523S	Cell Signaling Technology
phospho-Smad3 (Ser423/425) (C25A9)	9520T	Cell Signaling Technology
Smad2/3 (D7G7 XP)	8685	Cell Signaling Technology
Smad4 (D3M6U)	38454	Cell Signaling Technology
NF-κB p65 (D14E12 XP)	8242	Cell Signaling Technology
IRDye 680LT Donkey anti-Mouse IgG Secondary Antibody	926-68022	Li-Cor
IRDye 800CW Donkey anti-Rabbit IgG Secondary Antibody	92632213	Li-Cor
Anti-mouse IgG (H+L) (DyLight™ 680 Conjugate)	5470P	Cell Signaling Technology
Anti-rabbit IgG (H+L) (DyLight™ 800 4X PEG Conjugate)	5151P	Cell Signaling Technology

Table 10. Reagents used in western blot studies.

Name	Catalog number	Vendor
Pierce™ BCA Protein Assay Kit	23227	ThermoFisher Scientific
Nitrocellulose Membrane (0.2 μm)	10600004	GE Healthcare
APS	1610GR100	BioFroxx
TEMED	T22500-100	Sigma
Protogel (Ultra-Pure) %30	EC-890	National Diagnostics
Protogel stacking buffer	EC-893	National Diagnostics
Aprotinin (5mg/ml)	1278-25mg	Neofroxx
Leupeptin	1273-25mg	Neofroxx
Pepstatin	P5318-5MG	Sigma
PMSF	LSG36978-5G	Sigma
EDTA	E5134-500G	Sigma
EGTA (0,1M)	E3889-10G	Sigma
β-glycerolphosphate	50020-100G	Sigma
Na deoxycholate	30970-25G	Sigma
Na Vanadate	sc-3540A	Santa Cruz
NaF	S6776-100G	Sigma
Glycine	sc29096A-500g	Santa Cruz
SDS	8170341000	Merck
Tris	T1503-1KG	Sigma
Triton X-100	t8787-100ml	Sigma
BLUeye Prestained Protein Ladder	PM007-0500	GeneDirex
BLUelf Prestained Protein Ladder	PM008/0500	GeneDirex
Bovine serum albumin (BSA)	1126GR100	BioFroxx
Skimmed milk powder	70166-500G	Sigma
Tween-20	BP337-100	Fisher BioReagents
Ponceau S	P3504-10G	Sigma
Acetic acid	27225-2.5L-R	Sigma

Recipes of all the solutions and buffers used in western blot experiments are listed in Table 11.

Table 11. Buffers used in western blot studies.

Buffer	Recipe	
RIPA Lysis Buffer	Triton X-100 (10%)	5 ml
	Tris-Cl pH 7,4 (1M)	2,5 ml
	Na deoxycholate (10%)	2,25 ml
	NaCl (5M)	1,5 ml
	β-glycerolphosphate (0,5M)	1 ml
	NaF (0,5M)	1 ml
	EGTA (0,1M)	500 µl
	PMSF (0,1M)	500 µl
	Na Vanadate (0,1M)	500 µl
	Pepstatin (1mg/ml)	500 µl
	SDS (20%)	250 µl
	EDTA pH 8 (0,5M)	100 µl
	Leupeptin (5mg/ml)	100 µl
	Aprotinin (5mg/ml)	100 µl
	dH_2O to final volume	
Main Gel Buffer	72,684g Tris	
	16 ml of 10% SDS	
	- 180 mL ddH2O	
	- Adjust pH to 8,8 with HCl	
	- Fill up to 200 ml with ddH2O	
5X Laemmli Sample Loading Buffer	Tris pH 6,8 (1 M)	321.5 mM
	SDS	%10 (w/v)
	Glycerol	%25 (v/v)
	Bromophenol blue	%0,025 (w/v)
	β-Mercaptoethanol	0.5%
	ddH2O to final volume	
10X Running Buffer	250 mM Tris base	
	1,9 M Glycine	
	1% SDS	
	pH~ 8,3	
10X Transfer Buffer	250 mM Tris base	
	1,9 M Glycine	
	pH~ 8,3	
10X TBS	24 g Tris base	
	88 g NaCl	
	Dissolve in 800 ml of dH2O.	
	Adjust pH to 7.6 with 12N HCl.	
	Fill up to 1 L.	
1X TBS-T	Dilute 10X TBS to 1X with dH2O.	
	Add 1:1000 Tween-20.	
Blocking solution	5% Skimmed milk powder in 1X TBS.	
Antibody dilution solution	1% BSA (w/v) in 1X TBS-T	
Ponceau S Stain	0,05g Ponceau S	
	2,5 mL Acetic acid	
	dH2O up to 50mL	

5.1.8. Chromatin Immunoprecipitation

Chromatin immunoprecipitation experiments were conducted in collaboration with Erkek Lab (Izmir International Biomedicine and Genome Center). All the reagents and buffer recipes are listed in Table 12 and Table 13. Covaris PolyScience (SN004920) and SonoLab™ 7.2 Software were used for the sonication of the chromatin samples.

Table 12. Reagents and kits used in ChIP studies.

Name	Vendor
DNA Clean & Concentrator-25	Zymo Research
Formaldehyde	Sigma
Paro Fix	
Glycine	Sigma
Paro Rinse 1	
Protease Inhibitor Cocktail (PIC)	Roche
RNase A	
SDS	Thermo Scientific
NaCl	Sigma
Proteinase K	Thermo Scientific
Dynabeads® M-280 Sheep anti-Rabbit IgG	Thermo Scientific
tRNA	Sigma
Bovine serum albumin (BSA)	
EDTA	Sigma
Tris pH 8.0	Sigma
NaHCO3	Sigma
LiCl	Sigma
TERHITOL™ NP-40 (70%)	Sigma
DOC	Sigma
Hepes pH 8.0 (NaOH)	
EGTA	Sigma
Triton-X 100	Sigma
Hepes/KOH pH 7.5	
Covaris MilliTube	Covaris

Table 13. Buffers used in ChIP studies.

Buffer	Recipe
11x Formaldehyde Solution	Mix 7.1 ml Paro Fix and 2.9 ml formaldehyde stock (37%) Final concentration: – 50 mM Hepes pH 8.0 (NaOH) – 1 mM EDTA – 0.5 mM EGTA – 100 mM NaCl – 11% formaldehyde (add fresh before use)
Paro rinse 1	Mix 1 ml Tris, 10 ml EDTA, 0.5 ml EGTA, 1.25 ml Triton X-100 in 483 ml dH2O – 1M Tris pH 8.0, final conc. 10mM – 0.5M EDTA pH 8.0, final conc. 10mM – 0.5M EGTA, final conc. 0.5mM – Triton X-100, final conc. 0.25%
Paro rinse 2	Mix 0.5 ml Tris, 1 ml EDTA, 0.5 ml EGTA, 20 ml NaCl in 473 ml dH2O – 1M Tris pH 8.0, final conc. 10mM – 0.5M EDTA pH 8.0, final conc. 1mM – 0.5M EGTA, final conc. 0.5Mm – 5M NaCl, final conc. 200mM
DOC buffer	Mix 20 ul Tris, 0.5 ml LiCl, 10 ul NP-40, 0.1 ml DOC, 4 ul EDTA in 1366 ul dH2O – 1M Tris pH 8.0, final conc. 10mM – 1M LiCl, final conc. 0.25M – NP-40, final conc. 0.5% – 10% DOC, final conc. 0.5% – 0.5M EDTA, final conc. 1mM Freshly added protease inhibitors cocktail (PIC)
Lysis buffer	Mix 25 ml Hepes/KOH, 50 ml NaCl, 1 ml EDTA, 5 ml Triton X-100, 5 ml DOC, 2.5 ml SDS in 411.5 dH2O – 1M Hepes/KOH pH 7.5, final conc. 50mM – 5M NaCl, final conc. 500mM – 0.5M EDTA, final conc. 1mM – Triton X-100, final conc. 1% – 10% DOC, final conc. 0.1% – 20% SDS, final conc. 0.1% Freshly added protease inhibitors cocktail (PIC)
Elution buffer	Mix 50 ul SDS, 50 ul NaHCO3 in 400 ul dH2O – 10% SDS, final conc. 1% – 1M NaHCO3, final conc. 0.1M

5.1.9. Other Chemicals, Consumables and Commercial kits

Chemicals and commercial kits used in this study are listed in Table 14. Actinomycin D was kindly gifted by Prof. Dr. Bünyamin Akgül (İzmir Institute of Technology)

Table 14. Chemicals & Commercial kits

Name	Catalog Number	Vendor
Recombinant Human TGF-β1	100-21	Peprotech
SB-525334	S8220	Sigma
100bp DNA Ladder	N3231S	New England Biolabs
1kb DNA Ladder	N3232S	New England Biolabs
Gel Loading Dye, Purple (6X)	B7024S	New England Biolabs
SafeView™ Classic	G108	abm
Agarose	A9539-100G	Sigma
NucleoSpin® Gel and PCR Clean-up	740609.5	Macherey Nagel
NucleoSpin® Plasmid	740588.5	Macherey Nagel
NucleoSpin® RNA	740955.50	MN
Monarch Total RNA Miniprep Kit	T2010S	NEB
iScript™ cDNA Synthesis Kit	1708891	Bio-Rad
Dual-Glo® Luciferase Assay System	E2920	Promega
Deoxynucleotide (dNTP) Solution Mix	N0447S	New England Biolabs
RNase A	1263MG050	NeoFroxx
Formaldehyde	F1635-500	Honeywell
Ethanol	920.026.2500	Isolab
Isopropanol	24137-2.5L-R	Sigma

5.1.10. Hardware & Machines

Hardware and machines used in this study are listed in Table 15.

Table 15. Hardware & Machines

Machines	Vendors
Basic Power Supply	Biorad
MySpin™ 6 Mini Centrifuge	ThermoFisher Scientific
Water Bath and Lid	Nüve
pH Meter	Hanna
Microwave Oven	Beko
SimpliAmp Thermal Cycler	Applied Biosystems
Vortex Mixer	ThermoFisher Scientific
Electrophoresis Gel System	Biorad
Centrifuge 5810R	Eppendorf
Centrifuge MicroCL 17R	ThermoFisher Scientific
Incubator MaxQ 4000	ThermoFisher Scientific
Multiskan™ GO Microplate Spectrophotometer	ThermoFisher Scientific
Nanodrop 2000	ThermoFisher Scientific
GelDoc XR+ with Image Lab Software	Biorad
Axio Vert.A1 Inverted Microscope	ZEISS
Li-Cor Odyssey Imaging System	Li-Cor
ABI 7500 Fast thermal cycler	ThermoFisher Scientific
BD FACSAria™ III sorter	BD Biosciences
Centro XS3 LB 960 Microplate Luminometer	Berthold Technologies
Confocal Microscopy	Zeiss LSM880
Vilber Lourmart ECX-F20.L UV Transilluminator	

5.2. METHODS

5.2.1. Cell Culture Methods

Maintenance of cells

Huh7 and A549 cell lines were grown in DMEM high glucose medium supplemented with 10% FBS and 1% Penicillin-Streptomycin (complete DMEM).

Cryopreservation

Cells were frozen when they reached 80-90% confluency. The culture medium was discarded, and cells were washed with 1X PBS. Then, TrypLE or trypsin was added to cover the culture plate surface and incubated for 2-4 min at 37^{O}C. Cells were harvested with a complete growth medium in a falcon tube and centrifuged at 2000 rpm for 2 min in a tabletop centrifuge. The supernatant was discarded without disturbing the cell pellet. Then, the cell pellet was resuspended in +4-+2^{O}C freezing DMEM (72% incomplete DMEM, 20% FBS, and 8% DMSO) and transferred to cryovial tubes which are placed on ice right after. The cryovials were kept at -80^{O}C for short-term storage, and -196^{O}C for long-term storage.

Thawing

The cryovial tube was placed in a 37^{O}C water bath until a small piece of ice was left. Then, cells were transferred into 15 ml falcon tubes including 9 ml complete DMEM and centrifuged at 1300 rpm for 2 min in a tabletop centrifuge. Supernatants including DMSO were discarded. The cell pellet was gently resuspended in complete DMEM and transferred to a cell culture dish.

Subculturing of cells

The medium was discarded, and cells were washed with 1X PBS. Then, TrypLE or trypsin was added to cover the cell culture plate and incubated for 2-4 min at 37^{O}C. Cells were harvested with complete DMEM in a falcon tube and centrifuged at 2000 rpm for about 2 min in a tabletop centrifuge. If subculturing was only for maintenance, cells were split in a proper ratio based on the confluency and plated in a proper cell culture plate. Otherwise, the cell pellet was resuspended in a fresh complete DMEM, and 10µl of the cell-medium mix was loaded into both chambers of

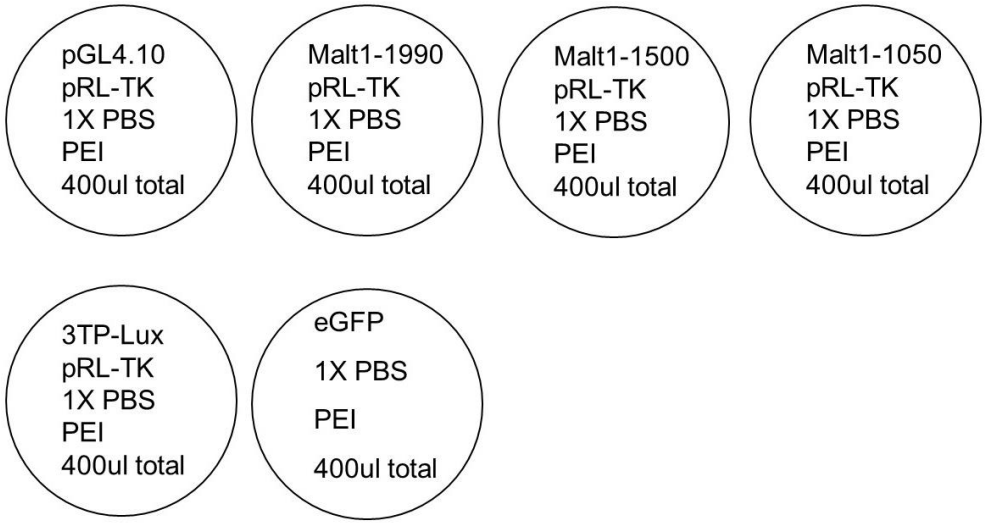
the hemocytometer to be counted. Then, an appropriate number of cells were seeded into a dish or plate.

5.2.2. Transfection

Huh7 cells were plated in a 60mm cell culture dish based on confluency. The day after plating, cells were transfected with PEI transfection reagent as shown below;

Microcentrifuge tube 1	Microcentrifuge tube 2
1X PBS (170ul)	1X PBS
PEI (30ul)	6 ug DNA
200 ul total	200 ul total

The combination in microcentrifuge tube 2 was prepared for each sample DNA and the mixture in tube 1 was prepared for total sample volume and delivered into sample tubes making the final volume 400 μl. After 25min incubation at room temperature, PEI+DNA+PBS mixture was dropped counterclockwise on plates including 2.6 ml DMEM. Transfection set up for Malt1 promoter reporters is shown below;



Huh7 cells were co-transfected with pRL-TK and the target vector. pRL-TK was used as an internal control which provides the Renilla luciferase luminescence to normalize the Firefly luciferase luminescence.

Following overnight transfection, the eGFP transfected plate was monitored under fluorescent microscopy to check transfection efficiency. Then, each 60mm plate was divided into 6-wells in a 24-well cell culture plate to set up TGF−β1 treated and untreated triplicates for each vector. The day after plating, cells wereleft either untreated (control group) or treated with 5 ng/ml TGF−β1 (treatment group) in the low-serum medium for 24h and Dual-Glo & Stop-Glo Luciferase Assay was performed as mentioned in the following sections.

5.2.3. Construction of shRNA, gRNA, and Overexpression Vectors

5.2.3.1. pLKO.1 shRNA construction

Annealing of oligonucleotide pairs

Annealing reaction mixture and conditions for forward and reverse shRNA oligonucleotide pairs are shown below.

Component	Volume (µl)
NEB Buffer 2 (10X)	5
Forward oligo	1,5
Reverse oligo	1,5
ddH2O	42

Annealing conditions:

4 min at 95°C

Ramp down to 25 °C (0,1 °C/s)

Digestion of pLKO.1 vector

AgeI and EcoRI restriction enzymes were used to digest 1 or 2 µg pLKO.1-Empty vector for 8h at 37°C.

Component
CutSmart Buffer (10X)
pLKO.1-Empty
AgeI-HF
EcoRI-HF
ddH2O

Digestion conditions:

8h at 37°C

Digested vector was run on agarose gel and purified by NucleoSpin® Gel and PCR Clean-up kit. Then, digested and purified backbone was ligated with annealed oligo pair in the following reaction;

Sample Reaction		Control Reaction 1		Control Reaction 2	
Component	**Amount**	**Component**	**Amount**	**Component**	**Amount**
T4 Ligase buffer	2 µl	T4 Ligase buffer	2 µl	T4 Ligase buffer	2 µl
Digested vector	50 ng	Digested vector	50 ng	Digested vector	50 ng
Oligo pair	1 µl	T4 ligase	0,5 µl	ddH2O	to 20 µl
T4 ligase	0,5 µl	ddH2O	to 20 µl		
ddH2O	to 20 µl				

Ligation condition:

2h

Room temperature

5.2.3.2. PX458 gRNA Construction

Annealing reaction mixture and conditions for forward and reverse gRNA oligonucleotide pairs are shown below. Dilute annealed oligos 1/20 in the end.

Component	Volume (10 µl)
T4 Ligation buffer (10X)	1
T4 PNK	0,5
Oligo 1 (100 µM)	1
Oligo 2 (100 µM)	1
ddH2O	6,5

Annealing conditions:

30 min at 37°C

5 min at 95°C

Ramp down to 25°C (5°C/min)

PX458 vector was digested and dephosphorylated as shown in the following reaction;

Component	Amount
FastDigest Buffer (10X)	6 µl
FastAP	3 µl
pX458 vector	5 µg
BbSI	3 µl
ddH2O	to 60 µl

Digestion conditions:

1 h at 37°C

Digested and dephosphorylated backbone vector was purified from agarose gel by NucleoSpin® Gel and PCR Clean-up kit. Gel purified backbone vector and annealed oligos were ligated as in the following reaction;

Component	Amount
T4 Ligation buffer (10X)	1 µl
T4 PNK	0,5 µl
Backbone vector	50 ng
Oligo pair	2 µl
ddH2O	to 10 µl

Ligation conditions:

2h at room temperature

5.2.3.3. pLV-EF1a-IRES-Puro Vector Construction

Digestion of pECPV and pLV-EF1a-IRES-Puro plasmids

pLV-EF1a-IRES-Puro was cut with BamHI-HF at single site to insert the Venus reporter gene and dephosphorylated to prevent recyclization in the following reaction;

Component	Volume (µl)
FastDigest Buffer (10X)	1 µl
FastAP	1 µl
Plasmid	1 µg
BamHI-HF	2 µl
dH2O	to 10 µl

Digestion conditions:

1h at 37°C

pECPV plasmid was digested with BamHI-HF to extract the Venus reporter gene in the following reaction;

Component	Volume (µl)
CutSmart Buffer (10X)	1 µl
Plasmid	1 µg
BamHI-HF	1 µl
dH2O	to 10 µl

Digestion conditions:

4h at 37°C

Digested and gel purified backbone and insert were ligated in a 3:1 (insert: vector) ratio in the following reaction;

Component	Amount
T4 Ligation buffer (10X)	1 µl
T4 PNK	0,5 µl
Backbone vector	50 ng
Oligo pair	1 µl
ddH2O	to 10 µl

Ligation conditions:

2h at room temperature

Digestion of TTi-GFP-Smad3 and pLV-EF1a-IRES-Puro plasmids

TTi-GFP-Smad3-WT was transformed into K12 dam- strain since BclI is a dam methylation-sensitive enzyme. pLV-EF1a-IRES-Puro was transformed into an Stbl3 strain.

First, Smad3 was excised from the 1 or 2 µg TTi-GFP backbone by digesting with BclI and EcoRI in the following reaction;

Component	Volume (µl)
CutSmart Buffer (10X)	1 µl
TTi-Smad3	7 µl
BclI	1 µl
EcoRI-HF	1 µl

Digestion conditions:

1.5h at 37°C with EcoRI

1.5h at 50°C with BclI

pLV-EF1a-IRES-Puro vector was digested with BamHI and EcoRI in the following reaction;

Component	Volume (µl)
CutSmart Buffer (10X)	1 µl
pLV-EF1a-IRES-Puro	5 µl
BamHI-HF	1 µl
EcoRI-HF	1 µl
dH2O	2 µl

Digestion conditions:

4h at 37°C

Smad3-WT and digested pLV-EF1a-IRES-Puro were ligated in a 3:1 (insert: vector) ratio with the vector being 50 ng in the reaction shown below;

Component	Amount
T4 Ligase buffer (10X)	1 µl
Digested vector	1.8 µl
Insert	6.7 µl
T4 ligase	0,5 µl
ddH2O	10 µl

Ligation condition:

2h

Room temperature

Digestion of pcDNA3 Smad3-3SA and pLV-EF1a-IRES-Puro plasmids

pcDNA3 plasmid including mutant Smad3 and pLV-EF1a-IRES-Puro empty backbone was cut with BamHI-HF and XbaI enzymes in the following reaction mixture separately;

Component	Volume (µl)
CutSmart Buffer (10X)	1 µl
Plasmid	1 µg
BamHI-HF	0.5 µl
XbaI	0.5 µl
dH2O	to 10 µl

Digestion conditions:

4h at 37°C

Agarose gel electrophoresis was performed to check if the restriction digestion was successful. Smad3-3SA and linearized pLV-EF1a-IRES-Puro were purified from the gel using NucleoSpin® Gel and PCR Clean-up kit according to the manufacturer's instructions.

Ligation

Digested insert and backbone were ligated according to the 3:1 insert: vector ratio with the vector being 50 ng in the following ligation reaction below. One control reaction for ligation was set up with 2x volume of backbone only.

Component	Amount
T4 Ligase buffer (10X)	1 µl
Digested vector	2.85 µl
Insert	2.34 µl
T4 ligase	0,5 µl
ddH2O	10 µl

Ligation condition:

2h

Room temperature

5.2.4. Transformation of Bacteria with Ligation Products

Ligated insert and backbone vector constructs were mixed with 40 µl of bacteria in a tube and placed on ice for 30 minutes. Then tubes were put into a 42°C water bath for 1 minute and on the ice again for 2 minutes.

After the heat-shock process, 1 ml LB media was added to the bacteria and plasmid mixture, then incubated at 37°C for an hour. Tubes were centrifuged at 11.000g for a min, and supernatants were discarded while keeping a small amount of liquid at the bottom to resuspend the pellet. Then, samples were spread on pre-warmed LB agar plates including 100 µg/ml Ampicillin and incubated overnight at 37°C. Then, colonies were picked, and colony PCR was performed if needed and inoculated based on mini- or midi-culture protocol.

5.2.5. Colony PCR

Colony PCR was performed to confirm the cloning efficiency. The protocol is given below. After preparing the reaction mixture, colonies were picked and swirled in PCR tubes, and then dropped in falcon tubes including LB medium with antibiotics.

Component	Amount
Taq Buffer (10X)	2 µl
dNTPs	0,4 µl
Forward Primer (10 µM)	0,4 µl
Reverse Primer (10 µM)	0,4 µl
Taq Polymerase (5U/µl)	0.1 µl
Nuclease-free water	6.7 µl

Thermocycler parameters for colony PCR are shown in Table 16;

Table 16. Colony PCR thermocycler parameters.

Step	Temperature	Time	Number of Cycles
Initial Denaturation	94°C	5 min	1
Denaturation	94°C	30 sec	
Annealing	60°C	30 sec	30
Extension	72°C	2 min	
Final Extension	72°C	5 min	1

The PCR products were run on 1% agarose gel.

5.2.6. Mini Culture & Midi Culture & Plasmid Isolation

Mini culture

Colonies were grown in 5 ml LB media with overnight ampicillin selection at 37^{o}C and 225 rpm shaker agitation. Mini-prep protocol of MN NucleoSpin® Plasmid kit was followed for plasmid DNA isolation.

Midi culture

First, mini culture was started in 5 ml LB media with ampicillin at 37^{o}C in a 225 rpm shaker for 8h. Then, 1 ml of mini culture was transferred into 80-100 ml of LB medium with ampicillin overnight at 37^{o}C in 225 rpm shaker as midi culture. Then, plasmid isolation was performed according to the midi-prep protocol of the MN NucleoSpin® Plasmid kit.

5.2.7. Virus Production

Hek293T cells were used for virus production. Cells were transfected with the target vector and helper plasmids using the PEI transfection reagent. For each target vector, transfection protocol with PEI was performed. PEI and 1X PBS were mixed in a microcentrifuge tube in a 1:10 ratio, respectively. Helper plasmid DNAs and target plasmid DNA (6 µg in total) were mixed in another microcentrifuge tube including 1X PBS. PEI+1X PBS and DNA+1X PBS mixtures were both 300 µl in final volume. Two tubes were combined,and then incubated for 25 minutes at room temperature. Hek293T cells with 80% confluency in a 60mm dish were washed with 1X PBS and 2.4 ml fresh complete DMEM was added. At the end of the incubation, 600 ul mixture was dropped counterclockwise into the medium, and mixed by gently shaking the plate clockwise. The medium with the virus of Hek293T cells was collected at 48h and 72h, combined and filtered with a 0.45µm PES membrane filter, then stored at -80^{o}C.

Lentivirus Production

psPAX2 and pMD2G are the helper vectors for lentivirus production. Target vector:psPAX2:pMD2G were mixed in a 4:2:1 ratio, 6 µg DNA in total.

Retrovirus Production

pLP/VSVG and pCL-ECO helper plasmids were used for retroviral packaging Target vector:pLP/VSVG:pCL-ECO, were mixed in a 1:1:1 ratio, 6 µg DNA in total. respectively.

5.2.8. Infection

Cells were plated into 60mm plates with 50% confluency. Infection was performed with polybrene that was diluted to a 1:1000 ratio in enough volume of DMEM depending on the sample size. DMEM with polybrene was distributed into falcon tubes if there were multiple different viral products. Lentivirus was diluted to 1:20 or retrovirus was diluted to 1:1 in the medium including polybrene, and then added to the cells to be infected for 48h. Two days after infection, cells were washed with 1X PBS twice and TrypLE was added onto cells to be transferred into a 100mm cell culture dish for puromycin selection or cell sorting based on GFP expression.

5.2.9. Cell Sorting

iBG Flow Cytometry Facility carried out the cell sorting. Briefly, cells were trypsinized and collected in falcon tubes. Then, cells were centrifuged, and supernatants were discarded. 1 ml FACS buffer was used to resuspend the pellet. Samples were then transferred into FACS tubes with a cell strainer cap and placed on ice. Additional FACS tubes were prepared including complete DMEM with 20% FBS and 2% pen/strep for cells to be sorted by FC-1072 Aria III Sorter.

5.2.10. Conventional gDNA Isolation

To validate Smad3 knock-out in targeted clones, cells were trypsinized and collected in microcentrifuge tubes, and then the pellet was rinsed with 1X PBS. Cells were then centrifuged at 1500 rpm for 2.5 min and the supernatant was discarded.

Pellets were lysed by 500 μl lysis buffer per sample at 55°C overnight. Then, 250 μl 6M NaCl was added per sample and centrifuged at maximum speed for 20 min. The supernatant was collected into a clean microcentrifuge tube. Samples were centrifuged at max speed for 15 min after vigorous vortexing upon 0.7x isopropanol per sample addition and the supernatant was discarded. 500 μl 100% EtOH was added per sample and vortexed. Then, tubes were centrifuged at maximum speed for 5 min and the supernatant was discarded. 70% EtOH has been added per sample and vortexed again. Samples were centrifuged and the maximum speed for 5 min and the supernatant was discarded. After the pellets were dried out, 75 μl pre-heated (55°C) Tris was used to dissolving the pellets and incubated at room temperature for 30 min. Concentration was measured by nanodrop.

Lysis Buffer	**Recipe for 25 ml**
– 10 mM Tris-Cl pH: 8.0	Mix;
– 0,1 M EDTA pH: 8.0	– 312,5 ul 0,8 M Tris-Cl
– %0,5 SDS	– 5 ml 0,5 M EDTA
– 100 ug/ml Proteinase K	– 0,125 g SDS
– 20 ug/ml RNase A	– dH2O to 25 ml
	Aliquot and freshly add Proteinase K and RNase A before use.

5.2.11. Quantitative Real-Time PCR (qRT-PCR)

RNA isolation

Cells were trypsinized, collected in a 1.5 ml tube, and centrifuged at 2000 rpm for 2 min. The medium was removed, and the pellet was washed with 1X PBS, and the centrifugation step was repeated. Macherey-Nagel NucleoSpin® RNA kit or Monarch Total RNA Miniprep Kit were used to isolate RNA molecules. After the RNA isolation, concentrations were measured by using Nanodrop and equalized to 100 ng/μl for each sample.

cDNA synthesis

BIO-RAD iScript™ cDNA Synthesis Kit was used for cDNA synthesis from extracted RNA. The reaction mixture for each reaction is shown below.

Component	Volume (µl)
iScript Reaction Mix (5X)	4
iScript Reverse Transcriptase	1
Nuclease free water	5
RNA template (1µg)	10
Total	20

Application	Duration	°C
Priming	5 min	25
Reverse transcription	20 min	46
Reverse transcriptase inactivation	1 min	95

Samples were diluted 4.2x by adding 64 µl of nuclease-free water and stored at -20°C.

qRT-PCR

Applied Biosystems® 7500 Fast Real-Time PCR System was used for qRT-PCR experiments. The reaction mixture per sample for the qRT-PCR reaction is shown below.

Component	Volume (µl)
SYBR Green Mastermix (2X)	5
Forward primer (10µM)	0,4
Reverse primer (10µM)	0,4
Template cDNA	4,2
Total	10

Gene expression calculation

The formula to calculate expression levels of a gene relative to an internal control based on the C_t values is shown below.

Relative expression = 2^ (C_t Control – C_t gene of interest)

5.2.12. Western Blot

Protein isolation

The whole process was performed on ice and ice-cold reagents or materials were used.

First, cells were washed with ice-cold 1X PBS once, and then, enough volume of ice-cold RIPA lysis buffer was added to the plates. Cells were scraped with a scraper and collected samples were transferred into clean 1.5 ml tubes. Each sample was vortexed roughly and incubated on ice for 30 min by vortexing every 10 min. At the end of the incubation and vortexing process, cells were centrifuged at full speed, at 4°C for 20-30 min in a tabletop centrifuge. After centrifugation, supernatants that contain proteins were transferred into new 1.5 ml microcentrifuge tubes and stored at -20°C for the short term, or at -80°C for long-term storage.

Determination of protein concentration by Bicinchoninic acid (BCA) assay

Bicinchoninic acid (BCA) assay was performed to determine protein concentration by using Pierce™ BCA Protein Assay Kit. Protein standards with concentrations of 1000, 500, 250, 125, 62.5, and 31.25 µg/ml were prepared by serial dilution with the RIPA buffer. First, albumin standards and samples were added to a 96-well plate as triplicates with a volume of 2 µl. Then, a working reagent was prepared by combining solution B and solution A by the ratio of 1:50 and given to the samples as 50 µl per well. Then, the plate was covered with aluminum foil to protect it from the light and incubated at 37°C for 30 min in the dark. To measure the absorbance at 562 nm, Multiskan™ GO Microplate Spectrophotometer was used. After the blank subtraction from each absorbance, a standard curve was plotted based on the average absorbance at 562 nm of the albumin standards vs. their concentration in µg/ml. The protein concentration of each sample was determined according to the standard curve which is automatically calculated by The Varioskan software.

Protein sample preparation

Determining protein concentrations were equalized about the lowest concentration with RIPA lysis buffer and each sample was mixed with 5X laemmli buffer including 30% β-Mercaptoethanol. Then, samples were boiled at 95°C for 5-7 min and stored at -20°C for the short term or at -80°C for the long term.

SDS gel preparation

Separating gel percentage was determined based on the protein size run in the gel. Separating and stacking gel recipes are listed in Table 16 and Table 17, respectively.

Table 16. 10 ml SDS-PAGE Separating gel recipe.

Components	6%	8%	10%	12%	15%
Protogel (ml)	2	2,7	3,3	4	5
ddH_2O (ml)	6,75	6,05	5,3	4,75	3,6
Main gel buffer (ml)			1,25		
10% APS (µl)			50		
TEMED (µl)			10		

Table 17. Stacking gel recipe.

Components	2 ml	3 ml	4 ml	5 ml	6 ml	7 ml	8 ml	9 ml	10 ml	11 ml	12 ml
Protogel (ml)	0,25	0,375	0,5	0,625	0,75	0,875	1	1,125	1,25	1,375	1,5
ddH_2O (ml)	1,25	1,875	2,5	3,125	3,75	4,375	5	5,625	6,25	6,675	7,5
Protogel stacking buffer pH:6.8 (ml)	0,5	0,75	1	1,25	1,5	1,75	2	2,25	2,5	2,75	3
10% APS (µl)	12,5	18,75	25	31,25	37,5	43,75	50	56,25	62,5	68,75	75
TEMED (µl)	2,5	3,75	5	6,25	7,5	8,75	10	11,25	12,5	13,75	15

Firstly, separating gel was prepared by mixing the proper volume of reagents based on the percentage. APS and TEMED were added at last, respectively. Then, mixture was poured between western blot glasses, and 60% isopropanol was added on top of the separating gel mixture to assure separating gel polymerizes as a flat line. After polymerization, isopropanol was discarded, and remaining were removed by dust-dry napkins. The comb was placed between the glass system after enough volume of stacking gel was prepared and poured on top of the separating gel. After the stacking gel polymerization, the comb was removed, and the running system was set with the gels in a 1X running buffer. 20 µg of protein sample was loaded.

Run and transfer proteins

Protein samples were run at 90V until passing stacking gel, then voltage was set at 120V during separation. The overall process lasts about 2.5 hours. At the end of running process, running buffer including dye with 2-mercaptoethanol was discarded in a proper chemical hazard waste. Glass pair with the gel inside was separated and stacking part of the gel was discarded. A sandwich system with filter papers, gel, and nitrocellulose membrane was prepared. A tightly clamped sandwich was placed in a tank filled with a 1X transfer buffer including 20% ethanol and the transfer was performed at 350 mA for 90 min.

Blocking and blotting of proteins

Membrane was stained with Ponceau S dye mixture to control if transfer of proteins was successfully done. Then, membrane was cut based on the target protein sizes and sliced membrane pieces were placed in an incubation tray for blocking and blotting processes. 5% skim milk powder dissolved in 1X TBS was used to block membranes at room temperature for 1h. Then, skim milk was discarded, and membranes were rinsed with 1X TBS-T 3 times to remove residual blocking reagent. Antibodies were diluted in 1% BSA in 1X TBS-T in an appropriate ratio. Membranes were blotted overnight at 4^{o}C or room temperature for 2h by shaking at 20 rpm. At the end of blotting, membranes were washed with 1X TBS-T 3 times for 10 min by shaking at 50 rpm. Secondary antibody incubation was performed at room temperature for 1 hour by shaking at 20 rpm. Then, membranes were washed with 1X TBS-T 3 times for 10 min and additionally with 1X TBS once for 5 min by shaking at 50 rpm.

Antibody dilutions and incubation parameters are listed below.

Name	Dilution	Incubation
MALT1	1:250	Overnight, 4°C and 2h, room temperature
β-actin	1:2000	Overnight, 4°C
Smad2	1:1000	Overnight, 4°C
phosho-Smad2	1:1000	Overnight, 4°C
Smad3	1:1000	Overnight, 4°C
phosho-Smad3	1:1000	Overnight, 4°C
Smad4	1:1000	Overnight, 4°C
IRDye anti-Mouse IgG Secondary Antibody	1:10.000	1h, room temperature
IRDye anti-Rabbit IgG Secondary Antibody	1:10.000	1h, room temperature
Anti-mouse IgG (H+L) (DyLight™)	1:10.000	1h, room temperature
Anti-rabbit IgG (H+L) (DyLight™)	1:20.000	1h, room temperature

Imaging of proteins

Membranes were imaged at 700 and/or 800 channels by Image-Studio software and Li-Cor Odyssey CLx imaging system.

5.2.13. Immunofluorescent Staining

Cells were trypsinized and counted by using a hemocytometer. 12mm coverslips were placed in each well of a 24-well cell culture plate and cells were plated on coverslips as duplicates by the number of 40.000 cells per well. The next day, the complete medium was replaced with a starvation medium including incomplete DMEM, 0,5% FBS, and 1% P/S and cells were starved for 24 hours. Then, the starvation medium was replaced with complete DMEM including 5ng/ml TGF−β1, and cells were treated for 2h. Then, cells were rinsed with 1X PBS twice and 4% formaldehyde in 1X PBS was used to fix the cells for 10 minutes at room temperature. Cells were washed with 1X PBS for 5 minutes 3 times by shaking and 0.5% Triton -100 in 1X PBS was used to permeabilize cell membranes for 5 minutes by shaking. After blocking with 5% BSA in 1X PBS for 1 hour at room temperature, cells were rinsed with 1X PBST and coverslips were centralized. NF-kB p65 primary antibody was diluted in the ratio of 1:500 in 1% BSA in 1X PBS. 10 µl of the primary antibody was loaded directly onto the coverslips and incubated for 1 hour at room temperature. At the end of the incubation, cells were washed 3 times with 1X PBS for 5 minutes by shaking. 1:500 ratio dilution of secondary antibody was in 1% BSA and 0.3% Triton X-100 in 1X PBS and it is also loaded in the center of the coverslips and

incubated for 1 hour at room temperature. Then, cells were washed 3 times with 1X PBS for 5 minutes by shaking and cells were stained with DAPI for 1 minute. Cells were rinsed with dH2O and mounted on glass. Confocal microscopy was used to visualize the cells.

5.2.14. Luciferase Assay

Dual-Glo Luciferase Assay System was used to perform reporter activity experiments.

Cells were counted and plated in a 60mm culture dish. The next day, 5ng/mL TGF−β1 treatment was started and lasted for 72h. At the end of the treatment, cells were washed with 1X PBS, trypsinized, and collected in 15 ml falcons to be counted by using a hemocytometer. A fixed number of cells were taken in 1,5 ml tubes and centrifuged for 2 min at 2000rpm. The supernatant was discarded and the pellet was resuspended with 1X PBS. Samples were centrifuged for 2,5 min at 2000 rpm and the wash step was repeated. In a tube, 75 ul of Dual-Glo Luciferase Reagent and 75 ul of 1X PBS were mixed for 1/2 dilution per sample. Lysis mixture was added onto samples and pipetted gently 5 times and incubated for 10 min at room temperature. Then, lysates were transferred into the Costar Clearbottom 96-well plate as 50 ul of triplicates. Microwin Software was used to measure luminescence. The luminescence of samples was normalized by number.

For experiments of Malt1 promoter vectors, Dual-Glo & Stop Glo Luciferase Assay System was used. Cells were plated based on confluency in a 60mm cell culture dish. The next day, cells were transfected with defined reporter plasmids for 16-18h. Transfected cells in 60mm culture dishes were trypsinized and transferred into 24-well cell culture plates as triplicates. The day after, cells were treated with 5 ng/ml TGF−β1 in low serum DMEM including 2% FBS and 1% pen/strep for 24h. Dual-Glo Luciferase Reagent and 1X PBS mixture were prepared as explained above. Additionally, the Stop-Glo mixture was prepared by diluting the RLuc substrate in RLuc Buffer by 1:100. Cells were washed with 1X PBS twice and a lysis mixture was added as 50 μl (25 μl Dual-Glo luciferase reagent + 25 μl 1X PBS) per well. The plate was incubated for 10 min at room temperature and lysates were

transferred into the Costar Clearbottom 96-well plate as 50 μl of triplicates. FLuc luminescence was measured first. Then, the Stop-Glo mixture was added as 25 μl per sample to stop FLuc luminescence and RLuc luminescence was measured. The luminescence of samples was normalized by RLuc.

5.2.15. Chromatin Immunoprecipitation and ChIP qRT-PCR

Huh7 cells were plated in 4x100mm cell culture dishes with confluency of 70-80%. Next day, cells were starved with DMEM including 0.5% FBS and 1% pen/strep for 24h. Then, 2 of 4 plates were treated with 5ng/ml TGF−β1 for 2h and others were incubated with complete DMEM as a control group. 10X formaldehyde solution was prepared with Paro Fix freshly and cells were crosslinked with 1X formaldehyde solution for 15 min at room temperature by shaking. Then, crosslinking was prevented with 0.125 M glycine at the final concentration for 10 min at 4°C. Cells were rinsed twice with 12 ml of ice-cold 1X PBS and then scraped with 2 ml of ice-cold 1X PBS. Collected cells were centrifuged for 5 min at 600 g(=1680 rpm) at 4°C. Pellets were resuspended in 12 ml Paro Rinse 1 with PIC and incubated for 5 min on ice, then centrifuged for 5 min at 600g at 4°C. Pellet was resuspended in 12 ml Paro Rinse 2 with PIC and incubated for 5 min on ice, the centrifugation step was repeated. After centrifugation, the pellet was lysed in 1 ml lysis buffer with PIC for 10 min on ice. 50 μl of the samples were saved as unsheared control for agarose gel electrophoresis. Then, the sonication step was performed 5 cycles per sample as 60 sec on 60 sec off for 10 min at 4°C in Covaris milliTube by using 6 sonicators and SonoLab 7.2 Software. After sonication, 50 μl of sonicated samples were saved as sheared control for agarose gel electrophoresis and input DNA. The rest of the samples were centrifuged at 14000g for 10 min at 4°C, supernatants were kept. Then, 150 μl of TE buffer was added to 50 μl of unsheared and sheared samples for reversal of the crosslinking step. Samples were incubated for 30 min at 37°C with 0.2 mg/ml RNase A as the final concentration. Later, 1% SDS, 100 mM NaCl and 200ug/ml Proteinase K were added to samples as the final concentration and incubated at 55°C for 2.5h, then at 65°C overnight. Samples were run on agarose gel if sheared chromatins are at the size of 200-500bp. Then, sonicated samples in Covaris milliTubes were transferred into 1.5 ml tubes to be centrifuged at 14000g for

10 min at 4ºC, and the supernatant was taken on the ice. 60 μl of Dynabeads (#11203D) per sample were mixed with 1 ml TE pH 8.0 by inverting and incubated for 1 min. Then, beads were magnetized by using Dynamag 2 stand (Invitrogen #12321D) and the supernatant was discarded. The previous step was repeated. Then, 1 aliquot of tRNA (Sigma) was denatured for 5 min at 95ºC. Magnetic beads were resuspended in 100 μl TE including 10 μl of 10 mg/ml BSA and 10 μl of 10 mg/ml tRNA. The mixture was incubated for 2h at 4ºC by overhead shaking. Then, beads were washed with 1 ml TE 3 times and 60ul TE per sample was added to the beads. 10 μl of beads per sample was mixed for pre-clearing of chromatins and incubated for 1h at 4ºC by overhead shaking, then supernatants were saved for the immunoprecipitation step. 18 μl Smad3 antibody per sample was mixed with sheared chromatin samples and incubated overnight at 4ºC by overhead shaking. 50 μl of pre-blocked magnetic beads per sample was mixed with chromatin samples and incubated for 3h at 4ºC by overhead shaking. Then, Smad3 bound chromatin pieces were collected by using Dynamag 2 magnetic stand, and the supernatant was discarded. Beads were resuspended in 1 ml lysis buffer with PIC and incubated for 5 min at room temperature by overhead shaking. The previous step was repeated. Then, beads were washed once with 1 ml of DOC buffer including PIC, and beads were collected by using a magnetic stand. The wash step was repeated with 1 ml of TE pH 8.0 including PIC and beads were collected by using the magnetic stand, and the supernatant was discarded. Bead bound samples were centrifuged for 3 min at 960g at 4ºC and residual supernatant was removed carefully by not pipetting beads up. Beads were resuspended in 100 μl of fresh elution buffer per sample and incubated for 15 min at 25ºC on the shaker incubator at 1000 rpm by vortexing every 5 min. Then, samples were centrifuged at 11000 rpm for 2 min at room temperature and 100 μl of supernatant was kept. Elution steps were repeated, and 200 μl immunoprecipitation was collected in total. Reversal of crosslink part was repeated for immunoprecipitated fractions. 4 μl of 10 mg/ml RNase A was added in 200 μl samples to reach 0.2 mg/ml concentration as final and incubated for 30 min at 37ºC. Then, 4 μl of 0.5 M EDTA, 8 μl of 1 M Tris pH 6.5 and 0.5 μl Proteinase K were added in 200 μl samples to reach 10 mM EDTA, 40 mM Tris, and 50 μg/ml Proteinase K as the final concentration. Samples were incubated for 2.5h at 55ºC

and overnight at 65ºC. Zymo DNA Clean&Concentrator kit was used to elute ChIP DNA at the end of the overnight incubation.

ChIP qRT-PCR

After elution, input DNA was diluted at 1:20, and ChIP DNA was diluted at 1:4 to use 2 μl DNA samples per well. FastStart Essential DNA Green Mastermix was used for the PCR mixture and samples were run with Applied Biosystems® 7500 Fast Real-Time PCR System.

5.2.16. Study Plan and Calendar

May 2019 – July 2019	Cell line validation Dose- and time-curve experiments Actinomycin D
July 2019 – September 2019	shTGFBR1 and shSmad clone generation TGF–β treatment experiments
September 2019 – March 2020	Smad3 KO experimental design Smad3 KO single cell clone generation Sanger sequencing Smad3 overexpression experimental design
June 2020 – September 2020	Overexpression vector cloning Clone generation Smad3 overexpression experiments
September 2020 – December 2020	Arrival of Malt1 promoter reporter vectors Luciferase assay
December 2020 – July 2021	Smad3 ChIP experimental design ChIP experiments
July 2021 – October 2021	shMalt1 cloning shMalt1 clone generation P65 IF experiments NF–κB target gene qPCR
October 2021 – March 2022	NF–κB reporter clone generation Reporter vector validation shMalt1 clone generation with reporter vector expressing cells Luciferase assay

5.2.17. Data Evaluation

GraphPad Prism 8 software was used to perform all statistical analyses. Student t-test was used to compare two independent groups. Significance was calculated by using the Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with Q = 1%. ANOVA was used to compare the multiple groups. p-value<0.05: *, p-value<0.01: **, p-value<0.001: *** represents the significance.

5.2.18. Limitation of the Study

No limitations to be reported.

5.2.19. Ethics Committee Approval

This study was approved by the IBG local ethics committee with ethics protocol number 2020-044.

5. RESULTS

5.2. Selection of Cancer Cell Line Models

As a first step, we decided on the cancer cell line models to be used in this study. Specifically, A549 and Huh7 cell lines, which are also known to be TGF−β responsive in the literature (47,48), were selected. To verify TGF−β signaling activation in these cell line models, cells were plated in 6-well plates for transfection with p3TP-Lux plasmid, a well-established TGF−β responsive reporter, which consists of 3TP promoter containing three consecutive TPA response elements (TREs) and a part of the plasminogen activator inhibitor 1 (PAI-1) promoter region (Figure 17). As shown in Figure 18, TGF−β1 (5 ng/ml) stimulation for 24h induces significant reporter activity in both cell lines.

Figure 17. Schematic representation of the reporter plasmid.

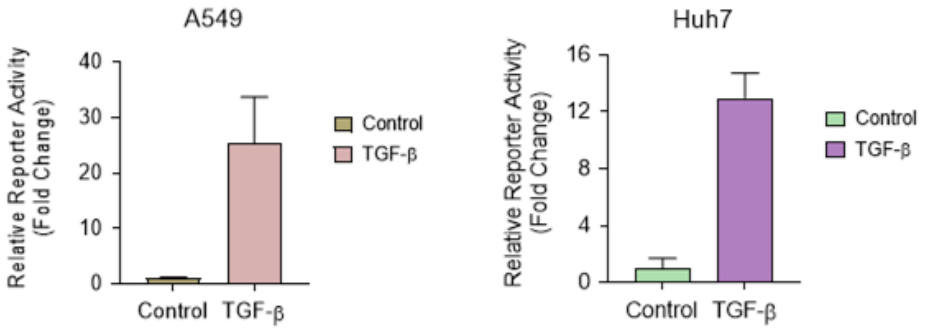


Figure 18. The reporter activity after TGF–β1 treatment in both cell lines.

5.3. TGF–β Upregulates Malt1 Expression

To evaluate the regulation of Malt1 expression by TGF–β pathway activation, we setup dose- and time-dependent experiments. First, both cell lines were plated in 60mm cell culture dishes and treated with TGF–β1 in varying doses for 72h. Expression levels of Malt1 were measured by western blot and qRT-PCR experiments We found that TGF–β treatment induces Malt1 expression in a dose-dependent fashion (Figure 19). Notably, C-terminal Smad2 phosphorylation (p-Smad2) served as an internal control for TGF–β pathway activation in all experiments throughout this study.

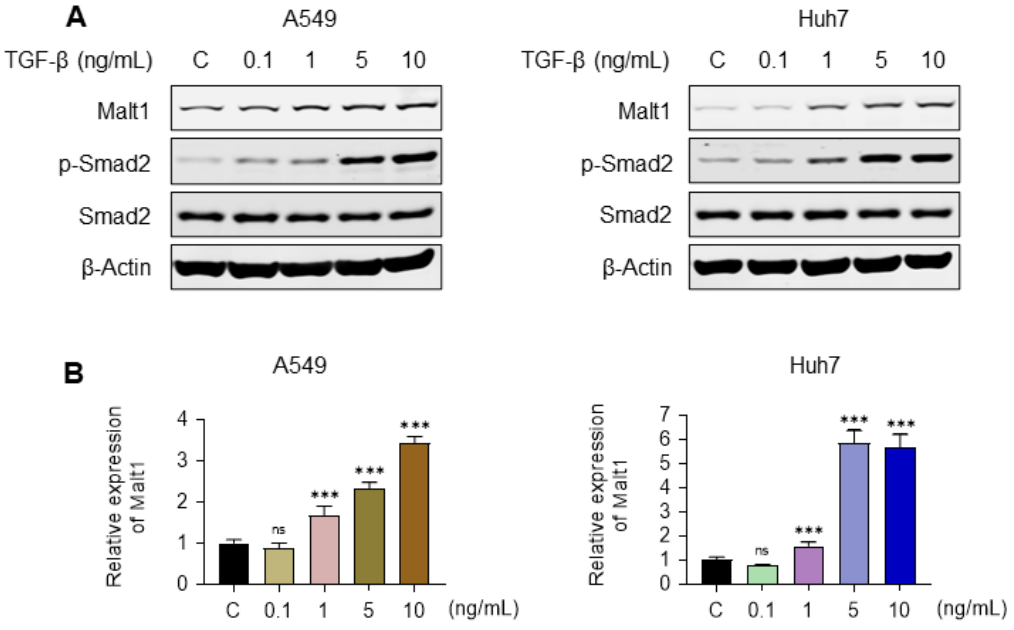


Figure 19. Malt1 expression levels at protein (A) *and RNA* (B) *levels in a dose-dependent manner.*

Since it is the optimal concentration that shows a significant effect in both cell lines, we continued the experiments with 5 ng/ml TGF−β1 treatment. As a first step, Malt1 levels were evaluated in a time-dependent manner. To this end, A549 and Huh7 cell lines were plated as 150.000 cells per 60mm cell culture dish at t=0 and treated with 5 ng/ml TGF−β1 until t=120h. As shown in Figure 20, Malt1 expression in A549 was increased by time whereas in Huh7 cell line, the upregulation was significantly faster at both protein and RNA levels. Overall, we conclude that TGF−β-mediated Malt1 induction is time-dependent.

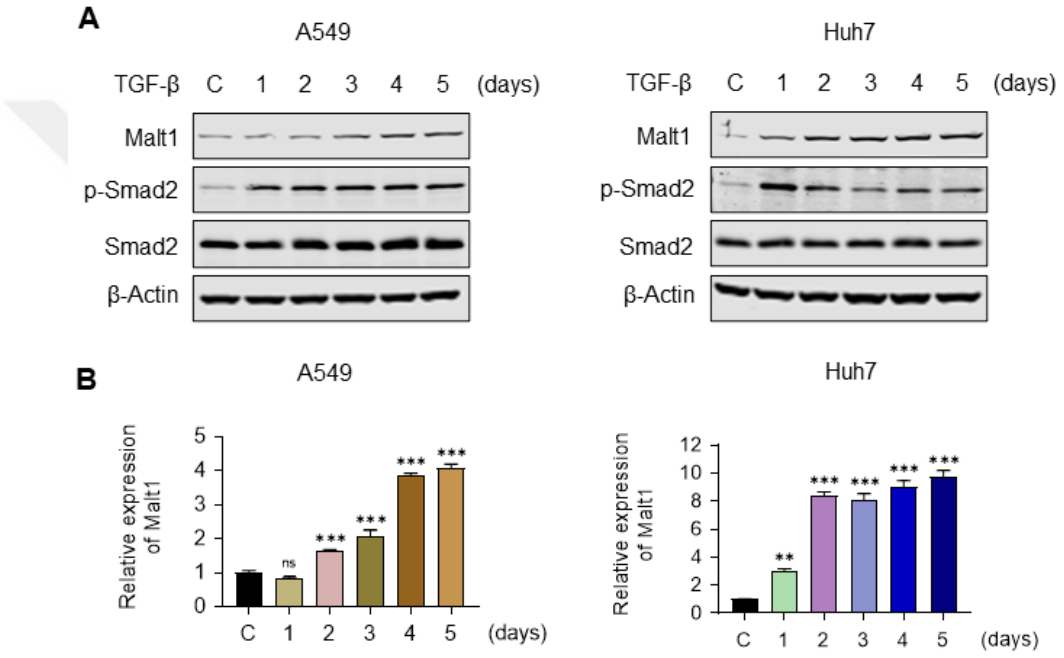


Figure 20. Malt1 expression levels at protein (A) *and RNA* (B) *levels in a time-dependent manner.*

5.4. Transcriptional Regulation of Malt1 Expression by Canonical TGF−β Signaling

To demonstrate transcriptional regulation of Malt1 by TGF−β pathway, we performed a cotreatment experiment in Huh7 cell line with TGF−β and Actinomycin D (Act D), a well-known transcription inhibitor. After plating, cells were treated with TGF−β1 (5 ng/ml) and Actinomycin D (5 μg/ml) individually or in combination. According to qRT-PCR results, ActD treatment attenuated TGF−β-mediated Malt1 upregulation. In this experimental setting, a well-known TGF−β target gene ADAM19

was used as a positive control (49) (Figure 21). These results indicate that TGF−β signaling pathway modulates Malt1 expression in a transcriptional manner.

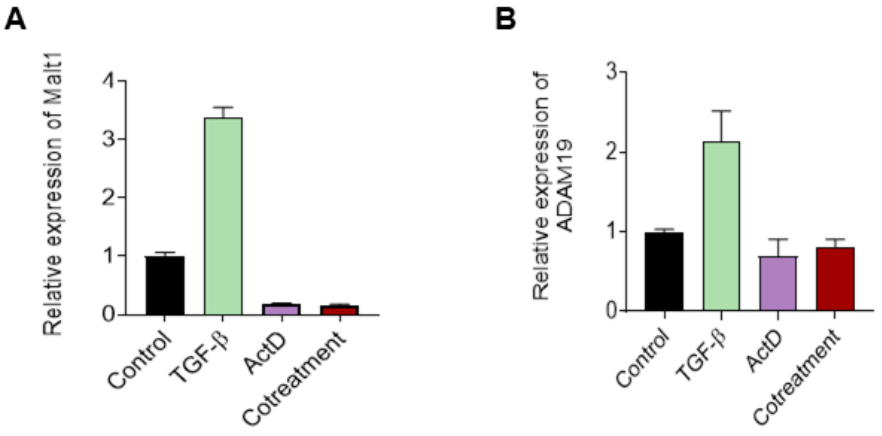


Figure 21. First, Act D treatment was performed for 4h, then cells were washed with 1X PBS and treated with TGF−β1 for 24h. qRT-PCR was performed for Malt1 (A) *and ADAM19* (B) *genes using the ABI 7500 Fast qPCR equipment.*

Having demonstrated that Malt1 expression is upregulated by TGF−β pathway, we then aimed to systematically dissect TGF−β signaling components in this context. So first, we silenced TGFBR1 (ALK5) using shRNA system. A549 and Huh7 cell lines were infected with pLKO.1-shSCR and pLKO.1-shTGFBR1 lentiviruses for 48h and cells were selected with 2.5 μg/ml puromycin for 72h. The knock-down level was evaluated by qRT-PCR for both cell lines (Figure 22). Then, cells were plated as 180.000 cells per 60mm cell culture dish and treated with 5 ng/ml TGF−β1 for 72h. Malt1 expression levels were measured by western blot and qRT-PCR experiments (Figure 23). TGFBR1 silencing abrogated TGF−β-mediated Malt1 upregulation significantly as compared to the control group at both protein and RNA levels.

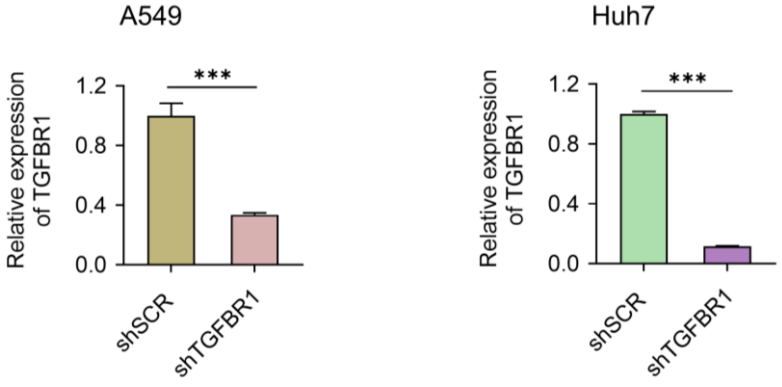


Figure 22. Validation of TGFBR1 knock-down at transcript level.

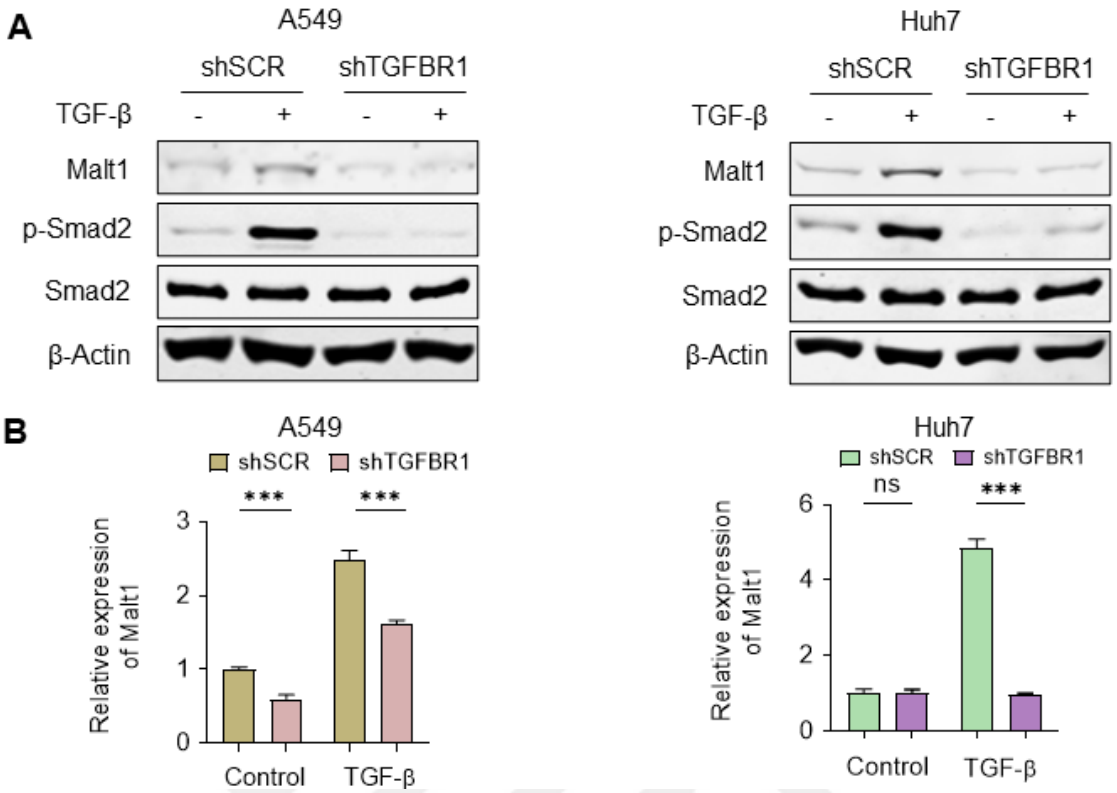


Figure 23. Silencing TGFBR1 abrogates Malt1 upregulation at both protein (A) *and RNA* (B) *levels.*

To corroborate these findings, we used a potent and selective small molecule inhibitor of TGFBR1 (SB525334) which blocks Smad protein activation A549 and Huh7 cell lines were plated in 60mm cell culture dishes and treated with TGF−β1 (5 ng/ml) and the inhibitor (5 μM) alone or in combination for 72h. Similar to shRNA results, TGFBR1 inhibition by the small molecule inhibitorblocked pathway activation as well as Malt1 stimulation (Figure 24).

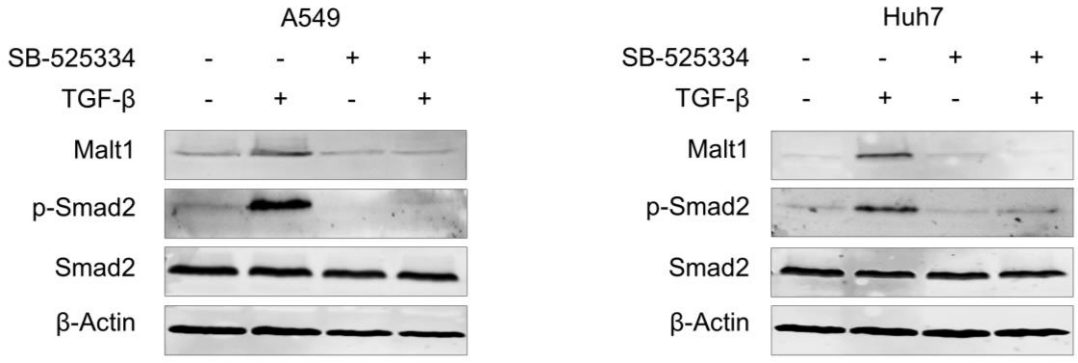


Figure 24. Malt1 expression level depends on inhibitor blockade of TGFBR1. Phospho-Smad2 represents the activation status of the pathway.

In the following experiments, we focused on downstream members of the TGF−β pathway namely Smad2, Smad3, and Smad4. In this experimental scheme, we employed the RNAi system for selective perturbation of each gene. In short, A549 and Huh7 cell lines were infected with retroviral particles expressing shRNAs against each target. Infected cells were enriched by GFP FACS sorting to obtain the clones with the maximum level of knock-down. After the recovery process, we validated knock-down levels by western blotting (Figure 25).

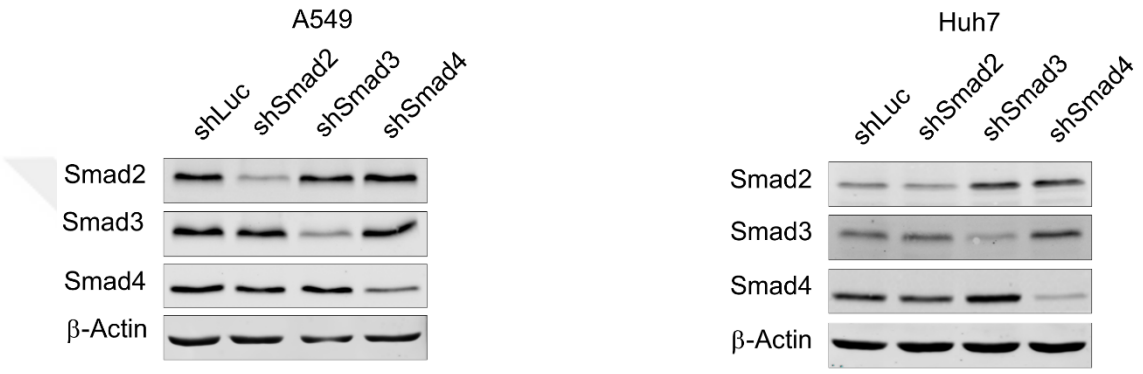


Figure 25. Knock-down levels of canonical pathway components at the protein level for both cell lines.

We then studied Malt1 expression in these clones at protein level. Cells were treated with TGF−β1 (5 ng/ml) for 72h. We found that TGF−β treatment failed to upregulate Malt1 expression in Smad3 and Smad4 knock-down clones when compared to the shLuc control clone (Figure 26). As the shared partner of Smad2 and Smad3, Smad4 n in the complex before translocating to the nucleus in the canonical signaling, it is unsurprising to see a major decrease in the expression level of Malt1 which led us to a correlation between Smad3 and Malt1.

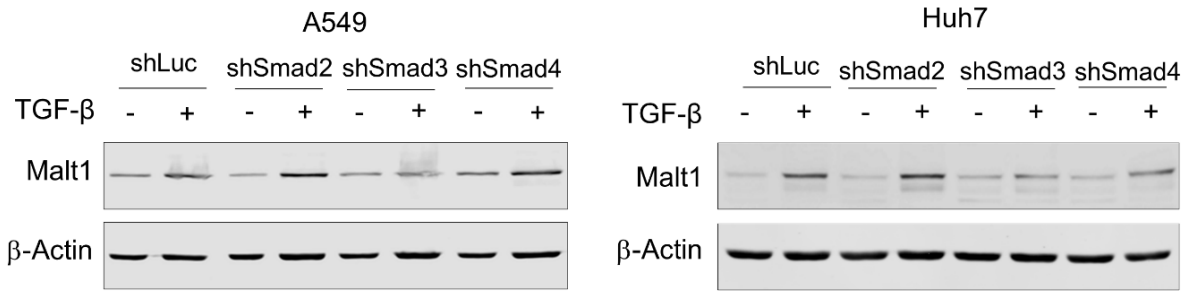


Figure 26. Silencing canonical pathway members affected Malt1 expression at the protein level. Cells were plated at a fixed number after the cell sorting and recovery process and treated with 5 ng/ml TGF−β1 *for 72h.*

We then evaluated Malt1 mRNA level in the Huh7 cell line. Confirming the western blot results, Malt1 mRNA levels were significantly attenuated, specifically in Smad3 and Smad4 knock-down clones. ADAM19 served as a positive control here (Figure 27).

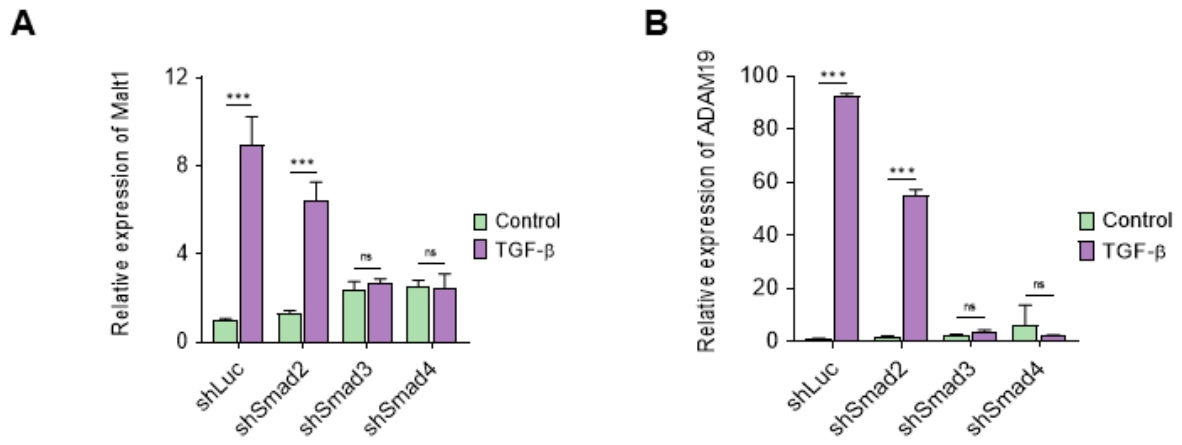


Figure 27. Malt1 (A) and ADAM19 (B) mRNA *levels after silencing Smad proteins in Huh7 cell line.*

5.5. Correlation Between Malt1 and Smad3 Expression

To corroborate the above findings, we conducted a correlation analysis between Malt1 and Smad3 genes in the TCGA datasets of lung adenocarcinoma, liver hepatocellular carcinoma, and all cancers using GEPIA2 (Gene Expression Profiling Interactive Analysis). We found significant co-expression correlation in all datasets (Figure 28), further implying a causal link between the two genes.

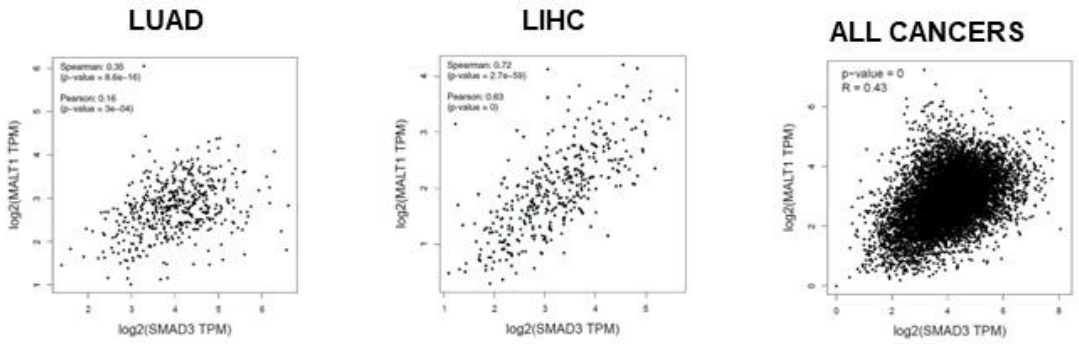


Figure 28. The correlation analysis between Malt1 and Smad3 genes. There is a significant correlation between Malt1 and Smad3 expressions based on R: Spearman correlation coefficient.

5.6. Smad3 Knock-out by CRISPR/Cas9 System

Following the correlation analysis, we wanted to further investigate if Smad3 was required for Malt1 regulation. To examine that, we targeted two exons of Smad3 gene, Exon2 and Exon4, using CRISPR/Cas9 system (Figure 29).

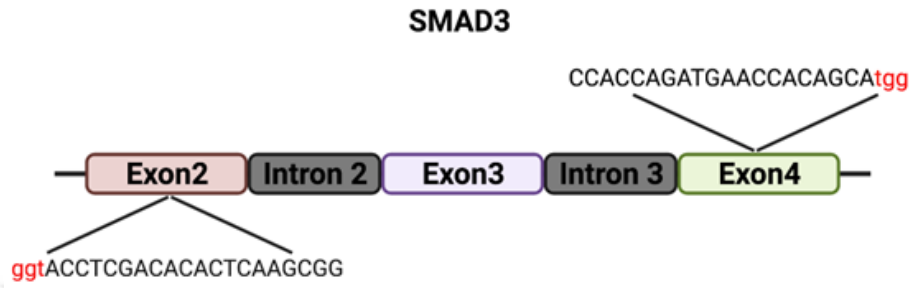


Figure 29. Schematic representation of Smad3 exons targeted by two gRNAs. PX458 backbone was used to introduce target gRNAs.

PX458 transfected cells were sorted by BD FACSAria™ III sorter in 96-well cell culture plates to obtain single-cell clones. The day after sorting, plates were scored for GFP signals, and each well was tracked for single cell clonogenicity. At the end, multiple single-cell clones were derived and validated for knock-out efficiency. We picked one clone for each exon in both cell lines for the following experiments (Figure 30).

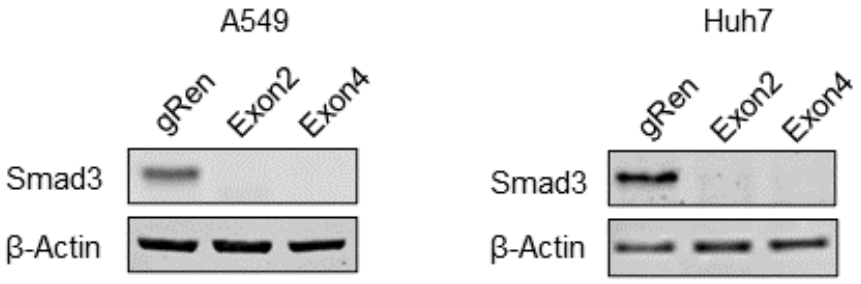


Figure 30. Western blot results of clones validated for each Smad3 exon together with the control gRen.

Next, genomic DNA isolation was performed for each knock-out clone, target regions were amplified by conventional PCR and subjected to Sanger sequencing. Analysis of mutations was performed by Inference of CRISPR Edits (ICE) tool (https://ice.synthego.com) and the indel efficiency was calculated. We note a

homozygous single nucleotide adenine insertion in Huh7 Exon4 clone while there is a heterozygous genotype with a single nucleotide adenine insertion and two nucleotide deletions in Huh7 Exon2 clone as well as in Exon2 and Exon4 clones of A549 cells, all leading to a frameshift mutation (Figure 31).

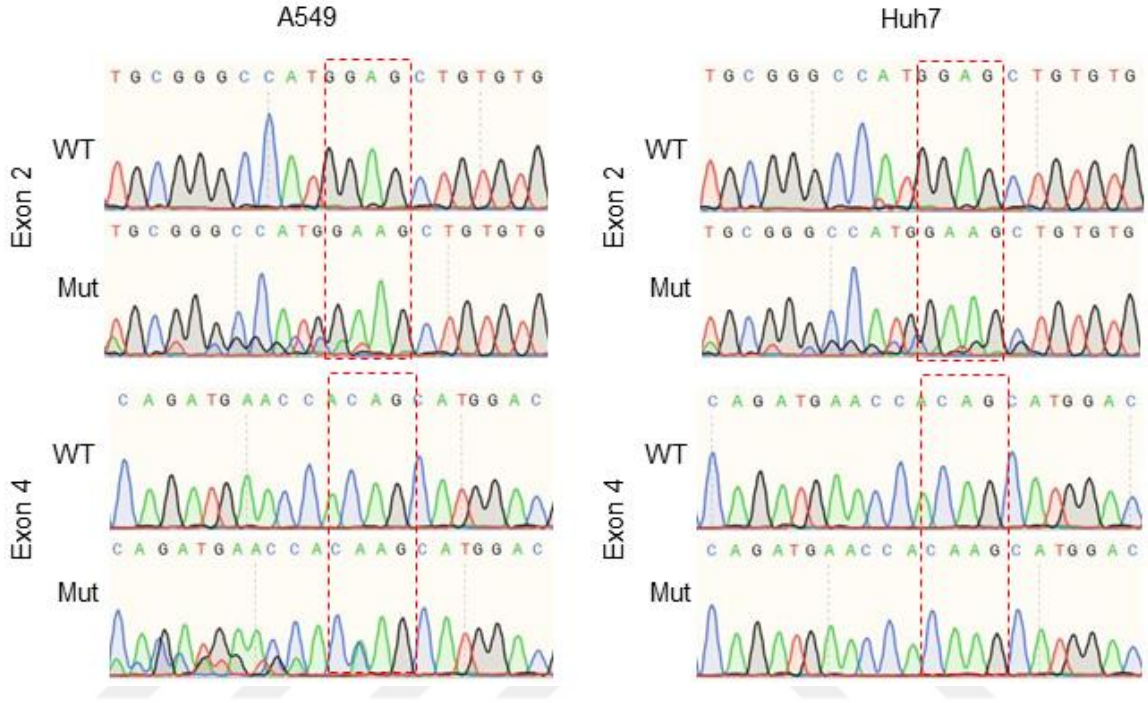


Figure 31. Sanger sequencing results of Smad3 knock-out samples for each exon.

5.7. Malt1 Expression is Regulated by Smad3 through the Canonical TGF–β Pathway

Following knock-out validation, we tested whether Malt1 was regulated by Smad3 through canonical TGF–β pathway. gRen clone was used as a Smad3 sufficient control. Cells were plated as 180.000 cells per 60mm cell culture dish and then, treated with TGF–β1 (5 ng/ml) or medium only for 72h. As expected TGF–β mediated Malt1 upregulation was entirely abrogated in Smad3 deficient clones when compared to gRen cells (Figure 32).

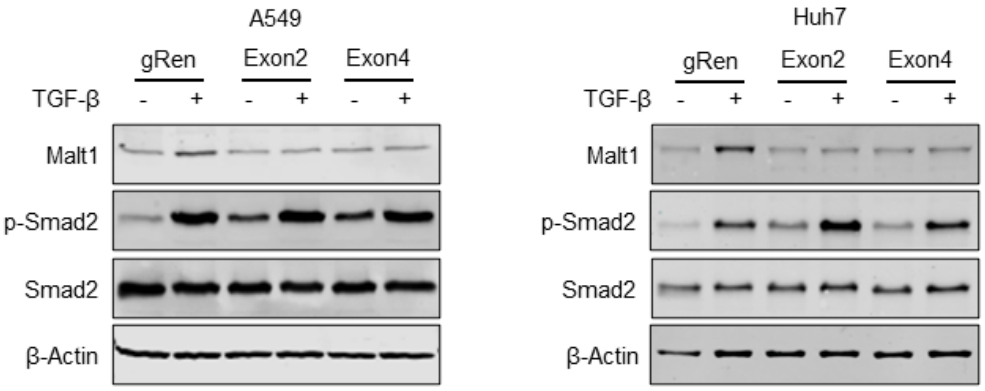


Figure 32. Malt1 expression increased only in TGF−β1 treated gRen samples in both cell lines which shows that Smad3 is needed for Malt1 expression. phospho-Smad2 shows canonical TGF−β pathway induction.

5.8. Smad3 Regulates Malt1 Expression through its C-tail Phosphorylation

TGF−β receptor-mediated phosphorylation of Smad2 and Smad3 at C-terminal serine residues in the SSXS motif, followed by Smad2 and Smad3 forming a complex with common factor Smad4 that translocates into the nucleus to regulate gene expression, is canonical TGF−β signaling. (50) In this context, we wanted to learn more about the relationship between Malt1 regulation and canonical Smad3 activation. As a result, we performed a rescue experiment with Smad3-deficient cells (Exon 4 clones in both cell lines) by ectopically producing wild-type Smad3 (Smad3-WT) or a physiologically inactive variant of Smad3 (Smad3-3SA). Smad3-3SA was produced by replacing three serine residues with alanines, yielding a phosphorylation incompetent dominant negative mutant (Figure 33).

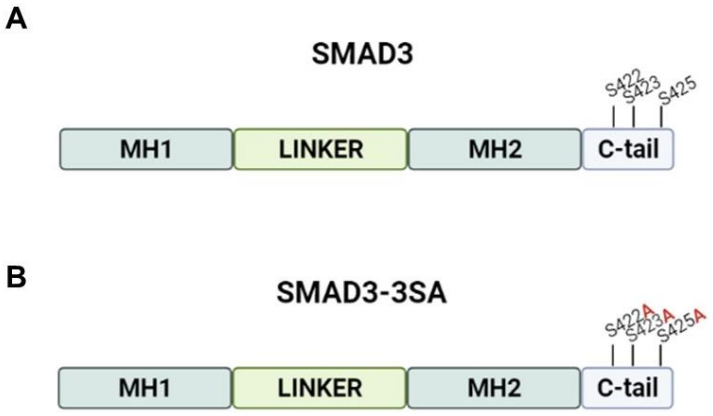


Figure 33. Schematic representation of Smad3 phosphorylation sites at its C-tail by TGFBR1. Wild-type Smad3 (A) and mutant Smad3-3SA (B).

Cells were infected with lentiviruses expressing wild-type and dominant-negative Smad3-3SA. Following infection, cells were selected with puromycin (2.5 μg/ml), and then plated as 150.000/60mm cell culture dish for TGF−β1 (5 ng/ml) treatment. After 72h, protein isolation and RNA isolation was performed. Venus is used as a negative control. Malt1 expression levels were increased at the protein and RNA levels in Smad3 wild-type overexpressing cells compared to Venus and mutant Smad3-3SA clones. A canonical TGF−β target gene ADAM19 was used as a control to demonstrate that the overexpression was functionally successful and the mutant Smad3-3SA was failed to perform its biological function as a transcription factor (Figure 34). Based on these results, we conclude that the canonical activation of Smad3 was instrumental for TGF−β-controlled Malt1 elevation.

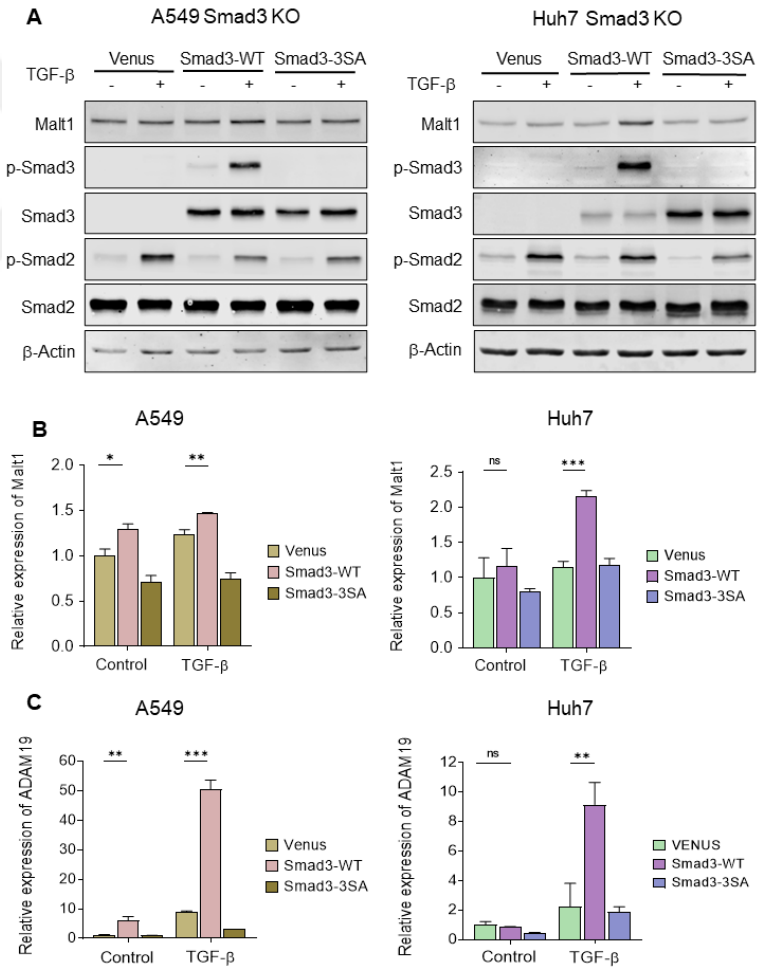


Figure 34. Malt1 expression at protein (A) and RNA (B) levels after o*verexpression of Smad3 wild-type and Smad3-3SA in the knock-out clones.* ADAM19 (C) was used as control. *The Licor Odyssey CLx machine was used to scan nitrocellulose membranes to visualize proteins. 7500 Fast qPCR machine was used to run the qRT-PCR reaction.*

5.9. Malt1 is Regulated at the Promoter Level by Smad3

Based on the results obtained so far, we wondered if Malt1 transcriptional regulation is mediated at the promoter level. Using JASPAR CORE 2018 vertebrates library on Eukaryotic Promoter Database (EPD), we detected multiple Smad binding elements (SBE) located on the -2000 bp upstream region of the transcription start site (TSS) of the Malt1 gene, suggesting that its regulation might essentially be governed through the promoter region (Figure 35).

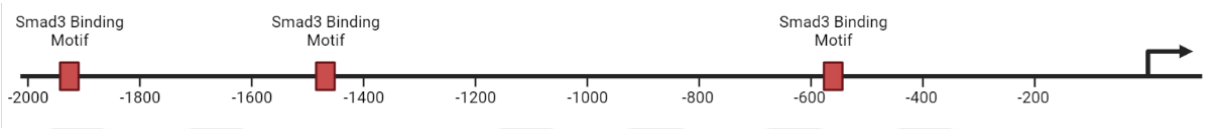


Figure 35. Schematic representation of the Smad-binding elements located in the 2 kb upstream region of the Malt1 gene.

In a study conducted by Nozawa T *et al.* (2021), the promoter region of the Malt1 gene from 701 bp downstream to 2500 bp upstream was amplified using Bacterial Artificial Chromosome and randomly truncated into different sizes. Then, truncated pieces were cloned in the pGL4.10 reporter vector. To examine the regulation at the promoter level, we sought to set up an experiment with a select number of these regions shown in Figure 36. Reporter vectors with the regions labeled as p-1050, p-1500, and p-1990 were kindly gifted by Takaomi Nozawa. Notably, these regions match with the SBEs detected on the Eukaryotic Promoter Database as mentioned above. Of particular importance, the region spanning the 701 bp downstream of the TSS did not include any detectable SBE for Smad3 in the EPD (Figure 37).

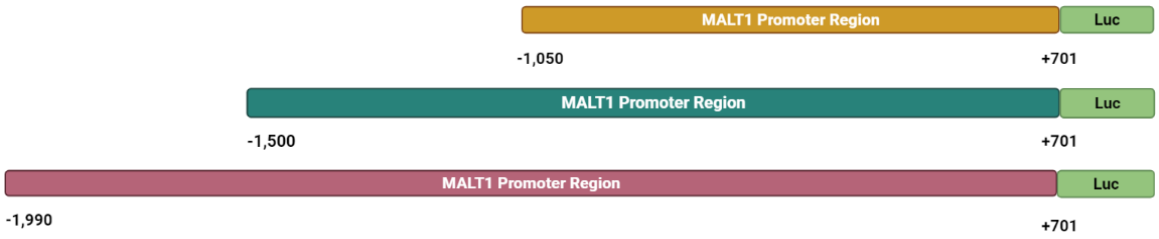


Figure 36. Truncated regions of Malt1 promoter cloned upstream of the luciferase gene in the pGL4.10 reporter plasmid.

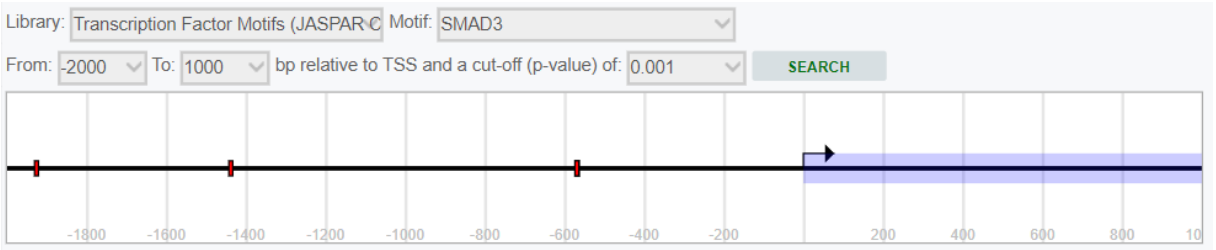


Figure 37. Smad-binding elements *located in the 2 kb upstream region* starting from TSS on *Malt1* promoter. Blue colored region in the downstream of TSS includes no SBEs based on the significance of p-value: 0.001 in EPD website.

Malt1 promoter experiments were conducted in the Huh7 cell line. Cells were plated in 60mm cell culture dishes (nearly 80% confluency) for transfection with reporter vectors. Plasmids were transfected together with pRL-TK as explained in the methods section. pcDNA3.1-eGFP was used as a transfection control and pGL4.10 empty vector served as a negative control (Figure 38)

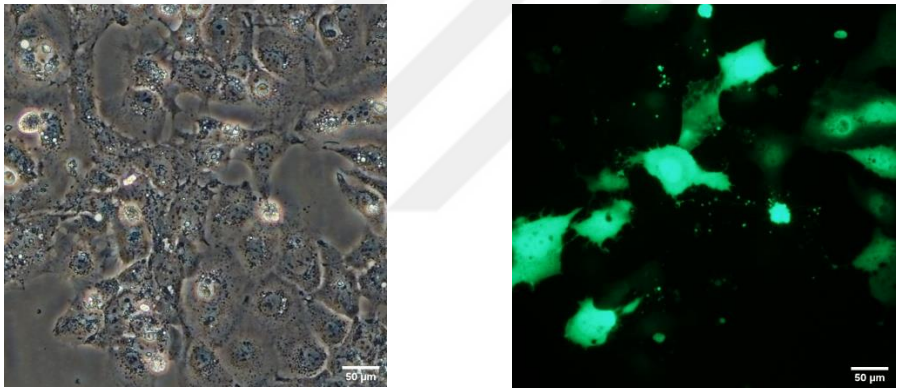


Figure 38. Cells were transfected with a pcDNA3.1-eGFP plasmid for 16-18h. Then, cells were visualized by fluorescent microscopy at phase contrast (left) and GFP channels (right).

At the end of transfection, cells were treated with TGF−β1 (5 ng/ml) in a low-serum medium for 24h, and then Dual-Glo&Stop-Glo Luciferase Assay was performed. There was a remarkable augmentation in the reporter activity of 2 truncated regions (-1500 and -1990) upon TGF−β1 treatment in contrast to the control vector (Figure 39).

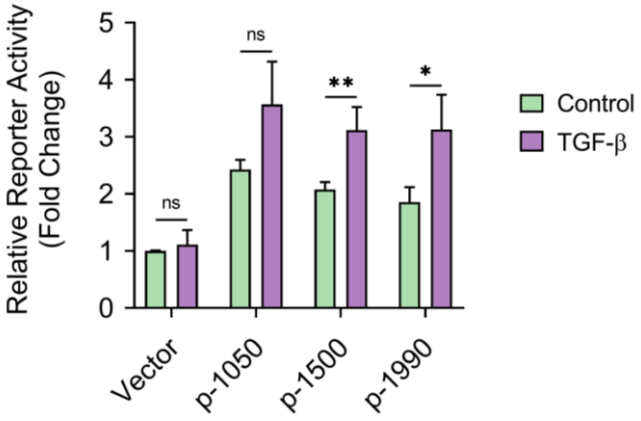


Figure 39. Vector represents pGL4.10 empty construct. Plasmids were labeled based on the sequence lengths they span in the promoter region of Malt1. Normalization of luminescence was calculated based on Renilla luciferase.

Based on the results obtained so far, we decided to search for available datasets with Smad3 ChIP-seq results (Figure 40).

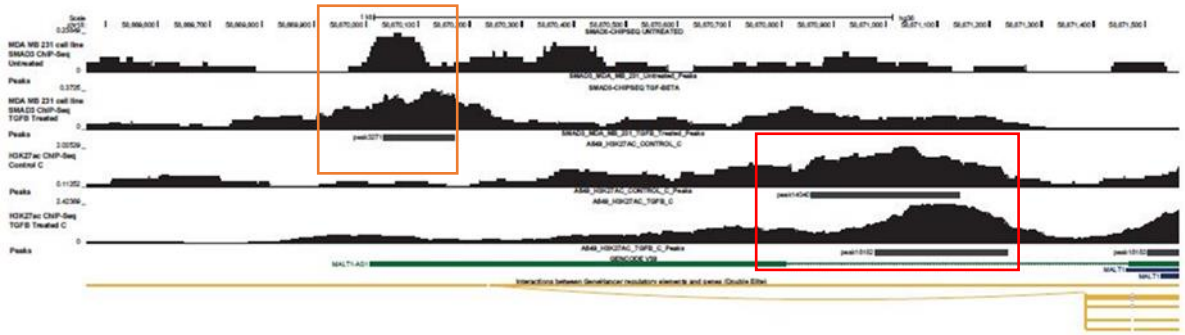


Figure 40. Datasets are placed in an order as untreated and treated from top to bottom. The first two datasets on the top belong to Smad3 ChIP-seq in the MDA-MB-231 cell line while the other pair of peaks belong to H3K27ac ChIP-seq in the A549 cell line.

Using Cistrome Database, we analyzed Smad3 ChIP datasets in the MDA-MB-231 cell line and found TGF−β-mediated differential enrichment of Smad3 peaks, highlighted with orange box. We also found 2 times greater acetylation peaks in the H3K27ac ChIP-Seq dataset performed in the A549 cell line (highlighted in the red box) treated with TGF−β which suggests that there can be a TGF−β-driven accessibility in Malt1 promoter. These promising findings prompted us to test Smad3 binding on Malt1 promoter by ChIP-qRT-PCR experiments. To this end, Huh7 cells were plated in 100mm cell culture dishes with 80% confluency and treated with TGF−β for different time points. Cells were collected, and ChIP protocol was followed as mentioned in the methods section. After ChIP DNA was obtained, ChIP-qRT-PCR

was performed with 2 positive control regions with well-defined Smad3 peaks (selected from Smad3 ChIP-seq datasets) and target regions belonging to Malt1 promoter (Figure 41). Unfortunately, we were not able to find any significant fold-enrichment even with the positive controls (Figure 42).

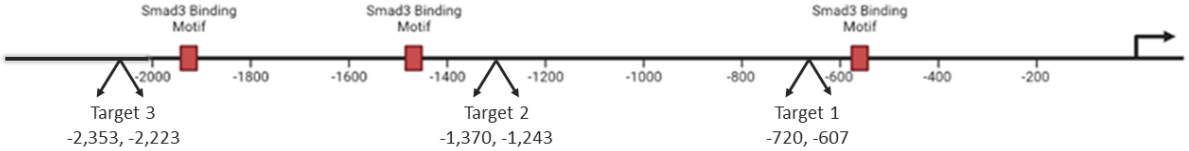


Figure 41. Schematic representation of ChIP qRT-PCR target regions (primer positions are highlighted) and SBEs belonging to Malt1 promoter.

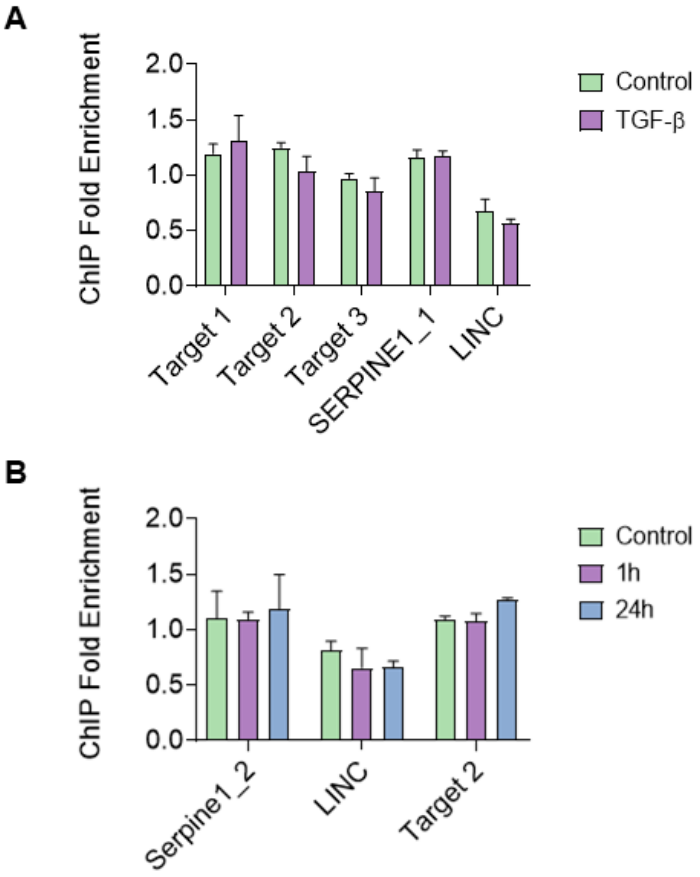


Figure 42. Smad3 ChIP qRT-PCR in Huh7 cell line. SERPINE1_1 (A) and SERPINE1_2 (B) are positive controls belonging to the same region but two different primer pairs. LINC is also positive control region (A-B). *Cells were treated with TGF−β1 for* 2h (A) while TGF−β treatment was experimented for *24h and 1h to catch a point in Smad3 binding on DNA but there was no fold-enrichment with any target* (B).

5.10. Malt1 Depletion Attenuates TGF–β-induced NF–κB Pathway Activation

As alluded to earlier, we hypothesized that Malt1 can be an intermediary protein between TGF–β and NF–κB signaling pathways. Having shown that Malt1 is a TGF–β target, we now examined whether Malt1 impacts NF–κB activation facilitated by the TGF–β pathway. To achieve this, Malt1 expression was blocked using the RNAi system (Figure 43) in A549 and Huh7 cell lines as explained in the methods section.

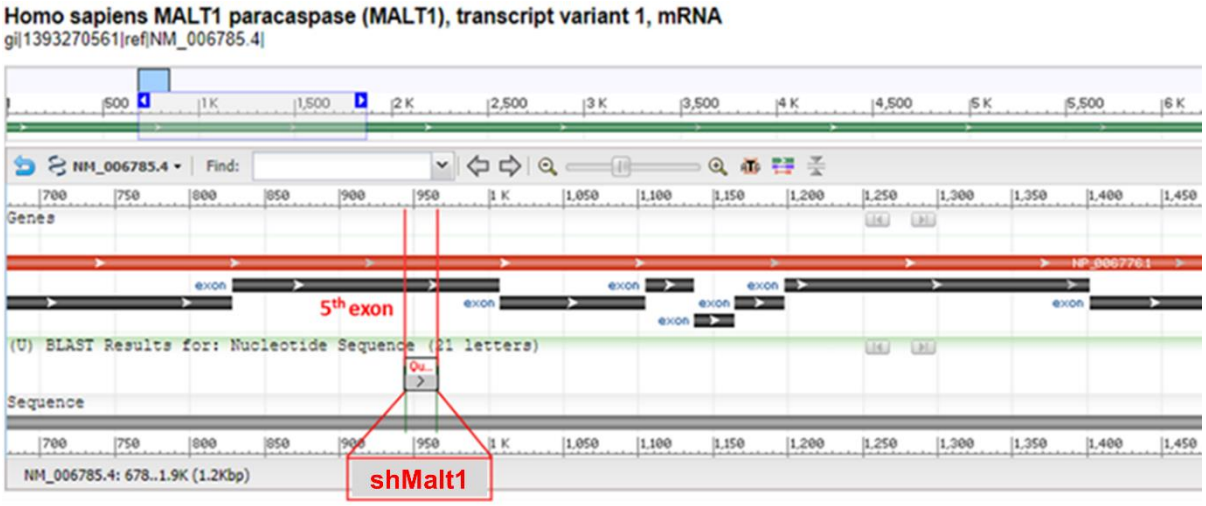


Figure 43. shRNA position on the Malt1 mRNA.

Following infection, cells were selected with puromycin (2.5 μg/ml) for 72h and plated as 150.000/60mm cell culture dish for control and Malt1 shRNAs after the recovery period. Then, cells were incubated with TGF–β1 (5 ng/ml) for 72h, and protein and RNA isolation was performed. Malt1 knock-down level was evaluated by western blot and qRT-PCR experiments. Specifically, we achieved more than 70% depletion in Malt1 expression. We also observed significant suppression in TGF–β-induced Malt1 expression (Figure 44).

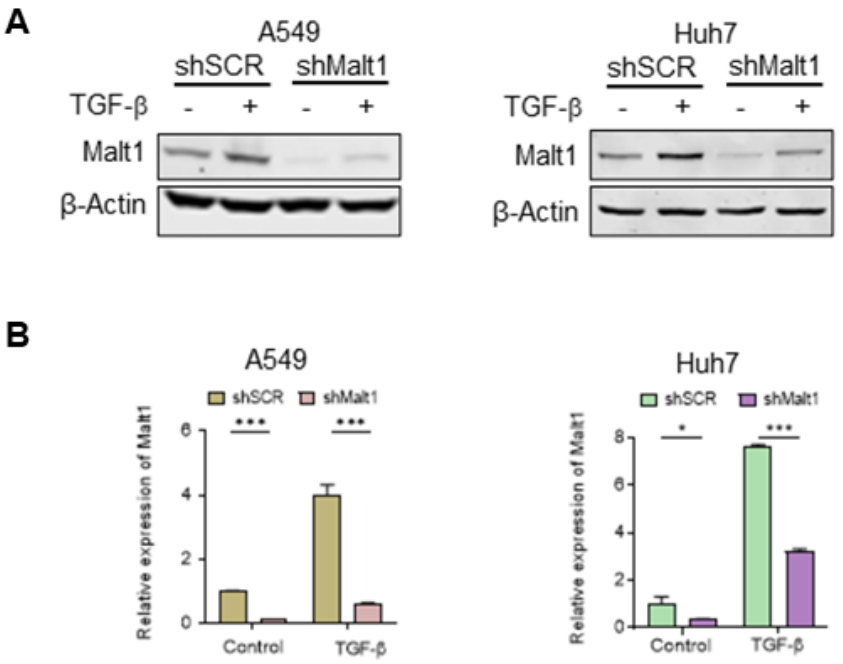


Figure 44. Malt1 expression at the protein level (A) and RNA level (B) after silencing by shRNA.

We then wondered how NF−κB activation was affected by Malt1 depletion. To evaluate that, we first assessed the translocation of p65 transcription factor to the nucleus by immunofluorescent (IF) staining. Cells were plated as 60.000/well on 12mm coverslips as duplicates in a 24-well cell culture plate. Following 24h serum starvation, treatment groups were incubated with a medium including TGF−β1 (5 ng/ml) for 2h while control groups were treated only with a complete medium. We found that TGF−β-mediated p65 translocation from cytoplasm to nucleus was abolished in Malt1 depleted cells whereas TGF−β treatment in the shSCR group was able to stimulate a dramatic translocation (Figure 45 A). Double-blind quantification (by two individuals) was performed to estimate nuclear p65 signal intensities. The analyses confirm that TGF−β-mediated nuclear translocation of p65 was significantly attenuated by Malt1 depletion in both cell lines (Figure 45 B).

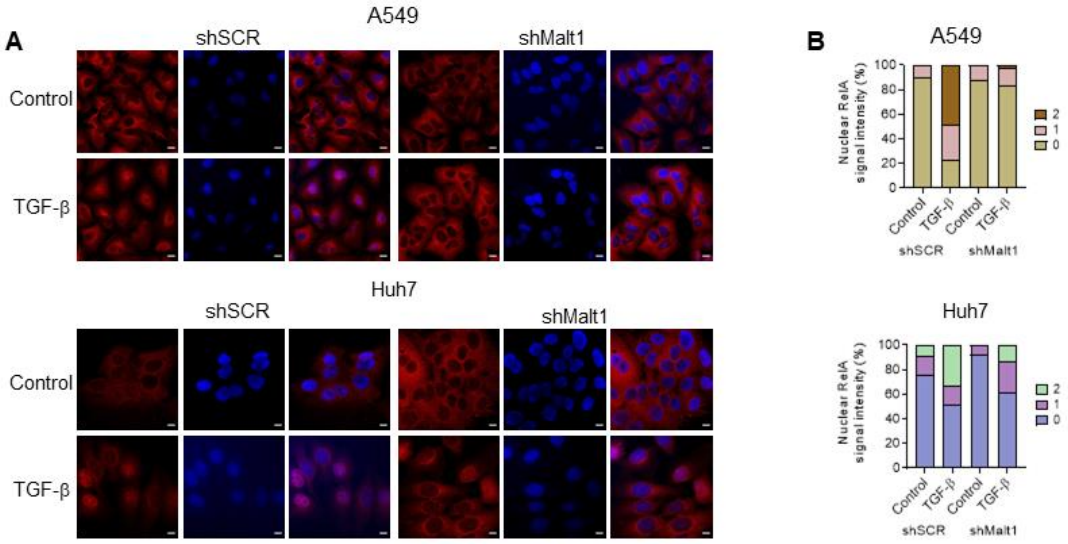


Figure 45. p65 translocation after Malt1 depletion in A549 and Huh7 cell lines upon TGF−β1 treatment (A). Signal intensity graphs were used for clear quantification of the p65 intensity in the nucleus (B). Scale bar=10 μM

To corraborate the IF results, we engineered NF−κB reporter cell line models using pHAGE NF−κB-TA-Luc-UBC-GFP-W reporter plasmid with 4X NF−κB consensus binding sequence and GFP as a traceable marker (Figure 46).

Figure 46. Schematic representation of the pHAGE NF−κB-TA-Luc-UBC-GFP-W reporter vector.

Cells were infected with the reporter plasmid and infection efficiency was visualized by microscopy imaging (>80% infection rate based on GFP intensity). Then, a luciferase assay was conducted for both cell lines. Upon TGF−β treatment, there was a notable rise in reporter activity in both cell lines, suggesting that nuclear translocation of p65 induces a transcriptional response (Figure 47).

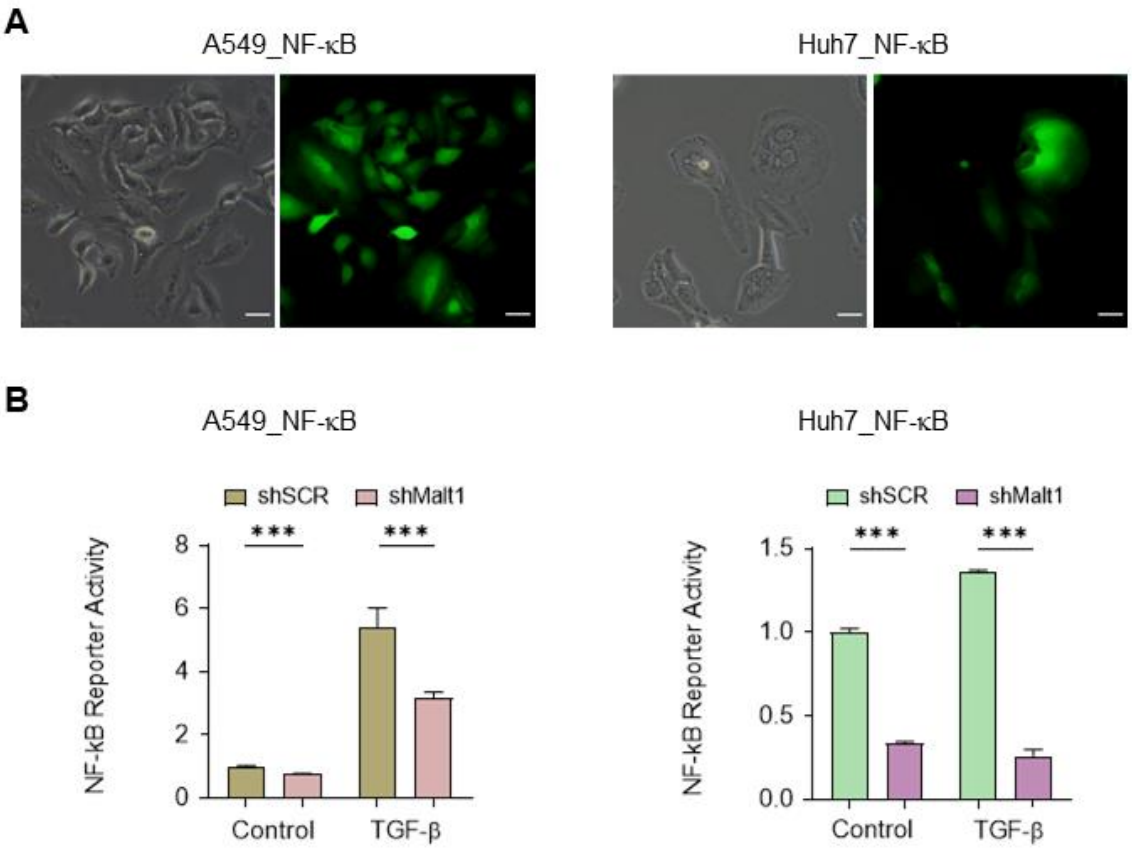


Figure 47. GFP intensity after infection with NF−κB reporter vector of A549 and Huh7 cell lines (A)*. Reporter activity was measured by luciferase assay in both cell lines* (B)*. Normalization of luminescence was calculated based on cell number.*

Next, Malt1 expression was depleted by shRNA system in reporter cell line models to examine the role of Malt1 in TGF−β-induced NF−κB reporter activation. Freshly infected and puromycin-selected cells were validated for knock-down efficiency before reporter assay was performed (Figure 48).

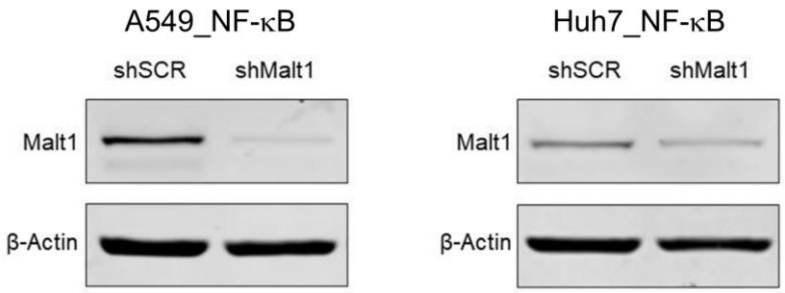


Figure 48. pHAGE NF−kB-TA-Luc-UBC-GFP-W reporter plasmid including A549 and Huh7 cell lines were plated for infection with shMalt1. Knock-down efficiency was evaluated by western blot.

Cells were plated as 150.000 cells per 60mm cell culture dish for TGF−β1 treatment after knock-down validation. At the end of 72h treatment, pellets were collected for Dual-Glo Luciferase assay as explained in the methods section. As shown in Figure 49, Malt1 depletion resulted in a significant attenuation of NF−κB reporter activity both in the absence and presence of TGF−β incubation.

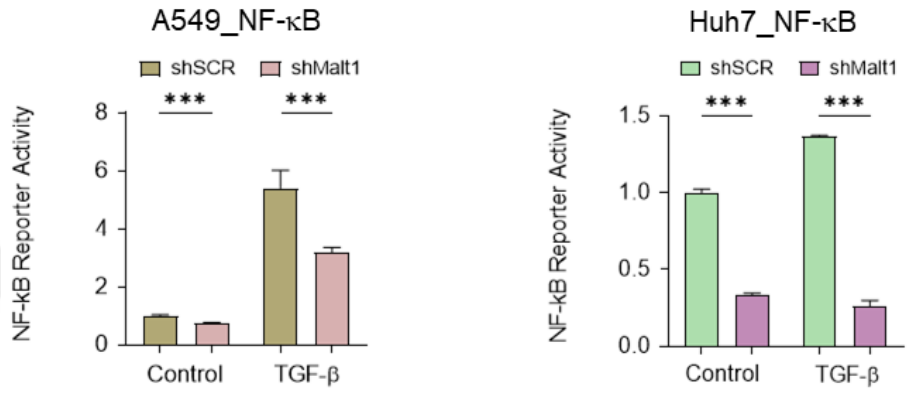


Figure 49. Reporter activity of NF−κB upon induction by TGF−β1 (5ng/ml) for 72h. Normalization of luminescence was calculated based on cell number.

Since Malt1 knock-down attenuated NF−κB reporter activity induced by the TGF−β pathway, we reasoned that the expression of NF−κB target genes could also be negatively affected. To address this statement, we studied a select number of targets by qRT-PCR. Specifically, we analyzed JUNB and CXCL1 expression in A549 cells (Figure 50 A), and JUNB and IL-1β in the Huh7 cell line (Figure 50 B). We observed significantly reduced elevation of target gene expression in Malt1 depleted cells exposed to TGF−β (Figure 50). Altogether, our data provides compelling evidence justifying the functional significance of TGF−β ligand induced Malt1 in the crosstalk of TGF−β and NF−κB signaling pathways.

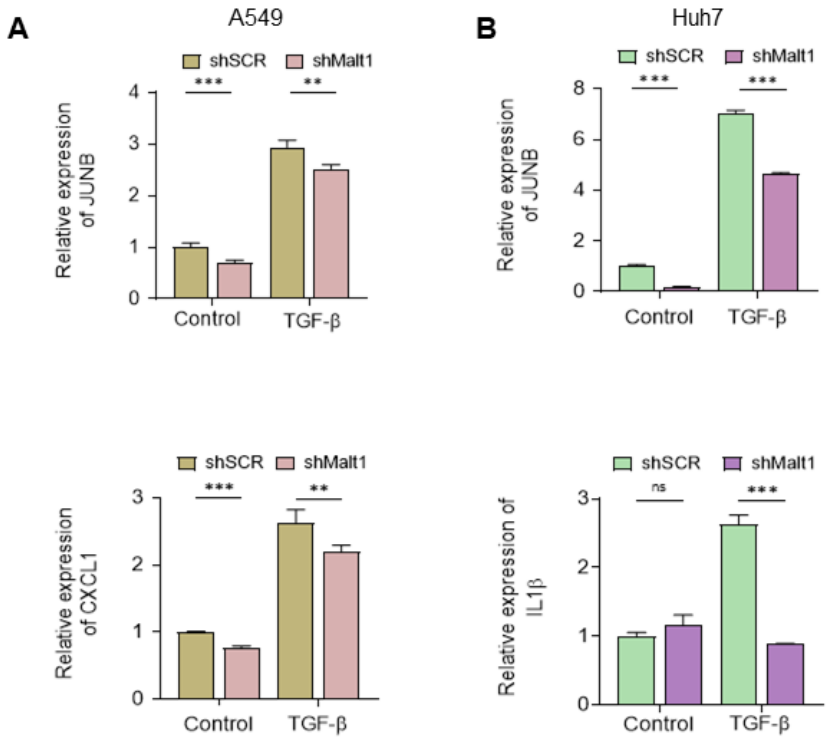


Figure 50. NF−κB target gene expression in Malt1 depleted cells treated with TGF−β. 7500 Fast qPCR machine was used to experiment.

6. DISCUSSION

The TGF−β pathway is known to elicit context-dependent functions in distinct cellular processes and homeostasis via its canonical- and non-canonical signaling branches. Dysregulation of TGF−β signaling is a frequent event in cancer. More significantly, this pathway has a dual function in cancer progression, behaving as a tumor suppressor in the early phase of cancer while switching to a promoting factor in the late stages of the disease. (51,52) Despite its conserved signaling mechanism through Smad proteins, there is an intricacy because of shared ligands and receptor interactions as well as the crosstalking mechanisms with several other signaling pathways, including the NF−κB cascade.

NF−κB is a collection of prominent family of transcription factors that have been intensively studied in immunological concepts. Its dysregulation is also a common feature in cancer progression. Although the NF−κB pathway is specifically stimulated

by immune system receptors or ligands such as TCR or BCR and the TNF family, its activation is not restricted to these *per se*. Having said that, NF−κB signaling can also be regulated via crosstalk mechanisms with various other signaling cascades, including TGF−β, and intermediate molecules, such as Malt1.

Malt1 is a paracaspase protein found in MALT lymphomas and has primarily been researched in T-cell and B-cell signaling, yet its function is not limited to the immune system. Malt1 is best known for its scaffolding activity in the CBM complex as well as its protease function in activating and stabilizing NF−κB signaling. Malt can be expressed in various cell types, and the CBM complex can be stimulated by a variety of immune system receptors as well as RTKs, EGFRs and GPCRs.

Accumulating evidence shows that the interaction of Malt1 and NF−κB signaling has mostly been analyzed in immune cells. (29) However, its widespread expression profile in other cell types further implies that Malt1 may also has a role in activation of NF−κB in other contexts. (28,31) More importantly, the established crosstalk between the TGF−β and NF−κB pathways, as well as common upstream proteins that interact with Malt1 protein, such as TAK1 and TRAF6, paved the way for further investigation. (38,39)

The purpose of this study was to examine the role of Malt1 in the crosstalk of TGF−β and NF−κB signaling pathways. To achieve this goal, we initially turned to studying whether TGF−β/Smad signaling pathway might regulate Malt1 expression. Accordingly, we conducted dose- and time-dependent TGF−β treatment experiments before silencing TGFBR1 using the RNAi system and blocking the pathway with a small-molecule TGFBR1 inhibitor. Our initial findings suggest that TGF−β signaling may be implicated in Malt1 expression. Specifically, we found that increasing doses of TGF−β or a time course treatment elevate Malt1 expression, but this mode of regulation is attenuated when TGFBR1 expression or kinase activity is blocked. At this point, we confirmed this mode of regulation by co-treating Huh7 cells with TGF−β and Actinomycin D, which is essentially a transcription inhibitor. Notably, Actinomycin D treatment blocked TGF−β-mediated upregulation of Malt1 transcript levels, suggesting that TGF−β signaling regulates Malt1 elevation transcriptionally. Of note,

we chose to employ Huh7 cells in this experimental scheme as this cell line provides an earlier transcriptional response to the TGF−β stimulation when compared to A549 cells.

To further investigate the functional significance of canonical TGF−β pathway components, Smad protein family (Smad2, Smad3 and Smad4 proteins) were silenced by shRNA vectors. Indeed, TGF−β-mediated Malt1 upregulation was abrogated in the case of Smad3 knockdown. Using the GEPIA2 database, we found a strong correlation between Smad3 and Malt1 expression in the TCGA datasets of lung adenocarcinoma, liver hepatocellular carcinoma, and all cancer types, further confirming the causal link between these two genes. To corroborate this finding, we engineered Smad3 knockout clones using CRISPR/Cas9 technology in both cell lines. Single cell isogenic knockout clones were generated by individually targeting two exons (Exon2 and Exon4) with sequence specific gRNAs. Clones were verified by western blot experiments and Sanger Sequencing. Much like shRNA results, knockout clones had entirely lost Smad3 regulatory activity over Malt1 expression. We then wondered whether the canonical activation of Smad3 through its C-terminal serine phosphorylation by TGFBR1 was required for Malt1 upregulation. To accomplish this goal, we performed a rescue experiment in Smad3 knockout clones with lentiviral constructs expressing wild-type Smad3 and phosphorylation incompetent mutant Smad3-3SA proteins. In summary, we found that TGFBR1-driven Smad3 C-terminal phosphorylation was required for TGF−β ligand-induced Malt1 expression. Our observations point to a canonical Smad3-dependent transcriptional regulation of Malt1. Thus, we aimed to study this regulation at the promoter level. To start with, we searched for putative Smad-binding elements (SBE) in the promoter region of Malt1 using Eukaryotic Promoter Database (EPD). We located three SBEs at 575, 1.443, and 1.931 bp upstream positions from TSS. Further supporting this information, we identified publicly available Smad3 ChIP datasets in MDA-MB-231 breast cancer cell line with TGF−β-promoted differential enrichment of Smad3 binding in the Malt1 promoter. We also found a H3K27ac ChIP dataset in A549 cell line which shows almost 2 times greater enrichment in TGF-β treated group, suggesting that TGF−β treatment may result in transcriptional

activation of Malt1 promoter. To further study these findings, we requested several reporter vectors including truncated Malt1 promoter regions spanning the SBEs from a research group in JAPAN. (53) Luciferase assays revealed significant increase in two vectors (-1500 and -1990) which together suggests a positive regulation of Malt1 by TGF−β pathway. (53) However, the shorter promoter construct albeit having a putative SBE did not exhibit any TGF−β responsive activation which may be explained by a misprediction or the presence of a silencing element. All these results encouraged us to perform Smad3 ChIP experiments in the Huh7 cell line. Despite several attempts and efforts, we failed to obtain promising results. We reason that this may be associated with the cell line model (Huh7). Besides, we were limited by the number of test conditions such as treatment duration and antibody concentration as well as positive control targets. Nonetheless, we believe to have obtained convincing results showing that Malt1 is transcriptionally regulated by Smad3 through canonical TGF−β cascade.

Within the context of crosstalking, we then examined how NF−κB signaling was affected by this regulation. To test this, we first depleted Malt1 expression in both cell lines using the RNAi system. To demonstrate whether Malt1 depletion inhibited NF−κB activation, we performed IF experiments for p65 protein, which translocates into the nucleus when NF−κB pathway is activated. We observed that there was an attenuated p65 translocation in Malt1 depleted clones compared to the negative control shSCR. The reason why signal intensity was not completely decreased in Malt1 depleted cells might be using shRNA system. There is no complete depletion of Malt1 in the cells which may still induce NF−κB signaling. There can be another stimulating mechanism apart from Malt1 such as TGF−β induced another intermediary component. (39,54) To strengthen these findings, we then generated clones with NF−κB reporter vector to perform the luciferase assay. Additionally, we further engineered these cells with shSCR or shMalt1 to manipulate Malt1 expression. As expected, we obtained decreased reporter activity in shMalt1 clones compared to shSCR. These results were also validated by qRT-PCR experiments showing that Malt1 depletion suppressed TGF−β-induced NF−κB target gene expression.

In this study, we demonstrated that Malt1 is regulated by TGF−β signaling pathway in a dose- and time-dependent manner. Moreover, we showed that Malt1 is a transcriptionally regulated Smad3 target. Finally, Malt1 is required for TGF−β-potentiated NF−κB pathway activation.

7. CONCLUSION AND FUTURE PERSPECTIVES

We showed by dose- and time-dependent TGF−β treatment experiments that Malt1 expression was upregulated by TGF−β signaling pathway. These findings were corroborated by TGFBR1 silencing and pharmacological inhibition. Silencing Smad proteins by RNAi system demonstrated that there is a canonical pathway driven regulation of Malt1 expression, specifically Smad3-dependent. This was further supported by GEPIA2 analyses which showed a correlation between Smad3 and Malt1 expression in both lung adenocarcinoma and hepatocellular carcinoma datasets as well as all cancer types. Smad3 knockout by CRISPR/Cas9 applications confirmed that Malt1 expression was regulated through Smad3, which was verified by rescue experiments. Particularly, the canonical pathway activated Smad3 dependency for Malt1 regulation was demonstrated by comparing wild-type Smad3 and its mutant version, Smad3-3SA. Furthermore, we identified multiple SBEs on the 2 kb upstream region of Malt1 starting from the TSS as a potential indication of transcriptional Malt1 regulation which was partially evidenced by reporter experiments.

Furthermore, we validated the role of Malt1 in the crosstalk by IF staining of p65 and NF−κB reporter experiments as well as the qRT-PCR results for NF−κB target genes in Malt1 knockdown clones stimulated by TGF−β1.

Collectively, we showed that Malt1 acts as an intermediate factor between TGF−β and NF−κB signaling pathways. Albeit this study does not provide a mechanistic explanation as to how Malt1 modulates this crosstalk, our findings warrant further investigation to enlighten if Malt1 functions as a scaffold protein or protease.

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9. APPENDIX

İZMİR BİYOTIP VE GENOM MERKEZİ
GİRİŞİMSEL OLMAYAN ARAŞTIRMALAR ETİK KURULU (İBG-GOEK)
KARARI

Toplantı Tarihi	: 25/12/2020	**Toplantı Günü**	: Cuma
Toplantı Sayısı	: 15	**Toplantı Saati**	: 10:30

Sayın Şerif ŞENTÜRK,

2020-044 Protokol No'lu; sorumlusu olduğunuz "NF-kB ve TGF-beta Sinyal Yolaklarının Çaprazlı Konuşmasında Malt 1'ın Rolü" başlıklı araştırmanın uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

Prof. Dr. H.Alper BAĞRIYANIK
Başkan

Pandemi süresince online ortamda gerçekleştirilen toplantımızda alınan kararlar tek imzalı olarak düzenlenmektedir. Pandemi sona erdikten sonra ıslak imzalı karar belgesi teslim edilecektir.

FATMA AYBÜKE MAZI

Kişisel Bilgiler

İletişim Bilgileri

İletişim Adresi

Telefon

E-posta

İnternet Sayfası

Öğrenim Bilgileri

08 Şubat 2019 - Şu Anda (3 yıl 4 ay)
Yüksek Lisans, Tezli Program, DOKUZ EYLÜL ÜNİVERSİTESİ, TÜRKİYE
İZMİR ULUSLARARASI BİYOTIP VE GENOM ENSTİTÜSÜ, MOLEKÜLER BİYOLOJİ VE GENETİK (YL) (TEZLİ) (İNGİLİZCE)
Diploma Numarası: -
Ağırlıklı Genel Not Ortalaması: 3.94 / 4.0

01 Eylül 2012 - 01 Haziran 2017 (4 yıl 10 ay)
Lisans, Anadal/Normal Öğretim, BAHÇEŞEHİR ÜNİVERSİTESİ, TÜRKİYE
FEN-EDEBİYAT FAKÜLTESİ, GENETİK VE BİYOİNFORMATİK BÖLÜMÜ
Diploma Numarası: 25599
Ağırlıklı Genel Not Ortalaması: 3.14 / 4.0

Deneyim / İşyeri Bilgileri

01 Ekim 2017 - 01 Mart 2018 (6 ay) (Tam Zamanlı)
MİSAFİR ARAŞTIRMACI, BAHÇEŞEHİR ÜNİVERSİTESİ TIP FAKÜLTESİ

Yabancı Dil Bilgileri

İNGİLİZCE (Okuma: İyi, Yazma: İyi, Konuşma: İyi)

Bilimsel Teknolojik Faaliyet Alanları

Bilimsel Teknolojik Faaliyet Alanı Bilgileri

Temel Bilimler -- Yaşam Bilimleri -- Moleküler Biyoloji ve Genetik -- Kanser Moleküler Biyolojisi

Ar-Ge Yetkinlik

Makaleler

D. DEMİRCİ, B. DAYANC, F. A. MAZİ & S. SENTURK, The Jekyll and Hyde of Cellular Senescence in Cancer, CELLS, 2021, 2073-4409, 10, 2.

Bildiriler

F. A. MAZI, E. ÇAKIROĞLU, A. KURDEN PEKMEZCİ, D. DEMİRCİ & Ş. ŞENTÜRK, TGF-??YA BAĞLI KANSER HÜCRE SEKRETOMUNUN REGÜLASYONUNDA MALT1?IN ROLÜ, Sözlü Sunum, Onkolojide İz Bırakanlar Zirvesi, 14 Kasım 2019, 17 Kasım 2019.

TÜBİTAK Burs ve Destekleri

Proje Bilgileri

118Z603, Pediatrik Nörogenetik Hastalıklarda Yeni Nesil Dizileme Uygulamalarının Tanısal Etkinliğini Arttırmaya Yönelik Bütüncül Bir Yaklaşım, Uluslararası, Burslu, Sonuçlandı, ARDEB, KBAG - Kimya Biyoloji Araştırma Destek Grubu, Projeye Katılma/Ayrılma Tarihleri: 19.07.2019 - 13.02.2020, Proje Başlangıç/Bitiş Tarihleri: 15.02.2019 - 15.08.2021.
119Z540, Egfr-Mutant Küçük Hücreli Dışı Akciğer Kanseri'nde Birinci Basamak Osimertinib Tedavisinde Gelişen Edinsel Dirençte Epigenetik ve Transkripsiyonel Faktörlerin Rolünün Araştırılması, Uluslararası, Burslu, Yürürlükte, ARDEB, KBAG - Kimya Biyoloji Araştırma Destek Grubu, Projeye Katılma/Ayrılma Tarihleri: 14.02.2020 - 15.11.2022, Proje Başlangıç/Bitiş Tarihleri: 15.11.2019 - 15.11.2022.

Panelistlik/İzleyicilik/Raportörlük Sayısı

Hakemlik/Panelistlik/Dış Danışmanlık Sayısı	ARDEB/BİDEB 0	TEYDEB 0	Toplam 0
İzleyicilik/Danışmanlık Sayısı	ARDEB/BİDEB 0	TEYDEB 0	Toplam 0
Raportörlük Sayısı	ARDEB/BİDEB 0	TEYDEB 0	Toplam 0