Role of YB-1 and NF-κB in TNFR signaling pathways:

deciding cell survival or death

Thesis

for the degree of

doctor rerum naturalium (Dr. rer. nat.)

approved by the Faculty of Natural Sciences of Otto von Guericke University
Magdeburg

by M.Sc. Aneri Shah

born on 19 March 1991 in Rampura, India

Examiner:

Prof. Dr. med. Peter R. Mertens

Prof. Dr. Birgit Schittek

submitted on: 31.08.2021

defended on: 05.04.2022

Table of Contents

Preamble

Results of the present work are published in:

Shah A, Plaza-Sirvent C, Weinert S, et al. YB-1 Mediates TNF-Induced Pro-Survival Signaling by Regulating NF-κB Activation. Cancers (Basel). 2020;12(8):2188. Published 2020 Aug 5. doi:10.3390/cancers12082188.

Shah A, Lindquist JA, Rosendahl L, Schmitz I, Mertens PR. Novel Insights into YB-1 Signaling and Cell Death Decisions. Cancers (Basel). 2021;13(13):3306. Published 2021 Jul 1. doi:10.3390/cancers13133306.

Text and figures from above mentioned publication were used in sections 4.1, 4.3, 4.4 and 5.

Publications:

This study has been published in the following papers:

Shah A, Plaza-Sirvent C, Weinert S, et al. YB-1 Mediates TNF-Induced Pro-Survival Signaling by Regulating NF-κB Activation. Cancers (Basel). 2020;12(8):2188. Published 2020 Aug 5. doi:10.3390/cancers12082188.

Shah A, Lindquist JA, Rosendahl L, Schmitz I, Mertens PR. Novel Insights into YB-1 Signaling and Cell Death Decisions. Cancers (Basel). 2021;13(13):3306. Published 2021 Jul 1. doi:10.3390/cancers13133306.

Additional publications during the PhD:

Stojanovska V, **Shah A,** Woidacki K, et al. YB-1 Is Altered in Pregnancy-Associated Disorders and Affects Trophoblast in Vitro Properties via Alternation of Multiple Molecular Traits. Int J Mol Sci. 2021;22(13):7226. Published 2021 Jul 5. doi:10.3390/ijms22137226.

Hessman CL, Hildebrandt J, **Shah A,** et al. YB-1 Interferes with TNFα-TNFR Binding and Modulates Progranulin-Mediated Inhibition of TNFα Signaling. Int J Mol Sci. 2020;21(19):7076. Published 2020 Sep 25. doi:10.3390/ijms21197076.

Liakopoulos V, Jeron A, **Shah A,** Bruder D, Mertens PR, Gorny X. Hemodialysis-related changes in phenotypical features of monocytes. Sci Rep. 2018;8(1):13964. Published 2018 Sep 18. doi:10.1038/s41598-018-31889-2.

Conference contributions:

Shah A, Lindquist JA, Gorny X, Buchbinder J, Lavrik I, Schmitz I, Mertens PR. Activation of NF-κB requires YB-1. Poster presentation. 22nd signal transduction meeting, Weimar, Germany, 2018

Shah A, Lindquist JA, Gorny X, Buchbinder J, Lavrik I, Schmitz I, Mertens PR. Activation of NF-κB requires YB-1. Best poster presentation prize (1st). 23rd signal transduction meeting, Weimar, Germany, 2019

A. Shah, C. Plaza-Sirvent, S. Weinert, J. Buchbinder, I. Lavrik, P. R. Mertens, I. Schmitz, J. Lindquist. YB-1 mediates TNFR-induced pro-survival signaling by regulating NF-kB activation. Poster presentation. 12th German meeting for nephrology, Berlin, Germany, 2020

Abbreviations

BAFFR	B-cell activating factor receptor
CCL5	CC chemokine ligand 5
CD40L	CD40 ligand
cFLIP	FLICE-like inhibitory protein
cIAP1/2	cellular inhibitor of apoptosis protein 1/2
CKII	Casein kinase II
CRDs	cysteine-rich domains
CRS	cytoplasmic retention signal
CSD	cold shock domain
CTD	C-terminal domain
CYLD	cylindromatosis
DNA	Deoxyribonucleic acid
ECD	extracellular domain
EGFR	epidermal growth factor receptor
eIF2α	Eukaryotic Initiation Factor 2α
ERK	Extracellular signal-regulated kinase
FRGY2	frog Y-box protein 2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HACE1	HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase1
HER-2	human epidermal growth factor receptor 2
HOIL-1	Heme-Oxidized IRP2 Ubiquitin Ligase 1
HOIP	HOIL-1-Interacting Protein
HuR	Human antigen R
IFNγ	Interferon γ

IGF-1	Insulin-like growth factor 1
IKK	IκB kinase
IL-1β	Interleukin-1β
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α
LCS	low complexity sequence
LPS	Lipopolysaccharide
LTβR	Lymphotoxin β Receptor
LUBAC	linear ubiquitin assembly complex
MAPK	mitogen-activated protein kinase
MDR	multidrug resistance
Met1	DNA (cytosine-5)-methyltransferase 1
MLKL	mixed lineage kinase domain-like protein
mRNA	messenger ribonucleic acid
mRNP	messenger Ribonucleoprotein
mTNF	transmembrane tumor necrosis factor
NEMO	nuclear factor-κB essential modulator
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF-κB inducing kinase
NLS	nuclear localization signal
OUTB1	OTU domain-containing ubiquitin aldehyde-binding protein 1
PABP	poly (A)-binding protein
PBs	processing bodies
PCNA	Proliferating cell nuclear antigen
PDGF	platelet derived growth factor

PKB/AKT	Protein kinase B
PKC	Protein kinase C
RANK	Receptor activator of NF-κB
RAP55	RNA associated protein 55
RIPK1	receptor-interacting serine/threonine-protein kinase 1
RNA	ribonucleic acid
RNP	ribonucleoprotein
RSK	Ribosomal S6 kinase
SCFβ-TrCP	Skp1-cullin-F-box β transduction repeat-containing protein
SGs	stress granules
SHARPIN	SHANK Associated RH Domain Interactor
sTNF	soluble tumor necrosis factor
TAK1	transforming growth factor (TGF)-β-activated kinase 1
TGF-β	transforming growth factor
TIA-1	T-cell Intracellular antigen-1
TIAR	TIA-1 related like protein
tiRNA	tRNA-derived stress-induced RNAs
TNF	tumor necrosis factor
TNFR1	tumor necrosis factor receptor 1
TRADD	TNF-receptor associated death domain protein
TRAF2	TNF-receptor associated factor 2
TRAF3	TNF-receptor associated factor 3
YB-1	Y-box binding protein-1

Summary

Y-box binding protein-1 (YB-1) belongs to the family of cold shock proteins that are characterized by an evolutionary and structurally conserved cold shock domain. The cold shock domain binds both RNA and single stranded DNA, therefore YB-1 has pleiotropic functions in cell proliferation, differentiation, stress response, DNA repair, and inflammation. Cell undergo stress in many ways, e.g., radiation, interferon release in response to viral infection, and lipopolysaccharide produced by bacteria. Binding of these factors to their receptors induces kinase activation, which phosphorylate YB-1. The same pathways also activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a well-known transcription factor, which regulates genes involved in immune responses, cell proliferation, and inflammation or apoptosis. Life or death decisions within cells are critical for development as well as in the defence against infectious diseases and cancer formation. Apoptosis has long been considered the only form of programmed cell death occurring during development and disease progression. Now, in addition to apoptosis, necroptosis (a programmed form of necrosis) can also be initiated by activation of death receptors. Survival, apoptosis, and necroptosis are all triggered by the same cell surface receptors e.g., tumor necrosis factor receptor 1 (TNFR1). Our novel findings showed a prominent role of YB-1 in the TNF-induced activation and nuclear translocation of NF-κB p65. We are the first to show that depleting YB-1 tips the balance from survival to enhanced apoptosis in TNFR1 signaling pathway.

1. Introduction

1.1 Y-box binding protein-1 (YB-1)

Y-box binding protein-1 (YB-1) belongs to the family of cold shock domain proteins, which was first recognized as a major messenger Ribonucleoprotein (mRNP) component [1]. YB-1 acquired its name from a DNA sequence called the Y-box to which it was bound [2]. YB-1 is composed of an alanine/proline-rich N terminus, the central cold shock domain (CSD), which mediates DNA and RNA binding, and a C-terminal charged zipper that comprises alternating positively and negatively charged amino acids (Figure 1) [3-5]. By binding to nucleic acids, YB-1 is involved in most DNA- and mRNA- dependent processes, including DNA replication and repair, pre-mRNA splicing, transcription, and mRNA translation. It packages and stabilizes mRNAs, realizes global and specific regulation of gene expression at various levels. It can both stimulate and inhibit transcription [4]. It is presumed that YB-1 regulates transcription through its direct interaction with the specific Y-box containing regions in gene promoters as well as with single stranded DNA regions, which are devoid of Y-box sequences.

The participation of YB-1 in DNA repair was first hypothesized three decades ago in 1991 when YB-1 was shown to have increased affinity for DNA with apurinic sites [6, 7]. This approach was supported by the evidence that elevated YB-1 has affinity for damaged DNA or for DNA with unpaired bases and for the ability of YB-1 to efficiently

2

1. INTRODUCTION

melt duplexes of such DNA [8-10]. In addition, YB-1 showed 3'-5' exonuclease and endonuclease activity on single and double stranded DNAs that may be actively dependent on the DNA sequence and structure [8, 10, 11]. It was shown that YB-1 interacts both *in vivo* and *in vitro* with several proteins involved in DNA repair and can influence the activity of some of them. Specifically, it interacts with proteins participating in base and nucleotide excision repair, in mismatch repair, in single and double strand breaks, and in DNA repair by recombination [1].
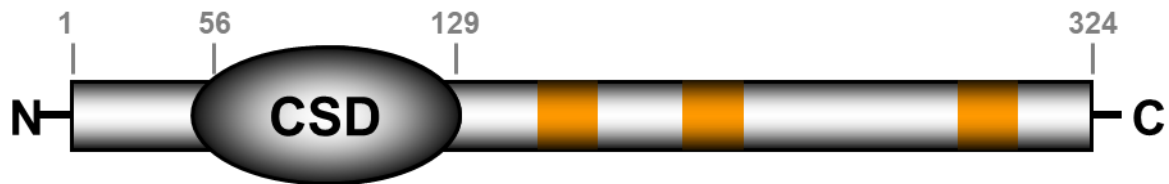

Figure 1. Structural organization of YB-1. *YB-1 is a 324 amino-acid protein comprising of the alanine/proline-rich variable N-terminal, a highly conserved nucleic acid binding cold shock domain (CSD), and a large disordered C-terminal containing alternating clusters of positively and negatively charged amino acid domains [1]. The nuclear localization signals are indicated by orange boxes.*

1.2 Transition from cytoplasm to nucleus

The YB-1 protein performs its functions both in the cytoplasm and the cell nucleus. Published experiments with YB-1 fragments described that it contains three sequences regulating its distribution between the nucleus and cytoplasm called nuclear localization signals (NLS) [12-14]. Long ago it was shown that YB-1 can move from the nucleus to the cytoplasm in complex with newly synthesized mRNA [15]. A

1. INTRODUCTION

decrease in the concentration of mRNA in the cytoplasm due to inhibition of its synthesis and/or enhancement of its degradation can promote mRNA release and nuclear translocation of these proteins, which activates the transcription process. By this mechanism, YB-1 maintains the required ratio between the synthesis rate of some mRNAs and the content of mRNAs in the cytoplasm. It was demonstrated that in mature oocytes from *X. laevis,* in which huge amount of masked mRNA is concentrated in the cytoplasm, and the transcriptional activity in the nucleus is low, the majority of frog Y-box protein 2 (FRGY2), the YB-1 homologue, is localized in the cytoplasm [16]. The above evidence supports the retention of YB-1 in the cytoplasm due to its binding to mRNA. Also, YB-1 selectively inhibits YB-1 mRNA translation, while poly (A)-binding protein (PABP) stimulates it [17]. First, the pre-dominant part of cytoplasmic YB-1 is found in complex with mRNA in HeLa cells [18]. Second, the substitute of phenylalanine and tyrosine residues in the ribonucleoprotein (RNP) - 1 consensus sequence of YB-1, extremely reduces the protein binding to RNA in cell lysates, resulting in the translocation of the mutant protein from the cytoplasm into the nucleus [12]. It is likely that YB-1 can be retained in the cytoplasm due to the interaction with its partner proteins. For e.g., FRGY2 fragments, which are incapable of binding to mRNA, still abide in the cytoplasm [19]. Transition of YB-1 from the cytoplasm to the nucleus is observed in the following cases: (1). It takes place at a definite moment of the cell cycle, specifically at the G1/S phase interface. Within the

1. INTRODUCTION

nucleus, YB-1 promotes transcription of cyclins A and B1 genes, which supports progression of cell cycle [20]. (2) The transition from cytoplasm to nucleus takes place during treatment of cells with UV-radiation, DNA damaging agents, and upon oxidative stress and hyperthermia [21-24]. In such cases, nuclear YB-1 stimulates transcription of multidrug resistance genes and is involved in cellular repair, thus enhancing cell viability. (3) YB-1 is also found in the nucleus after cell infection with adenovirus [25]. (4) The transition of YB-1 to the nucleus can be promoted by some of the growth factors and cytokines, such as fetal bovine serum, IFNγ, and HSc025 [26-28].

Several experiments showed that YB-1 translocation from the cytoplasm to the nucleus was accompanied by a gradual increase or depleted amounts of mRNAs or proteins encoded by many genes responsible for cell division, apoptosis, immune response, differentiation, multiple drug resistance, stress response, and tumor growth [1]. In the case of YB-1, two research groups have demonstrated that deletion of mouse *Ybx1* has dramatic consequences [29, 30]. Firstly, disturbances in neurotubule formation during early embryonic development was observed. Secondly, fetal growth retardation i.e., hypoplasia and frequent prenatal deaths were reported. Despite of the fact, YB-1 knockout in embryonic fibroblasts caused no global changes either in the transcriptome or the proteome, it rather made the cells more sensitive to stress, e.g. hypoxia, cisplatin treatment, and temperature [29, 31]. This signifies the involvement

of YB-1 in triggering selective expression of genes required for cell (de)differentiation,

proliferation, and stress response, rather than in global regulation of gene expression.
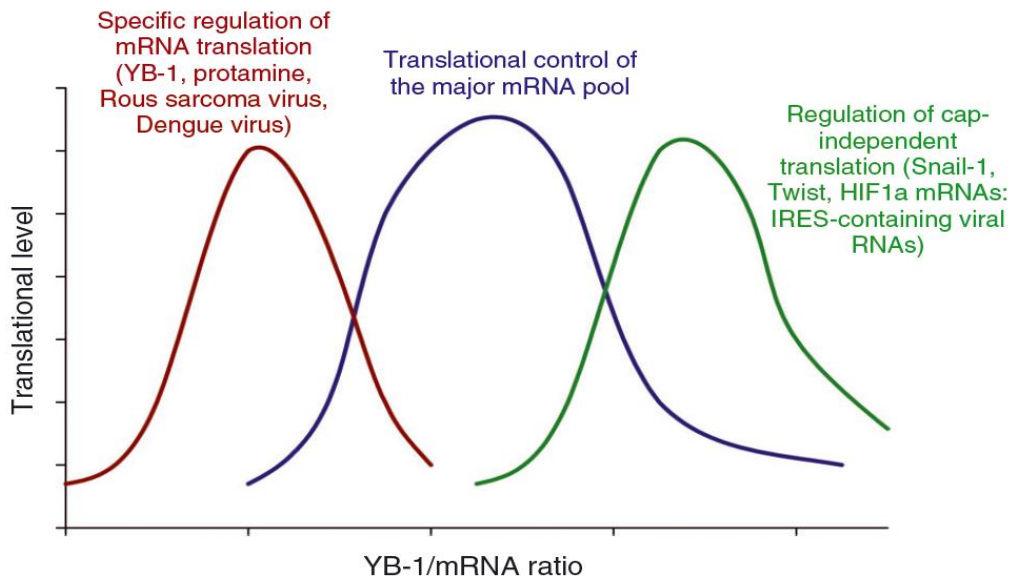

Figure 2. The mRNA translational activity is dependent on YB-1/mRNA ratio. A curve for translational level dependence on the YB-1/mRNA ratio is bell-shaped. At a relatively low YB-1/mRNA ratio, monomeric YB-1 interacts with its cold shock domain (CSD) and C-terminal domain (CTD) and results in messenger ribonucleoprotein (mRNP) winding. At a higher ratio, YB-1 undergoes multimerization with its CTD, while its CSD remain mRNA-bound [32].

1.3 Stress granules

YB-1 can be found within cytoplasmic granules. Currently, localization of YB-1 has

been identified in two kinds of such granules – stress granules (SGs) [33] and

processing bodies (PBs) [34]. SGs are produced in the cell in response to hypoxia,

heat shock, oxidative stress, viral infection, etc. Initiation of SG formation occurs

through eIF2α phosphorylation, which results in the inhibition of protein synthesis at

the initiation stage [35]. It is presumed that these granules represent a type of mRNA

storage. These mRNAs could be used for translation upon restoration under favourable conditions. PBs are present in the cell almost continuously and involved in mRNA degradation. The composition of proteins varies greatly from that of SGs. In normal growth conditions they also contain YB-1, but in lower amounts then SGs [33, 34]. Following stress, a great part of YB-1 is associated with SG. It has been reported that several mRNA-associated proteins with the so-called low complexity sequence (LCS) form reversible filaments. A specific agent, biotinylated 5-aryl-isoxazole-3-carboxyamide, stimulated fibril formation, and its addition to cell lysates gave precipitates like the SGs [36, 37]. YB-1 was detected within these RNA granules as well; due to its ability to form reversible fibrils, YB-1 may participate in their formation together with LCS proteins [38]. YB-1 was detected among specific tiRNA-binding proteins and appeared to be the only one essential for tiRNA-induced translation inhibition and SG formation. The A/P domain, CSD, and a fragment of C-terminal domain (205-281) are required to localize YB-1 within PBs, whereas the CSD (44-128) is required to localize it within SGs. In contrary, RNA associated protein 55 (RAP55) moves swiftly from SGs to PBs upon cessation of stress. It is likely that in this way YB-1 supports the restoration of translation and facilitates the transition of mRNAs from SGs to polysomes [34]. Studies showed that formation of a great number of exceedingly small SGs are formed, and then they merge into larger granules. The merging process is active and depends upon the assembly and disassembly of

microtubules. It is possible that basic mRNPs proteins, such as YB-1 and PABP, facilitate association of stress granules with microtubules [39].

1.4 Secretion and Extracellular YB-1

YB-1 can be secreted from cells under inflammatory stress when treated with lipopolysaccharide (LPS), hydrogen peroxide, platelet derived growth factor (PDGF- BB) or transforming growth factor (TGF)-β [40]. The secretion of YB-1 occurs by a non-classical pathway and not by classical protein secretion, i.e., not by trafficking through the endoplasmic reticulum and Golgi apparatus. This is evident that brefeldin A, which interferes with the classical secretion pathway, did not reduce, but instead, increased the secretion of YB-1. In inflammatory stress, YB-1 is co-localized with the protein, a marker of vesicular transport. Similar to some pro-inflammatory proteins like IL-1β, MIF, HMGB1 and FGF2, YB-1 is secreted by a non-classical mechanism within endolysosomal vesicles. The secretion of YB-1 depends on the presence of lysine residues 301 and 304 in its CTD, and the substitution of these lysine by alanine entirely inhibits YB-1 export from the cell [40, 41]. It is assumed that acetylation of lysine residues 301 and 304 plays a major role in YB-1 secretion from the cell. Extracellular YB-1 functions as a growth factor, which when added to the cultured cells stimulates proliferation and migration of rat mesangial and human kidney cells [40]. Acetylation and ubiquitination of YB-1 have both been shown to play roles in regulating secretion as well as intracellular stability [42-45]. Over the years, there have been first reports

1. INTRODUCTION

on YB-1 species in the sera of patients suffering from carcinomas and haematological malignancies [46-48]. Secreted YB-1 was shown to be chemoattractive and participates in monocyte/macrophage recruitment, and differentiation after LPS stimulation *in vitro* and in an animal model of kidney inflammation *in vivo*, supporting its role in immunomodulation [49, 50]. Also, YB-1 supplementation to melanoma cells with reduced YB-1 secretion escalates the metastatic capacity of several melanoma cells within the primary tumor [51]. YB-1 can be proteolytically cleaved and extracellular YB-1 and/or fragments thereof are found in serum of patients, bind to cell surface receptors, and exert extracellular activities like augmenting proliferation, and induces migration of immune cells [14, 47, 48, 52-56]. Rauen et al., identified and characterized an interaction of extracellular YB-1 with Notch-3 receptor, leading to intracellular signaling [53]. Notch-3 was observed to be upregulated in human kidney diseases and plays a role in modulating inflammation and fibrosis in tubulointerstitial kidney injury [57]. It has been shown that extracellular YB-1 can downregulate *Notch-3* expression and regulates Notch-3 signaling [58]. Recently, a new activity for extracellular YB-1 was identified by Hessman CL et al., namely an activity influencing TNFR1 signaling [59]. Within YB-1, five sites of Ser phosphorylation were identified upon TNF stimulation (Table 1), including Ser165 and Ser176 [60]. Prabhu et al. and Martin M et al. showed that phosphorylation at Ser165 and Ser176 within YB-1 is required for NF-κB activation following IL-1βR activation [61-63]. Moreover,

phosphorylation at these sites promotes tumor formation by regulating the expression of NF-κB target genes. Additionally, YB-1 was also reported to be ubiquitinated and functionally interact with other proteins upon TNF stimulation [60]. Thus, several pro-inflammatory cytokines as well as bacterial toxins, like LPS, induce YB-1 secretion, which extracellularly binds to TNF receptors and the Notch-3 receptors [53].

Protein ID	Site of phosphorylation	Amino acid
P67809	174	S
P67809	176	S
P67809	165	S
P67809	167	S
P67809	209	S
	Site of ubiquitination	
P67809	264	K
P67809	137	K
P67809	170	K

Table 1. **Known post-translational modifications of YB-1** (adapted from [60])

1.5 Role of YB-1 in Cancer and Inflammation

As mentioned above, YB-1 is a multifunctional protein and is activated by the phosphorylation at Ser102 [64]. Upon phosphorylation, YB-1 shuttles from cytoplasm to the nucleus where it turns into an oncogenic transcription factor by inducing the expression of growth-promoting genes, such as HER-2, EGFR, PCNA, Cyclin A, and Cyclin B [9, 20, 64, 65]. High levels of YB-1 expression level were reported to be exceedingly associated with the cancer cell progression and proliferation [66]. YB-1

1. INTRODUCTION

was found to be correlated with the expression of several crucial genes including genes responsible for infinite growth, drug resistance, cell cycle, and monitoring gene transcription and translation in cancer cells [67, 68]. Many genes that are overexpressed in cancer have a Y-box sequence in their promoter or enhancer region [69, 70]. YB-1 binds to the Y-box sequences of these genes and trans-activates their expression [71]. For example, YB-1 trans-activates the Multidrug Resistance (MDR) 1 gene, comprising a Y- box sequence in its promoter region resulting in the overexpression of the MDR1 gene. The high MDR1 expression is associated with many kinds of human cancer, such as osteosarcoma, lung, breast, prostate, and synovial sarcoma cancer [72-79]. Besides strong corelation with MDR1, the nuclear YB-1 level was reported to co-regulate growth promoting genes as well [67]. As described by Hanahan and Weinberg the pleotropic protein YB-1 has multiple effects on cancer cells by regulating all of the nine "Hallmarks of Cancer" [1, 80]. To tweak the cell signaling pathways to maintain or promote malignancy in cancer cell, YB-1 plays critical role in every trait of cancer that includes uncontrolled proliferation signaling, evading growth suppressors and cell cycle checkpoints, resisting cell death and eluding immune destructions [81-83]. YB-1 expression is upregulated in cancer and nuclear localization indicates a poor prognosis [84]. The YB-1 decline causes growth repression and apoptosis in a huge number of cancer cells like colon, lung, breast, and prostate cancer [83]. YB-1 inhibits the Fas-mediated pathway at multiple

1. INTRODUCTION

points to prevent apoptosis [85]. YB-1 promotes rampant cancer cell proliferation by activating the E2F pathway which is a prevalent event in most types of cancer [86]. YB-1 blocks the expression of RB (gatekeeper protein) in several ways and is reported to inhibit the expression of the p53 gene in cancer cells, thus reducing its tumour suppression activity [87, 88]. p53 alone does not only bind to DNA, but exerts its interactions with other transcription factor and regulators. Mutation in the p53 gene is associated with changes in chromatin structure, leading to genetic instability and modifications in cell cycle regulation as well as cellular metabolism. Mutant p53 has been reported to act downstream of the TNF receptor to extend and enhance NF-κB activation, thus driving tumor-promoting inflammation and enhancing chemokine secretion. There are p53 pathway inhibitors available that reactivates p53 function which in turn enhances its anti-proliferative activity and thereby sensitizing the cells to apoptosis [89].

Inflammation is a complex phenomenon that is mainly induced to combat injuries and infection. The process encompasses cell recruitment. Over the last years, YB-1 is shown to play a major role in inflammatory processes [90], regulating proliferation, chemotaxis, and matrix protein synthesis and therefore is a candidate protein involved in the orchestration of inflammatory responses [32, 80, 83, 91-93]. Furthermore, in hearts recovering from ischemia/reperfusion injury, YB-1 upregulation occurred transiently in a situation of sterile inflammation [90]. It was demonstrated that this

upregulation was accompanied by enhanced activation of YB-1 through a posttranslational phosphorylation at S102 during the early phase of inflammatory process [49]. It was previously shown that in mesangial and vascular smooth muscle cells that undergo cellular stress, YB-1 is a major regulator of chemotaxis and that it differentially regulates CCL5 in monocyte/macrophages [94, 95]. The chemokine CCL5 plays a central role in recruiting immune cells, such as macrophages, which strongly express the cognate receptor C-C chemokine receptor type 5.

1.6 TNF signaling pathway

TNF was first isolated and characterized in 1984. The 17 kDa secreted form of this molecule has been recognized as a potent inflammatory cytokine. It is known to play a critical role in the pathogenesis of chronic inflammatory diseases [96]. In the beginning, TNF was discovered as a serum factor that induced cell death in tumour cells and therefore was believed to be a potential targeting in cancer. Later, it was realised to be involved for the treatment of inflammatory diseases, mainly for rheumatoid arthritis [97]. TNF is primarily produced by monocytes/macrophages, however T and B lymphocytes, natural killer cells, neutrophils, mast cells, fibroblasts and osteoclasts can also secrete TNF in minor quantities [98]. TNF is produced as a 26 kDa transmembrane protein (mTNF), which is expressed on the cell surface. Cleavage via TNF-converting enzyme (TACE) produces a 17 kDa soluble (sTNF) homotrimer, which is then released and is detectable in blood plasma [99]. TNF

1. INTRODUCTION

performs cellular functions by its two receptors: TNFR1 is expressed in all human tissues, while TNFR2 is expressed mainly in immune cells, neurons and endothelial cells [100, 101]. Although both the receptors are similar in their extracellular structures at the mTNF and sTNF-binding sites, they possess distinct intracellular structures, which then bind to several adapter proteins [102]. Both receptors share a similar structural arrangement with an N-terminal extracellular domain (ECD) composed of four cysteine-rich domains (CRDs), an α-helical transmembrane domain and a cytoplasmic domain [103]. However, they are most divergent in the cytoplasmic domain, where TNFR1 consists of a death domain that is missing in TNFR2 [104].

Survival, apoptosis, and necroptosis are all triggered by the same cell surface receptor TNFR1. Signaling via this receptor promotes pro-inflammatory responses and induces cell death, but it can also result in cell survival [105]. Binding of TNF-α to TNFR1 leads to the sequential recruitment of various adaptor proteins including TNF-receptor associated death domain protein (TRADD), receptor-interacting serine/threonine-protein kinase 1 (RIPK1), and TNF-receptor associated factor 2 (TRAF2) to the plasma membrane (complex 1). Here, TRAF2, an E3 ubiquitin ligase, plays a central role in the activation of NF-κB [106, 107]. TRAF2 recruits the cellular inhibitor of apoptosis protein (cIAP1/2), which ubiquitinates RIPK1. Ubiquitinated RIPK1 serves as a scaffold for the recruitment of the linear ubiquitin assembly complex (LUBAC), composed of Heme-Oxidized IRP2 Ubiquitin Ligase 1 (HOIL-1L)/HOIL-1-Interacting

1. INTRODUCTION

Protein (HOIP)/SHANK Associated RH Domain Interactor (SHARPIN). In addition, ubiquitinated RIPK1 also serves as a platform for the assembly of downstream signaling proteins like transforming growth factor (TGF)-β-activated kinase 1 (TAK1), TGF-beta activated kinase binding protein (TAB) 2 and TAB 3. LUBAC generates M1-linked linear ubiquitin chains, which recruit the IKK complex (IKK1/IKK2 and NEMO). The recruitment of these kinase complexes leads to the activation of NF-κB and mitogen-activated protein kinase (MAPK) signaling [108, 109]. In most cells, TNFR1 engagement promotes cell survival *via* the induction of NF-κB activation [110, 111]. Following the activation of NF-κB, which then orchestrate gene transcription of cellular FLICE-like inhibitory protein (cFLIP). Protein cFLIP eventually translocates to complex IIa to prevent caspase-8 activation. Though the late NF-κB-dependent check point is disrupted and cFLIP levels are subsequently reduced, apoptosis is initiated by complex IIa (Figure 3). The early check point occurs immediately after binding of the ligand, initiated by the ubiquitination of RIPK1 by cIAP and LUBAC [112, 113]. When RIPK1 remains non-ubiquitinated, complex IIb is formed; for this process to occur, the cylindromatosis tumor suppressor protein (CYLD) enzyme deubiquitinated RIPK1, thus allowing it to disassociate from complex I and forms complex IIb, whereby TRADD is replaced by RIPK3, upon degradation of cIAP1 and 2. This leads to the cleavage of pro-caspase 8 to caspase-8, and activation of the caspase signaling cascade results in apoptosis [114, 115]. To ensure that apoptosis and not necrosis (unregulated cell

death) is induced, which mostly results in unwanted inflammation and damage to surrounding tissues, caspase-8 in complex with cFLIP is essential to cleave RIPK1 and 3 [114]. However, RIPK1 and RIPK3 may sometimes remain uncleaved, leading to their aggregation and formation of complexes, with the activation of mixed lineage kinase domain-like protein (MLKL) and the induction of regulated necrotic cell death, termed as necroptosis [116, 117].

Based on the current understanding of inflammatory diseases, novel treatment strategies and drugs have been developed that target cytokines or interferes with signaling cascades. Popular approaches include cytokine blocking antibodies (Infliximab: TNF-α), soluble TNF receptors (Onercept/Etanercept) and IL-1β receptor (Anakinra) [118, 119]. It has been observed that rheumatoid arthritis patients treated with TNF-α blocking drugs showed improved clinical symptoms such as preventing or controlling joint damage. Similarly, patients suffering from chronic kidney diseases demonstrated a beneficial effect on kidney function [120]. Although all anti-TNF-α therapies have well-established efficacy, the enhanced risk of lymphomas and reactivation of latent infection remain major drawbacks [119, 121-123]. Therefore, drug development has more recently been focused on interference with cytokine receptors, thereby blocking the activation of cytokine-induced signaling pathways, thus elimination of an inflammatory milieu. There is strong evidence for the role of macrophage-derived TNF-α in the advancement of atherosclerosis [124-127]. In

1. INTRODUCTION

inflammatory models, TNF-α has been shown to be important in the upregulation of

adhesion molecules that are critical in extravasation of monocytes. This role is

mediated by the NF-κB pathway [128]. Recently it was shown that mast cells derived

TNF directly primes circulating neutrophils by TNFR1 while being vital for endothelial

cell activation [129].

Like YB-1, TNF also possesses the ability to auto-regulate its own activity. TNFR

stimulation activates NF-κB, amongst other factors, which induces the expression of

TNF mRNA. TNF stimulation of YB-1 deficient cells failed to activate NF-κB, as was

previously shown for both IGF-1 and IL-1β [61, 62, 130]. An inability to activate YB-1

and thus NF-κB, negatively effects cell survival in monocytes, macrophages and T

cells [130-132]. Nevertheless, this is not the only stage where YB-1 may affect TNF

functionality. The TNF mRNA possesses similar regulatory sequences that are found

in the mRNA of GM-CSF, which is a known target of YB-1 [133-135].

In addition, RNA binding proteins, such as TIA-1, TIAR, and HuR, which are

components of stress granules, regulate TNF mRNA. As mentioned above YB-1

regulates stress granule formation and is found together with TIA-1, TIAR, and HuR

within these structures, there arises a strong possibility of YB-1 contributing to the

regulation of TNF mRNA [136-138]. In addition to enhancing the expression of TNF

mRNA, NF-κB also induces TRAF2 expression [119]. TRAF2 is a common signaling

component TNFR1 and TNFR2 that plays a key role in regulating canonical *versus*

non-canonical NF-κB activation, as well as survival and death signaling [139]. An induction of TRAF2 would amplify the canonical NF-κB (p50/p65) pathway. The phosphorylation of TRAF2 by protein kinase C regulates its ability to recruit as well as activate IKKα, which then phosphorylates inhibitor IκBα, targeting it for its degradation and thus activating NF-κB (p50/p65) [139, 140]. It has been reported that TRAF2 mediates the recruitment of ubiquitin ligases to TNFR1 complex 1, thereby promoting NF-κB activation [141]. Moreover, YB-1 has been shown to directly interact with p65 and act as transcriptional co-activator [142]. Several kinases (AKT, ERK, RSK, PKC, CKII) have been identified that phosphorylate YB-1 within its cold shock domain, resulting in nuclear translocation [64, 143]. Therefore, it is foreseeable that the TNF receptor oligomerization following ligand occupancy, induces the recruitment of adaptors, such as TRAF2, which in turn recruit kinases and ubiquitin ligases that activate both NF-κB and its *trans*-activator YB-1.
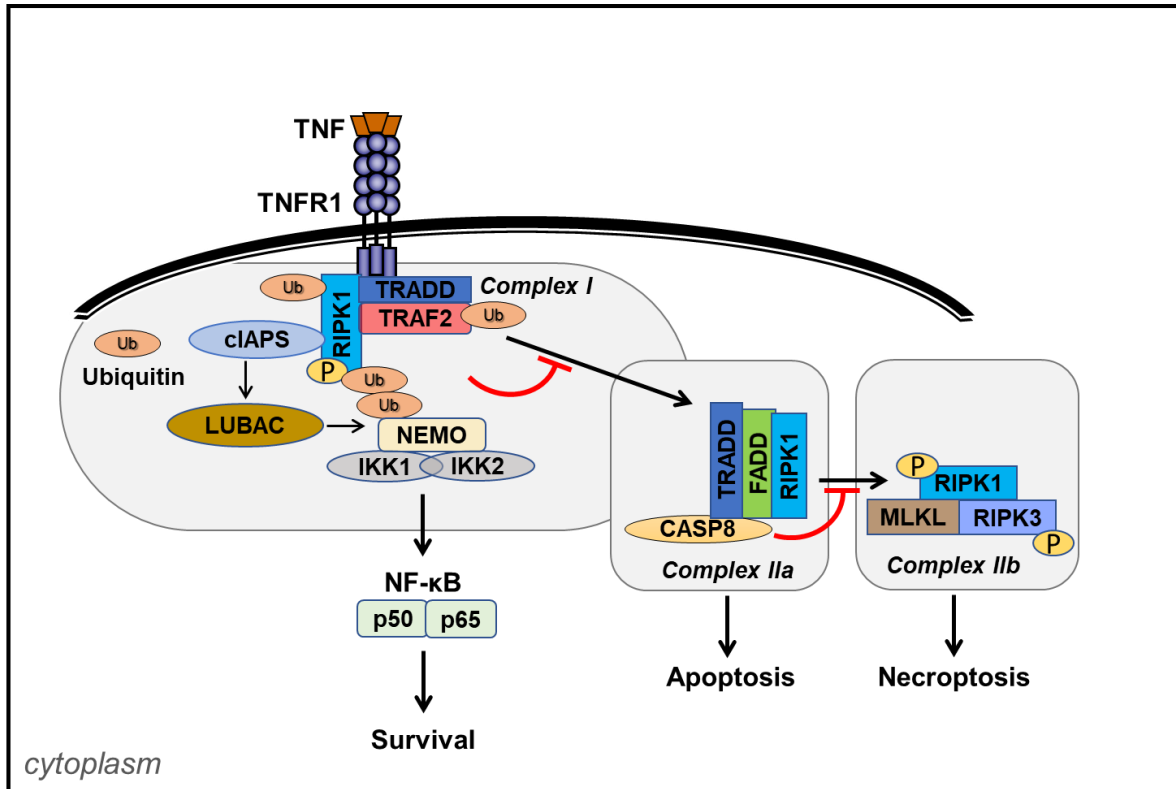
Figure 3. Formation of protein complexes following TNFR1 signaling. *Upon binding of TNF to TNFR1, TNFR1 recruits TNFR1-associated death domain (TRADD), which then binds to receptor- interacting protein kinase 1 (RIPK1), TNFR-associated factor 2 (TRAF2), and cellular inhibitor of apoptosis protein 1 or 2 (cIAP1/2) to form complex I [106, 107]. cIAP1/2 and the linear ubiquitin chain assembly complex (LUBAC) add Met1 and Lys63-linked polyubiquitin chains to RIPK1. This linkage specific ubiquitination stabilizes RIPK1, amplifying its signal. Lys63 linked chains on RIPK1 recruit the transforming growth factor-β (TGFβ)-activated kinase 1 (TAK1) complex, comprising of TGFβ -activated kinase 1 and mitogen-activated protein kinase (MAPK)-binding protein 2 and 3 (TAB2 and 3) and TAK1. The TAK1 complex phosphorylates the IκB kinase (IKK) complex. This results in the translocation of transcription of NF-κB into the nucleus, leading to the transcription of genes [108-111]. RIPK1 is deubiquitinated by cylindromatosis tumor suppressor protein (CYLD), facilitating its dissociation from complex I. Recruitment of Fas-associated protein with death domain (FADD), and caspase 8 by TRADD, results in the formation of complex IIa that triggers apoptosis by activating caspase, although additional recruitment of RIPK1 forms complex IIb that is a potent inducer of apoptosis [114-117]. In the absence of caspase 8 protease activity, RIPK3 and MLKL (mixed lineage kinase domain-like pseudokinase) are recruited to complex IIb and together triggers necroptosis that is also RIPK1 dependent.*

1.7 The NF-κB pathway

Sen and Baltimore first described in 1986 the NF-κB pathway [144]. These days, it is

clear that this tightly regulated pathway synchronizes, the transcription of a wide range

of genes encoding both pro- and anti-inflammatory cytokines, costimulatory and adhesion molecules, chemokines, and modulates apoptosis or cell proliferation [145]. The activation of NF-κB involves two major signaling pathways, denoted as canonical and noncanonical (or alternative) pathways. Both are crucial for regulating immune and inflammatory responses despite their differences in signaling mechanisms [146, 147]. Functional NF-κB proteins are dimers, possessing Rel homology domain at their N-terminus, hence their classification as NF-κB/Rel proteins. There are five proteins in the NF-κB family: RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). NF-κB1 and NF-κB2 are synthesized as large precursors (p105 and p100), which are proteolytically cleaved to p50 and p52, respectively. NF-κB is found in the cytoplasm of resting cells as an inactive complex. NF-κB members bind the inhibitory IκB proteins that maintain the associated NF-κB dimers in the cytoplasm.

1.7.1 Canonical NF-κB pathway

In the canonical (or classical) NF-κB pathway activation, a variety of cell membrane receptors (TNFR1, IL-1R, Toll like receptor etc.) lead to the phosphorylation, ubiquitination, and subsequent proteasomal degradation of IκBα. Activation of the canonical NF-κB pathway occurs by pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin (IL)-1β [148-150]. Phosphorylation of IκBα is catalyzed by IκB kinases (IKK). IKK is composed of IKKα, IKKβ and the regulatory subunit NEMO (NF-κB essential modulator; IKKγ). Phosphorylation of IκBα is

mediated by the IKK complex; predominantly by IKKβ. The phosphorylated IκBα is ubiquitinated by the E3 complex SCFβ-TrCP for the lysine 48 (K48)-linkage which mediates IκBα proteasomal degradation. This results in proteolysis of IκBα by the 26S proteasome. This in turn frees the NF-κB dimers, which translocate into the nucleus where they bind to NF-κB sites on the DNA and activate gene transcription. A noteworthy feature of NF-κB activity is the strong interaction with other signal transduction pathways, such as MAPK signaling. Phosphorylation of TAK1 not only leads to the activation of the IKK complex, but also activation of MAPKs [151]. The classical pathway is important at multiple stages of development and for homeostasis of the immune system, and when perturbed, in the induction and progression of autoimmune pathologies [148, 152-154]. These signaling pathways are tightly controlled processes regulated by reversible post-translational modifications, mainly phosphorylation and ubiquitination. A schematic representation of the canonical NF-κB pathway is shown in Figure 4.

1.7.2 Non-canonical NF-κB activation

The non-canonical pathway is largely for activation of p100/RelB complexes. It mainly regulates lymphoid organ development, survival of B lymphocytes, and generation and maintenance of effector and memory T-cells. This pathway proceeds through an IKK complex that contains two IKKα subunits (but not NEMO). This type of activation is triggered by signals of the TNF receptor family, like TNFR2, BAFFR (B-cell activator

factor receptor), CD154, RANK (receptor activator of NF-κB), and LTβR (Lymphotoxin β receptor) [155]. In comparison to the activation of the canonical NF-κB pathway, that requires the phosphorylation of the IKK complex, the non-canonical pathway requires the stabilization and accumulation of NF-κB-inducing kinase (NIK). In resting situation, NIK is constantly bound by a complex TRAF3 (TNFR associated factor 3), TRAF2 and cIAP, which mediates NIK K48 poly ubiquitination (pUb) and consequently, its proteasomal degradation. When stimulated, the complex gets recruited to the receptor. The binding with the receptor induces the activation of cIAP, which acts as E3 ubiquitin ligase, transferring the K48 pUb chains to TRAF3, initiating its proteasomal degradation and disrupting the complex. Henceforth, NIK is no longer linked to TRAF3 and begins to accumulate in the cytoplasm and phosphorylates IKKα. This further induces the proteasomal processing of p100 to p52 [155, 156]. Heterodimers of p52 and RelB can then migrate to the nucleus to initiate gene transcription.
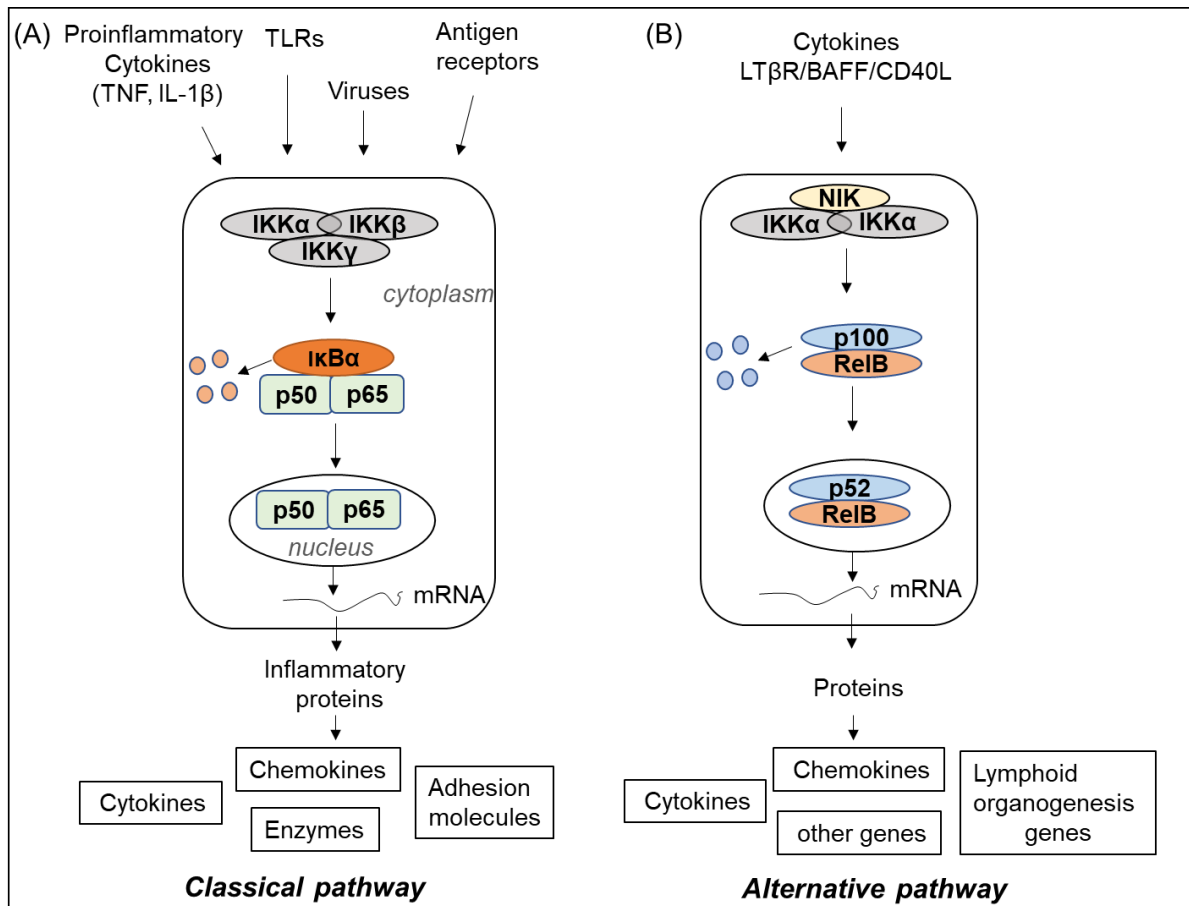
Figure 4. Overview on classical and alternative NF-κB pathway. *(A) The classical NF-κB pathway is activated by a variety of inflammatory signals. The proinflammatory cytokines IL-1β and TNF-α activate NF-κB, inducing the expression of inflammatory proteins. (B) The alternative pathway of NF-κB results in nuclear translocation of p52-RelB dimers, which is dependent on IKKα homodimers, activated by LTα1β2, BAFF, and CD40L by NIK [148].*

1.8 Ubiquitination

Ubiquitin is a 76 amino acid protein, highly conserved among eukaryotic organisms from yeast to human. The process of ubiquitination is an energy-dependent post-translational modification in which one or more ubiquitin molecules are attached to a substrate protein. Ubiquitin has seven lysine residues, each of which can be used to form a unique type of polyubiquitin chain that is based on its position K6, K11, K27, K29, K33, K48, and K63, resulting in distinct linkage-specific effects (Table 2) [157].

In addition, a donor ubiquitin can also be attached to a recipient ubiquitin at the amino terminal methionine (M1) leading to M1 or linear Ub linkages. The process of ubiquitination comprises of a cascade of three sequential steps that are activation, conjugation, and ligation. These are each catalysed by different enzymes known as E1, E2, and E3, respectively. The first step is the activation of the ubiquitin molecule by E1 ubiquitin-activating enzyme in an ATP-dependent manner, resulting in the formation of thioester linkage between ubiquitin and E1.
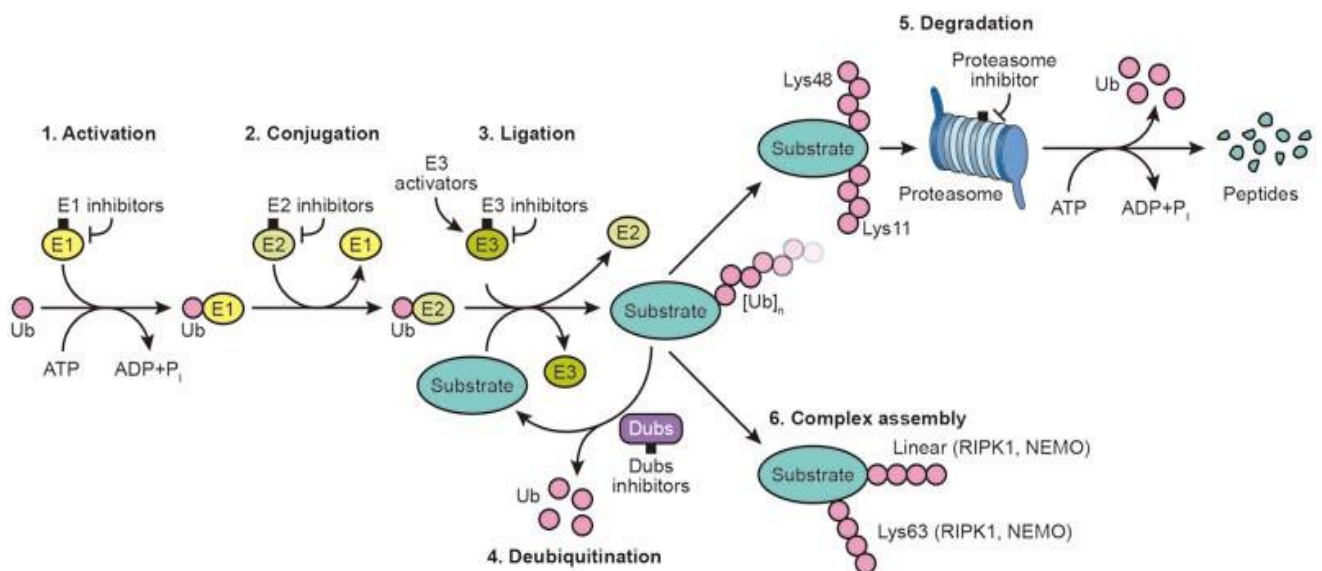

Figure 5. Process of ubiquitination. *Ubiquitination is an ATP-dependent process carried out by three types of enzymes. (1). E1 activating enzymes form a thioester bond with ubiquitin, following binding of ubiquitin to E2 conjugating enzymes (2). The formation of an isopeptide bond between the carboxyl-terminal glycine of ubiquitin and a lysine residue on the substrate protein, (3) requires E3 ubiquitin ligases [158].*

There are only two E1 enzymes encoded in the human genome and they do not show specificity for specific E2 enzymes. E2 ubiquitin-conjugating enzymes can bind ubiquitin with its catalytic cysteine residue. There are about 40 different E2 enzymes,

whose primary function are to determine the kind of polyubiquitin chains that are catalysed by the E3 ligases. Lastly, the E3 ubiquitin ligases bind both E2, and the substrate, an isopeptide bond is formed between the C- terminal glycine of ubiquitin and a lysine residue within the target protein. The E3 ligase is responsible for substrate specificity. Therefore, E3 ligases are the most abundant enzymes in human cells [159, 160]. The ubiquitination process is reversible and ubiquitin molecules can be removed from the substrate by the deubiquitinating enzymes (Figure 6). Residues can be modified with a single ubiquitin molecule (monoubiquitination), many single ubiquitin proteins (multi-monoubiquitination), or a chain of ubiquitin molecules (polyubiquitination). Polyubiquitin chains are usually formed through covalent binding of C-terminal glycine of one ubiquitin molecule to an internal lysine residue of another ubiquitin molecule. Different linkages are linked to different physiological functions [161]. Within the past years, a plethora of data has emerged regarding the ubiquitin linkage types and their function [162, 163].

1. INTRODUCTION

Type of Ubiquitination	Function
K6 PolyUb	Cell cycle, DNA repair, Histone modification [164-166]
K11 PolyUb	Cell cycle, membrane trafficking, TNFR1 signaling [165, 167-169]
K29 PolyUb	ER stress-mediated apoptosis, Lysosomal degradation, mRNP disassembly, Wnt/β-Catenin signaling [170]
K33 PolyUb	Protein trafficking, TCR signaling [171, 172]
K48 Poly Ub	Proteasomal degradation, TNF signaling, Transcription regulation [173, 174]
K63 Poly Ub	Autophagy, Development, DNA replication and repair, Innate and adaptive immunity, Signal transduction, Spliceosome function [175-178]
M1 Poly Ub	Innate immunity [179]
Mono-ubiquitination	DNA replication and repair, Endocytosis, Protein localization, Signal transduction, Transcription regulation [180, 181]

Table 2. Ubiquitin linkages and their function

Multiple sites of ubiquitination have been identified within YB-1 [182]. Interestingly, three of these sites were also identified to be ubiquitinated upon TNF stimulation [60]. The homologous to E6AP C-terminus (HECT) domain and ankyrin repeat containing E3 ubiquitin ligase 1 (HACE1) is a central gatekeeper for TNFR1-induced cell death. HACE1 is required for the ubiquitination of TRAF2 and thereby forming the apoptotic complex [183]. HACE1 has been shown to interact with YB-1. This involves the polyubiquitination of the YB-1 protein through K27-linked ubiquitin chain for protein secretion [45]. In addition, to HACE1, YB-1 also interacts with the deubiquitinase

otubain-1 (OTUB1) [44]. OTUB1 is responsible for removing K48-linked ubiquitin chains from YB-1 and thereby stabilizes YB-1 expression [44].

1.9 Interactions of YB-1 and NF-κB

The crosstalk between the proteins have been known. Thus, we hypothesize that there might be an interaction directly or indirectly. There is an overlap within the genes and or proteins regulated by both YB-1 and NF-κB. The group of genes and proteins regulated by both or any one of them are listed below (Table 3).

1. INTRODUCTION

Target	Reference for YB-1	Regulation (YB-1)	Regulation (NF-κB)	Reference for NF-κB
Cytokine, chemokine and receptors PDGF-BB	Stenina, et. al., 2000	↑	↑	Khachigian et. al., 1995
VEGF	Coles, et. al., 2002	↓	↑	Chilov et. al., 1997
RANTES/CCL5	Krohn R, et.al., 2007	↑	↑	Wickremasinghe et. al., 2004
Tyrosine-phosphatase 1B (PTP1B)	Fukada and Tons, 2003	↑↓	↑	Zabolotny et. al., 2008
GM-CSF	Diamond P, et.al., 2001	↑↓	↑	Schreck and Baeuerle, 1990
IFN α and β	Yan C and Tamm I, 1991	↑	-	
Receptors Thyrotropin receptor	Ohmori et al., 1996	↓	-	
EGFR	Wu J, et. al., 2006	↑	↑	Nishi et. al., 2003
FAS (CD95/Apo-1)	Lasham, et. al., 2000	↓	↑	Chan et. al., 1999, Singh et. al., 2006
Notch-3	Rauen T, et. al., 2009	↑	-	
Matrix and structure proteins Gelatinase –A (MMP2)	Mertens, et. al., 1997	↑↓	-	
MMP12	Samuel S, et. al., 2005	↓	-	
MMP13	Samuel S, et. al., 2007	↓	-	
α-smooth muscle actin	Zhang A, et. al., 2005	↓	-	
COL1A1	Norman J T, et. al., 2005	↓	-	
COL2A2	Higashi K, et. al., 2005	↓	↑	Nieto, 2007
Proliferation and apoptosis DNA-polymerase-α	En-Nia, et. al., 2005	↑		
p53	Okamoto, et. al., 2000	↑	↑	Schumm et. al., 2006
Cyclin A and B	Zhang H, et al., 2014, Jurchott K, er.al., 2003	↑		
Cyclin D1	Harada M, et. al., 2014	↑	↑	Guttridge et. al., 1999
Sox2	Jung K, et. al., 2014	↓		

Antigen presentation MHC-1, MHC-II	Didier, et.al., 1988	↓		
Transportprotein, Detoxification MDR-1	Bargoa, et.al., 1997	↑	↑	Wang et. al., 2007
MDR-2	Geier, et.al., 2003	↓		
Mrp-1	Stein, et.al., 2001	↑		
Mrp-2	Kaufmann, et.al., 2001	↓		
Grp78	Li, et.al., 1997	↓		
Viral promoters RSV	Swamynathan, et.al., 1997	↑		
HTLV-1	Kashanchi, et.al., 1994	↑		
HIV	Ansari, et.al., 1999	↑	↑	Nabel and Baltimore, 1987
JC polymavirus	Safak, et.al., 1999	↑	↑	Ranganathan and Khalili, 1993

Table 3. Interactions of YB-1 and NF-κB.

2. AIM

The immune system constitutes the first line of defense against invading pathogens. Phagocytic cells, like monocytes/macrophages, combat and clear pathogens and coordinate immune responses via the secretion of cytokines, such as tumor necrosis factor (TNF), interleukin (IL)-1β, IL-6, 1L-8, and IL-12. The cold shock protein YB-1 is important for monocyte/macrophage differentiation and phagocytic function [184]. Macrophage polarization is impaired when YB-1 is genetically deleted [184]. Of the secreted cytokines, especially TNF-α and IL-1β are known to activate NF-κB transcription factor. In turn, their expression is controlled by NF-κB, hence resulting in a positive feedback loop. Therefore, the NF-κB signaling pathway plays pivotal role in activating myeloid cell function during inflammation. Like YB-1, NF-κB is an important transcription factor involved in inflammatory and autoimmune diseases, viral infection and improper immune development [147]. Nevertheless, the crosstalk between YB-1 and NF-κB signaling in the orchestration of monocyte/macrophage function is still unclear.

2. AIM

To obtain more insights into the role of monocyte/macrophage in inflammation, I aim to

1. identify the molecular mechanism of YB-1 mediated NF-κB activation.

2. determine the cellular function of YB-1 upon TNF stimulation in monocytes/macrophages.

3. look for differential regulation of TNF complex formation in the absence and presence of endogenous YB-1.

4. elucidate the cellular phenotype in immune cells, especially monocytes/macrophages, that have been manifested to deplete endogenous YB-1.

3. MATERIALS AND METHODS

3. Materials

Cell culture

Cell culture was performed under sterile conditions, within a laminar flow hood. Before use, all the cell culture media were pre-warmed at 37°C in water bath. Cells were cultured in an incubator at 37°C, 5% CO_2 and 60% of water vapour. Plastic consumables like plates, flasks, plastic pipettes, for cell culture were purchased from Greiner Bio-One (Frickenhausen, Germany) and Carl Roth (Karlsruhe, Germany).

Cell lines

The cell lines HEK293T, THP-1 (TIB-202) and U937 (CRL-1593) were obtained from the American Type Culture Collection (ATCC). HEK293T, human embryonic kidney cells were cultivated in DMEM media with 10% FCS and 1% P/S under humidified conditions at 37°C and 5% CO_2. THP-1 cells were cultured in Rosewell Park Memorial Institute (RPMI) medium and supplemented with 10% FCS, 1% P/S, and 50 μM β-mercaptoethanol, and U937 cells were cultured in RPMI medium and supplemented with 10% FCS and 1% penicillin under humidified conditions at 37°C and 5% CO_2. Media were replaced every 2-3 days.

3. MATERIALS AND METHODS

Chemicals:

RPMI 1640 medium	Gibco
DMEM medium	Gibco
Fetal Calf Serum	PAN-Biotech
Penicillin/Streptomycin (P/S)	Thermo Fisher Scientific
Sodium Pyruvate	Thermo Fisher Scientific
Dulbecco's Phosphate-Buffered Saline (DPBS)	Thermo Fisher Scientific
Trypsin	Thermo Fisher Scientific
Dimethyl sulfoxide (DMSO)	Applichem
β-Mercaptoethanol	Sigma
M-CSF 10ng/mL	Peprotech
Human TNF-α	R & D biosystems
Murine TNF-α	R & D biosystems
IL-1β	R & D biosystems
Cycloheximide	Sigma
BV6	Adooq Bioscience, Canada
z-VAD	Merck
Nec-1	Calbiochem, USA

3. MATERIALS AND METHODS

Propidium iodide	Bio Chemica A2261
Annexin V	BD Biosciences
7-AAD	Enzo Life Sciences, Germany
Hoechst 33,342	Thermo Fisher Scientific
Retronectin solution	TaKaRa
Chloroquine	Sigma
Hydroxyethyl piperazineethanesulfonic acid (HEPES) buffer	Gibco, Dublin, Ireland
Sodium butyrate	Sigma, Darmstadt, Germany
Polybrene	Sigma
Puromycin	Calbiochem
MG132	Sigma

Western blotting:

1x TBST	Tris 1 M, pH 8.0, Nacl 5 M, Tween 20 1 mL (Chem cruz, sc-29113)
10x Running buffer pH 8.6	Tris 0.25 M, Glycin 1.92 M, SDS 1%
5x reducing sample buffer	Tris-HCL (Applichem), pH 7.4, 150 mM, Glycerol, 30%, SDS 12%, β-

3. MATERIALS AND METHODS

	mercaptoethanol 24% (Roth), Bromophenol blue (Merck, 1.081.220.005)
BSA (Bovine serum albumin)	Roth, 8076.3
Complete mini protease cocktail	Roche, Mannheim, Germany
Dyna Beads	Sigma
Ethanol 70%	Fischar
Filter paper	Thermo scientific
Milk powder for blotting	Roth, T145.3
Nitrocellulose membrane	Thermo scientific
Page Ruler Pre-stained Ladder (26619)	Thermo Fischer Scientific (Waltham, USA)
Phospho-stop	Roche
Pierce ECL western blotting substrate	Thermo scientific, 32106
RIPA lysis buffer	50 mM Tris Base, 150 mM NaCl (Roth, 3957.2), 1 mM ethylenediamine-tetraacetic acid (EDTA) (Biorad, 161-0729), Nonidet (NP-40), 0.25% sodium deoxycholate

3.   MATERIALS AND METHODS

SDS (Sodium dodecyl sulfate)-polyacrylamide resolving gel	Distilled water 8-10%, acrylamide 30% (Serva, 10688.01), Tris 1.5 M (Roth), 0.1% ammonium persulphate (Roth, 9592.2), SDS 0.1% (Roth), TEMED (Roth), pH 8.8
SDS-polyacrylamide stacking gel	Distilled water 5%, acrylamide 30%, Tris 0.5 M, ammonium persulphate 0.1%, SDS 0.1% TEMED, pH 6.8
shRNA (pLKO and pLKO-YB-1)	Sigma-Aldrich
Transfer buffer	Tris 25 mM, Glycin 190 mM (Roth, 3790.3), Methanol 20% (Roth, CP43.3), Water, pH 8.2-8.4
DISC lysis buffer	Tris 1M pH 7.4, 5M NaCl, Glycerol, Triton X-100

3. MATERIALS AND METHODS

Antibodies for western blotting

Anti-NF-κB p65	Cell signaling, Frankfurt am Main, Germany, #6956
Anti-phospho NF-κB p65	Cell signaling, #3033
Anti-TRAF2	BD Biosciences, Heidelberg, Germany, #558.890
Anti-RIP	BD Biosciences, #610.459
Anti-IκBα	Cell signaing, #9242
Anti-p-IκBα	Cell signaling, #9246
Anti-TNFR1	Santa Cruz, CA, USA, #8436
Anti-Vinculin	Santa Cruz, #59.803
Anti-mono-and polyubiquitinated protein	Enzo, clone FK2
Anti-cleaved caspase-3	Cell signaling, #9661
Anti-caspase-8	Cell signaling, #4790
Anti-cleaved caspase-8	Cell signaling, #9496
Anti-pIKKα/β	Cell signaling, #2697
Anti-YB-1 (DGKETKAADPPAENS)	Eurogentec, Liege, Belgium

3. MATERIALS AND METHODS

Instruments

FACS Canto^{TM} II Flow Cytometer	BD Biosciences
Protein system	Bio-Rad
Centrifuge	Sigma
Centrifuge Mikro	Eppendorf
CO_2 Incubator	Thermo Scientific/VWR
Imaging flow cytometer	BD Biosciences
ECL western blot developer	Intas
Laboratory balance	Acculab
Laminar flow hood	Herasafe
Microscope	Leica
Nanodrop	Analytik Jena
Neubauer Chamber	Thermo Scientific
pH meter	Omnilab
PowerPac	Consort/VWR
Spectrophotometer	GE Healthcare/VWR
Thermomixer	Eppendorf
Tube roller	Omnilab
Vortex	Scientific Industries

3. MATERIALS AND METHODS

Methods

Cell culture stimulation

Cells were stimulated with recombinant TNF-α (20 ng/mL; R&D Systems) for the indicated time points and were subsequently washed with ice-cold phosphate-buffered saline. Cells were lysed with Radio-Immunoprecipitation Assay (RIPA) lysis buffer (50 mM Tris Base, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Nonidet (NP-40), 0.25% sodium deoxycholate) supplemented with complete mini protease cocktail inhibitor cocktail (Roche, Mannheim, Germany) Phospho-stop (Roche). Lysates were cleared by centrifugation at 14,000x g.

Bone Marrow-derived Macrophages

Bone marrow-derived macrophages (BMDMs) were generated from wild type or *Ybx1*$^{\Delta LysM}$ deficient mice by flushing femur and tibia with sterile Dulbecco's phosphate-buffered saline (DPBS). Erythrocytes were lysed under hypotonic conditions and were cells seeded with 2 x 10^6 cells/mL in RPMI growth media supplemented with 10% FCS, 1% penicillin/streptomycin, and murine macrophage colony-stimulating factor (10 ng/mL; M-CSF, 315-02, Peprotech). Cells were cultivated under humidified conditions at 37°C and 5% CO$_2$. Cells were fed every 2 days until fully differentiated (after 7 days) [185]. Animals were maintained according to the FELASA guidelines (Federation of European Laboratory Animal Science Association) in a 12 h/12 h light/dark cycle at

3. MATERIALS AND METHODS

22°C in the Central Animal Facility of the Otto-von-Guericke University Magdeburg under specific pathogen-free (SPF) conditions using individual ventilated cages (IVC, Techniplast, Buguggiate, Italy) with free access to food and water. All experimental procedures were conducted in accordance with the German National Guidelines for the Use of Experimental Animals (Animal Protection Act) and approved by the State of Saxony-Anhalt (AZ UniMD 42502-2-1401).

Lentiviral Transduction of YB-1

The plasmids pLKO and pLKO-YB-1 shRNA were fromSigma-Aldrich. For knockdown, YB-1 shRNA and scrambled shRNA were used. (shRNA:CCGGCCAGTTCAAGGCAGAAATATCTCGAGATAT TTACTGCCTTGAACTGG-TTTTTG). Human embryonic kidney cells (HEK293T; 8×10^5) were seeded in 6-well plates the day before viral transduction and co-transfected with 2 μg YB-1 construct plasmid, 1 μg psPAX2, and 1 μg pVSV-G with calcium phosphate precipitates. Chloroquine (25 μM) was added to the cells immediately before transfection. After 1 day, the medium was exchanged with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS, 1% penicillin, 10 mM hydroxyethyl piperazineethanesulfonic acid (HEPES) buffer (Gibco, Dublin, Ireland), and 1 mM sodium butyrate (Sigma, Darmstadt, Germany). Virus-containing supernatants were harvested and filtered with 0.45 μm filter, and 4 μg/mL polybrene was added, and the target cells (THP-1 and U937) were infected. After 6 h

of transduction, an equal volume of fresh medium was added, and the medium was exchanged with fresh medium the following day. Stably transduced cell lines were selected using puromycin (1.5 µg/mL) for 7–14 days. Cells were harvested 3 days after transduction for Western blot analysis.

SDS-PAGE and Western Blotting

Protein quantification was carried out photometrically using the Bradford reagent (Sigma). Protein samples were boiled in sample buffer at 95°C for 5 mins. Equal amounts of protein were loaded onto the SDS gels. Proteins were resolved using 10% sodium dodecyl sulphate (SDS) polyacrylamide gel and blotted onto nitrocellulose membranes. The membranes were blocked with 5% dry milk in Tris-buffered saline-Tween. Primary antibodies were incubated overnight at 4°C and diluted according to the manufacturer's instructions. Secondary antibodies coupled to horseradish peroxidase (Southern Biotech, Birmingham, AL, USA) were used for immuno-detection. The detection was performed using Pierce ECL Western blotting substrate (32106, Thermo Fischer Scientific, Waltham, MA, USA).

3. MATERIALS AND METHODS

NuPAGE

The NuPAGE gradient gels (4-12%) offers maximum stability of proteins and gel matrix during electrophoresis and better band resolution than other gel systems due to the discontinuous SDS-PAGE. Samples are prepared in 4X Lithium dodecyl sulphate (LDS) sample buffer and 4% β-mercaptoethanol. Protein samples were cooked at 70°C for 10 mins and loaded onto the gels. The transfer of the protein was ran at 30 V, for 1 h on ice. Further steps were carried out analogously to the western blot protocol as mentioned above.

Imaging Flow Cytometry

BMDMs and THP-1 cells were cultured and stimulated as described above. Cells were centrifuged at 500x g at 4°C for 5 min and washed twice with 0.5 mL incubation buffer [5 g/L bovine serum albumin (BSA) in phosphate buffer saline (PBS)]. After washing, the pellet was suspended in 50 µL incubation buffer and stained with anti-NF-κB phospho-S529 p65 phycoerythrin (PE) antibody (clone REA348, Miltenyi). After 1 h incubation at RT in the dark, the cells were washed with 0.5 mL incubation buffer again and suspended in PBS. 7AAD (BioLegend) was added at least 5 min before measuring. The cells were measured with an Amnis FlowSight (Merck Millipore). 7AAD was measured in channel 5 (642–745 nm) and PE in channel 3 (560–595 nm). The nuclear translocation of NF-κB phospho-p65 was determined by the overlap between phospho-p65 and 7AAD.

3. MATERIALS AND METHODS

Cell Death Assays

To quantify apoptosis, we seeded THP-1 and U937 cells at 1×10^6 cells per well in 24-well plates. Cells were pre-treated with pan caspase inhibitor z-VAD (1:1000) and RIPK1 inhibitor Nec-1 (50 µM, Calbiochem, USA) or BV6 (5 µM, Adooq Bioscience, Canada) for 1 h and then stimulated with TNF (20 ng/mL) and cycloheximide (10 µg/mL) for the indicated time points. To detect dead cells, the samples were stained with propidium iodide (10 µg/mL) and measured by flow cytometry. To assess apoptosis, we stained untreated and treated cells (as mentioned above) with annexin V (BD Biosciences) and 7AAD (Enzo Life Sciences, Germany) to discriminate between apoptotic and necrotic cells. The cleavage of caspase-3/7 was determined using Caspase-3/7 Green Reagent (Invitrogen). After stimulation (as mentioned above), the cells were incubated with diluted reagent (1:5000) for 10 min and measured with flow cytometry.

Time-Lapse Microscopy

For each stimulation condition, we separated a Fluorodish into two small and one big volume using 2-well silicone inserts. The two small compartments were coated with a Retronectin solution, overnight. The compartments were washed with PBS, and 28,000 cells of each control and knockdown U937 cells were seeded into small compartments using RPMI medium, supplemented with 10% fetal calf serum, 1% penicillin and Hoechst 33,342 (5 µg/mL), and propidium iodide (1 µg/mL). After 2 h of

adhesion, non-adherent cells were washed away, and the surrounding compartment was filled with same medium along with and without TNF (20 ng/mL) and cycloheximide (10 µg). Subsequently, the silicone insert was removed thereby both control and knockdown cells were exposed to the surrounding cell culture medium. Within each of the four fields, fields of view were defined. Repetitive images of the Hoechst 33,342 and propidium iodide (PI) staining were acquired along with a phase contrast for all the 20 fields of view for every 150ms for 18 h.

Image Segmentation

To analyze cell death in U937 cells, we exported the 26 k single channel images as unaltered 8-bit tiff files. The images were analyzed in cell profiler software using pipeline consisting of an Otsu two class threshold, with a size exclusion for the diameter of 10–40 pixels (6.5–26 µm) for the healthy nuclei, to analyze cell death in U937 cells, we exported the 26 k single channel images as unaltered 8-bit tiff files. The images were analyzed in cell profiler software using pipeline consisting of an Otsu two class threshold, with a size exclusion for the diameter of 10–40 pixels (6.5–26 µm) for the healthy nuclei, an Otsu two class threshold for PI-positive objects as well as PI-positive nuclei fragments per image. The number of healthy nuclei was normalized to nuclei number in the first frame.

3. MATERIALS AND METHODS

TNFR Immunoprecipitation

50×10^6 cells were taken per sample (in PBS) on ice. Cells were stimulated with 5 µg of Flag TNF for 10 mins, 37°C in water bath. Thereafter cells were centrifuged at 400 xg, 5 min and 4°C. The supernatant was aspirated, meanwhile lyse the pellet with 1 ml of DISC lysis buffer on ice. For unstimulated samples flag TNF was added post lysis (control). Take 50 µl aliquots as Input. Centrifuge at 14,000 rpm, 10 mins, and 4°C to remove nuclei. Samples were stored at -20°C until use. To the remaining lysate, add 3.12 µl anti-FLAG antibody (F7425, Sigma) and incubate at 4°C with rotation for 30 mins. Add 30 µl washed Dynabeads (10004D, Invitrogen) and continue incubation for 2 hours. Supernatant was removed using the magnet and stored at -20°C. Wash the pellet 2x with cold PBS, use a syringe to carefully remove the fluid without losing the pellet. Add 50 µl FLAG peptide to the pellet and incubate 30 mins at RT with occasional gentle mixing. Pellet should stay at the bottom of the tube (Figure 4). Centrifuge and transfer the supernatant to new tubes. Samples were treated according to the NuPAGE protocol.
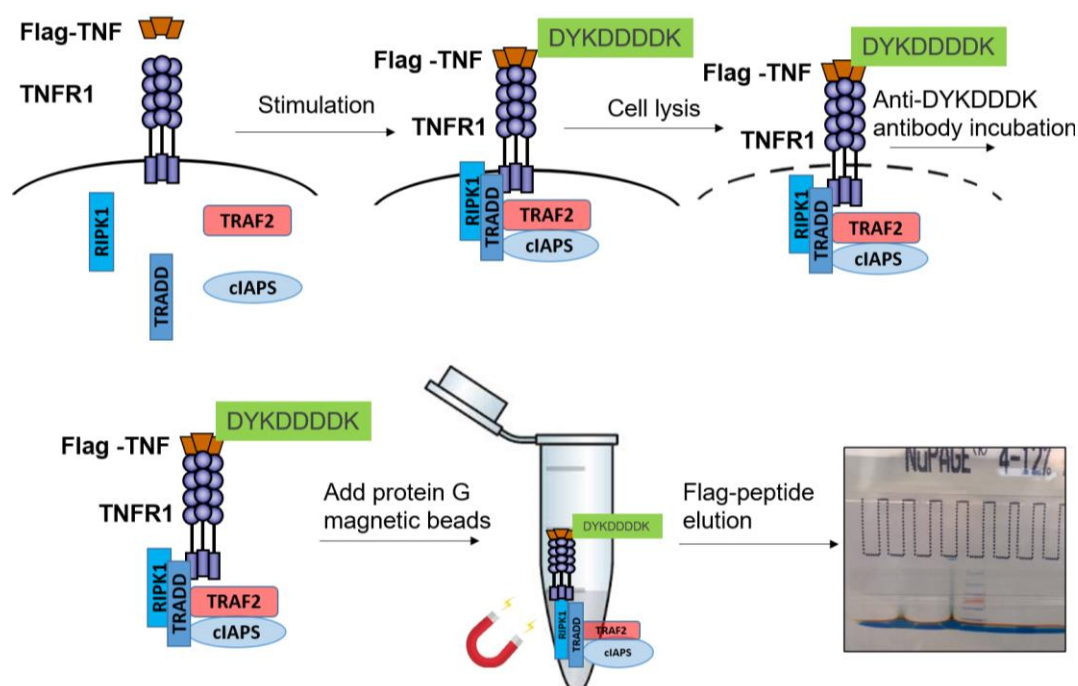
Figure 4. Schematic representation of TNFR Immunoprecipitation. *Cells were stimulated with Flag TNF, thus forming a receptor-complex at the cell membrane. Flag antibody was used to isolate the complex (proteins). Magnetic beads were added to the samples to separate the complex. Lastly, eluates were resolved using NuPAGE gels.*

Mass spectrometric analysis

For Mass Spectrometry samples were prepared as stated above in addition to

N-ethylmalemide (NEM) to the lysis buffer to block free cysteine amino acid.

4. Results

4.1 Endogenous YB-1 expression is required for TNF-induced NF-κB Activation

Note: Results of this part of the thesis has been published in:

Shah A, Plaza-Sirvent C, Weinert S, et al. YB-1 Mediates TNF-Induced Pro-Survival Signaling by Regulating NF-κB Activation. Cancers (Basel). 2020;12(8):2188. Published 2020 Aug 5. doi:10.3390/cancers12082188.

To investigate the role of YB-1 within the TNFR signaling pathway, we isolated primary bone marrow-derived macrophages (BMDMs) from either wild type (WT) or *Ybx1^(ΔLysM)* knockout (KO) mice [184]. After 10 days in culture with granulocyte/macrophage-colony stimulating factor (GM-CSF), mature macrophages were harvested and stimulated with TNF [20 ng/mL]. Imaging flow cytometry was used to observe the nuclear translocation of NF-κB phospho-p65 (pp65) (Figure 5). Wild type cells showed the expected translocation of NF-κB into the nucleus after 60 min of TNF stimulation, whereas in KO cells, no NF-κB translocation was detected (Figure 5B). Western blot analysis of the BMDMs showed that the loss of YB-1 did not influence the expression of either TNFR1 or NF-κB p65, only its activation (Figure 6A). The data obtained in BMDMs were confirmed in human knockdown cells i.e., THP-1 and U937 (Figure 5C, D). To validate this observation in human cells, we suppressed YB-1 expression in two

monocytic cell lines using specific lentiviral short-hairpin RNA (shRNA), as previously

published [186]. Western blot analysis showed that in the absence of YB-1, both p65

and TNF receptor 1 expression remained unchanged in both cell types (Figure 6C).

This shows that although YB-1 is a transcriptional and translational regulator, it does

not influence the expression of p65 and TNFR1. Quantification of knockdown (KD)

cells showed that a ~75% reduction in YB-1 expression is achieved compared to

control cells (Figure 6D). Following TNF stimulation, knockdown cells also showed a

compromised phosphorylation of the NF-κB inhibitor IκBα.
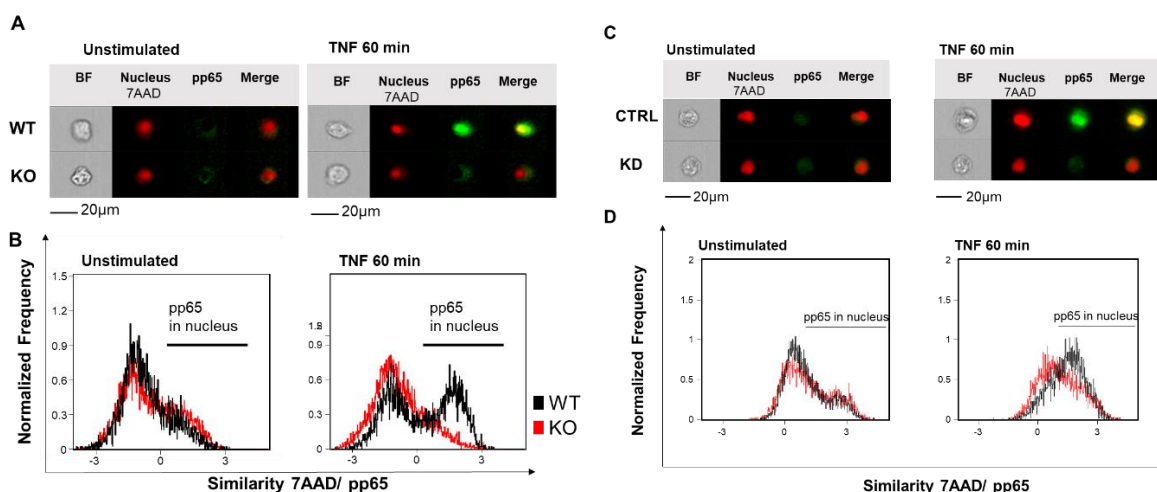
Figure 5. YB-1 is required for TNF-induced NF-κB nuclear translocation in bone marrow-derived macrophages (BMDMs) and THP-1 cells. *(A), (C) Imaging flow cytometry showing one representative cell. The nucleus stains positive for 7AAD (red). Staining for NF-κB p65 activation (pp65) is shown with and without stimulation (green) in BMDMs. Colocalization (yellow) indicates nuclear translocation. (B), Normalized frequency of WT (black) and YB-1 KO (Ybx1^{ΔLysM}) cells (red) and (D) CTRL (black) and KD (red) THP-1 cells showing nuclear translocation of activated NF-κB p65 with and without TNF stimulation [20 ng/ml]. Figure taken from published manuscript [130].*

As a result, the TNF-induced phosphorylation and activation of NF-κB p65 was

significantly reduced in knockdown (KD) cells compared to control cells. The protein

kinase upstream of IκBα, i.e., IKKα/β, also showed compromised phosphorylation. Thus, we hypothesized that the recruitment of components to the receptor complex may be affected. Therefore, we investigated the expression of the TRAF2 protein (Figure 6C), since it is important for the activation of NF-κB upon TNF stimulation [110, 187]. Unstimulated cells showed unaltered TRAF2 expression. However, following TNF stimulation of YB-1 KD cells, we observed a significant reduction of TRAF2 protein (Figure 6E). This observation supports our hypothesis and points towards a defect in TNF receptor complex formation.
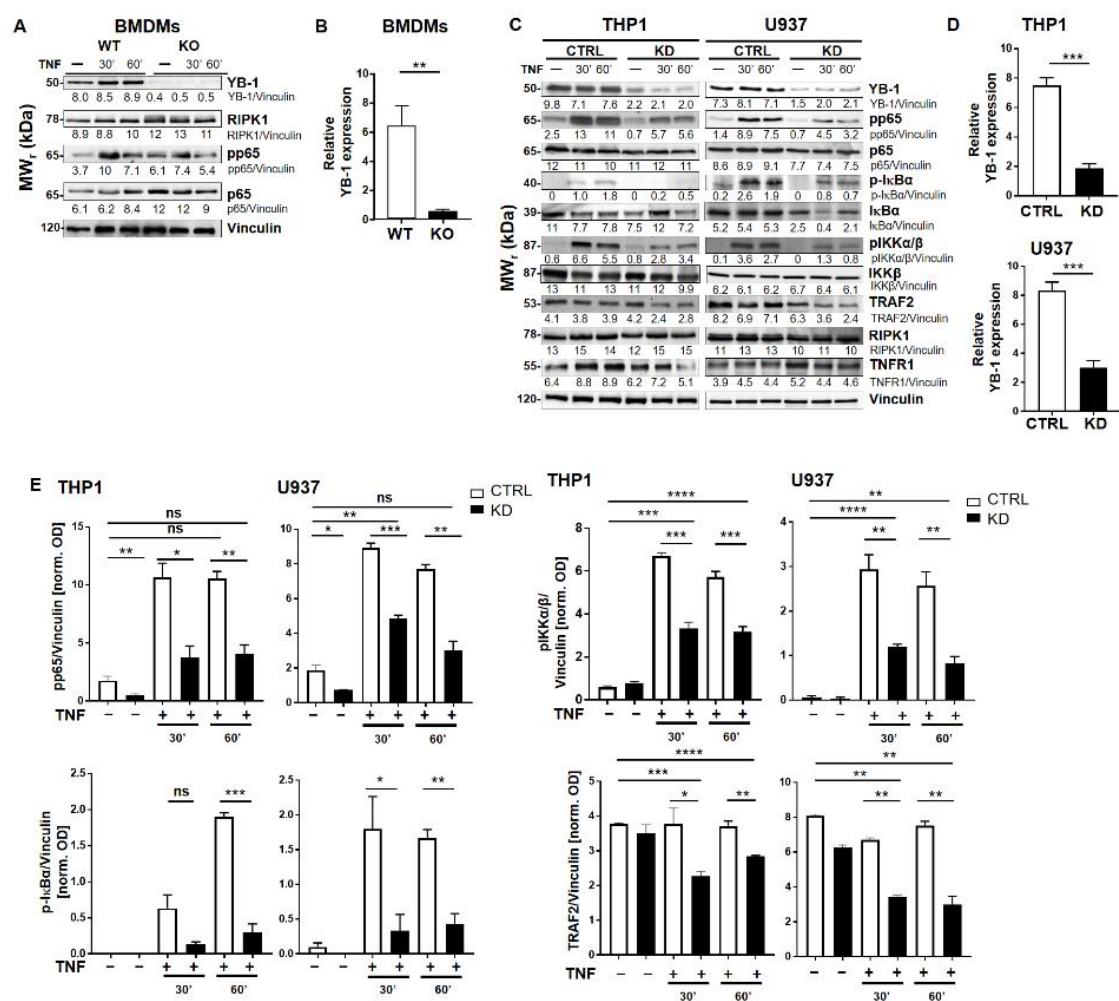
Figure 6. YB-1 is required for TNF-induced NF-κB activation. *(A) Bone-marrow derived macrophages (BMDM, WT and YB-1 KO) were stimulated with TNF [20 ng/ml] for the indicate time periods to activate the NF-κB signaling pathway. Protein expression was analyzed by immunoblotting using the indicated antibodies. Vinculin was used as the loading control. The relative band intensities are indicated. (B) Relative YB-1 expression is shown for three independent experiments. WT (white) and KO (black bars). (C) Control and YB-1 knockdown (KD) cells (THP-1 and U937) were stimulated with TNF [20 ng/ml] to activate TNFR1. Proteins were analyzed by immunoblotting for expression of the indicated proteins. Vinculin was used as loading control. Control (CTRL; white) and knockdown (KD; black bars). (D) Relative YB-1 expression in KD cells is shown for three independent experiments. (E) Relative pp65, p-IκBα, pIKKα/β, and TRAF2 expression in WT and KD THP-1 and U937 cells are shown for three independent experiments. Control (CTRL; white) and knockdown (KD; black bars). Error bars specify the standard error of the mean (SEM). Statistical significance was calculated using an unpaired t-test, n=3. Data represent the mean ±SEM. *p<0.05, **p<0.01, ***p ≤ 0.001. Figure taken from published manuscript [130].*

4.2 MG132 stabilizes the expression of TRAF2

The YB-1 KD cells showed that upon TNF stimulation, expression of TRAF2 protein is

significantly reduced compared to the control cells (Figure 6C). To investigate the

degradation pathway of TRAF2, THP-1 cells were treated with MG132 (a potent

proteasome inhibitor) alone or combined with TNF. Expression of TRAF2 protein upon

TNF stimulation in YB-1 KD cells was successfully rescued with MG132. This clearly

shows that the degradation of TRAF2 in the absence of YB-1 is carried out by ubiquitin

proteasome pathway. YB-1 certainly stabilizes the expression of TRAF2 in THP-1 cells

upon TNF stimulation.
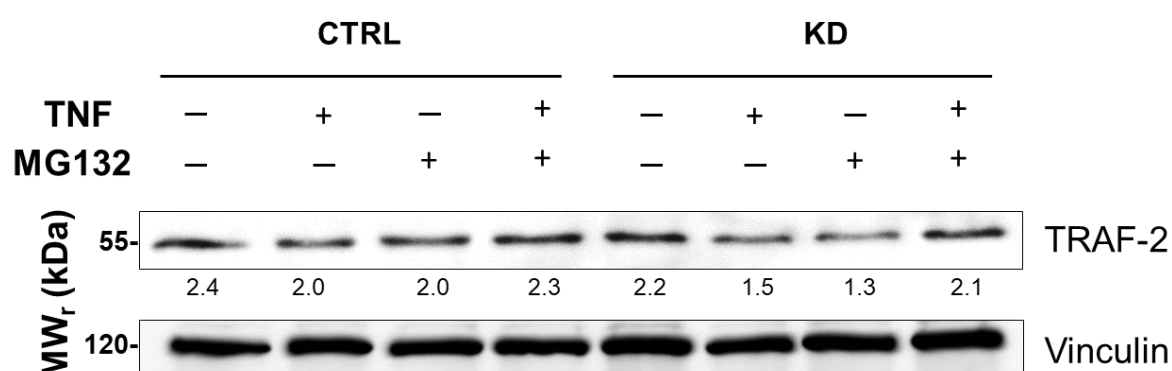
Figure 7. MG132 stabilizes the expression of TRAF2. *THP-1 cells (Control and KD) were treated with TNF [20 ng/ml] or MG132 [20 µM] and combined for 60 mins. TRAF2 expression was analyzed by immunoblotting. Vinculin was used as loading control.*

4.3 In the absence of YB-1, TNFR activation induces apoptosis

Frequently, NF-κB signals for survival, and therefore inactivation of NF-κB leads to enhanced apoptotic cell death [111]. Taking into consideration that TNF-induced NF-κB activation is defective in YB-1-deficient cells; we hypothesized that the loss of YB-1 might sensitize cells to TNFR-induced apoptosis. To investigate this, the monocytic cell lines (THP-1 and U937) were left untreated, treated overnight with either TNF or cycloheximide (CHX) alone, or combined. CHX inhibits protein synthesis, in particular the pro-survival gene product c-FLIP [14]. Cells were then analyzed by flow cytometry using annexin V and 7-amino-actinomycin D (7AAD) staining to distinguish apoptotic and/or necrotic cells (Figure 8A, B). Compared to WT, untreated YB-1-deficient cells appeared to show an increased basal rate of cell death.
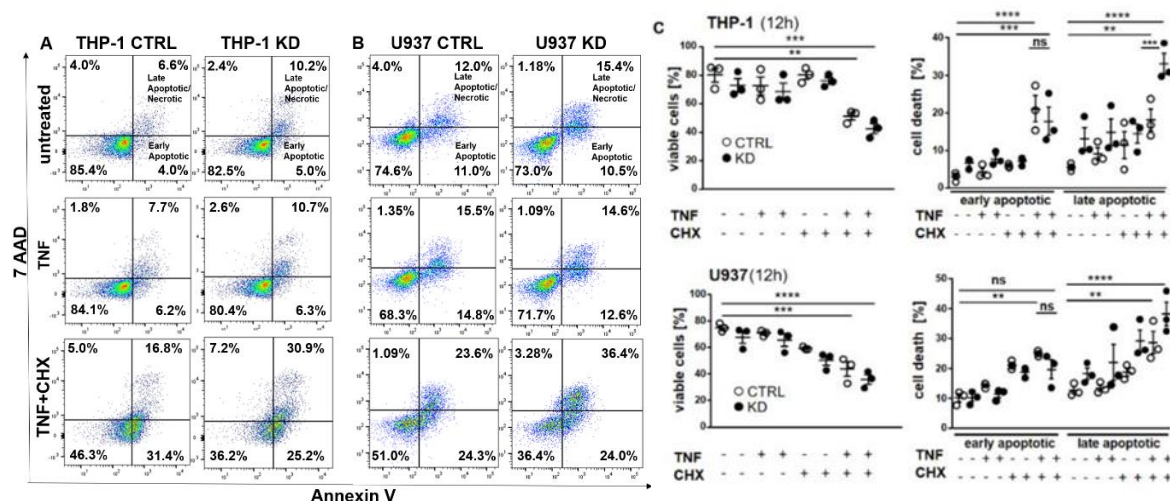

Figure 8. YB-1 is protective against cell death in vitro. *(A-B) Representative flow cytometry dot plot analyses of THP-1 (A) and U937 cells (B). Cells were treated with or without TNF [20 ng/ml] and cycloheximide [10 ng/ml] alone or together overnight. Cells were harvested and stained for Annexin V and 7AAD. (C) Graphs representing flow cytometry analysis of live and dead cells in THP-1 and U937 cells. Statistical significance was calculated by two-way ANOVA with Bonferroni post-hoc test, n=3. Data represents the mean ±SEM. **p<0.01, ***p ≤ 0.001, ****p ≤ 0.0001. Figure taken from published manuscript [130].*

The population of early apoptotic cells that were annexin-positive and 7 AAD-negative remained unaltered. TNF or CHX alone did not induce cell death in either the presence or absence of YB-1. However, we observed an ~50% increase in late apoptotic/necrotic cells upon combined treatment. The percentage of late apoptotic cells was higher in KD compared to control cells. Visual observation of the cells (Figure 9A) supported the flow cytometry results, suggesting that YB-1 deficient cells are more susceptible to cell death.
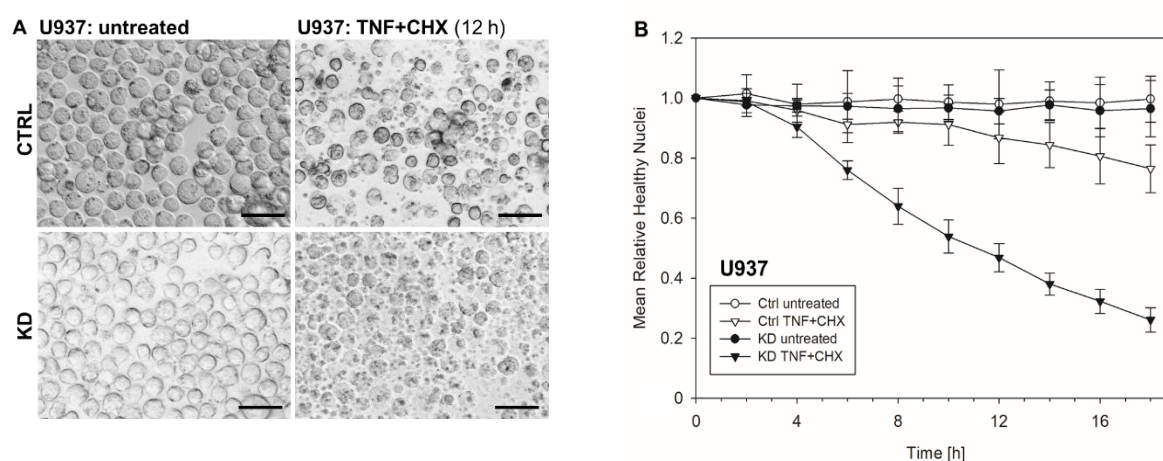
Figure 9. Enhanced apoptosis upon TNF+CHX stimulation of YB-1-deificient cells.
(A)The graph showing the normalized number of healthy nuclei per field of view over indicated period of time. The total number of healthy nuclei decreases after 4 h of TNF+CHX treatment. (B) Representative images show increased cell death after 12 h of TNF+CHX treatment in CTRL and YB-1 KD cells. Scale bar 100 µm. Figure taken from published manuscript [130].

To confirm this, we performed time lapse microscopy (Figure 9B), which showed that YB-1 deficient cells died faster than control cells upon TNF+CHX-treatment. In addition, representative single images show enhanced PI-positive knockdown cells

treated with TNF+CHX (Figure 10). Since we observed enhanced cell death, we next investigated whether we could also detect enhanced caspase activation. As, enhanced caspase activation leads to apoptosis.

Effector caspase-3/7 are activated by initiator caspases, such as caspase-8, and cleave several different target proteins that play an important role in mediating apoptotic cell death. Activation of caspase-3/7 was enhanced in the YB-1 KD cells (Figure 11A, B).
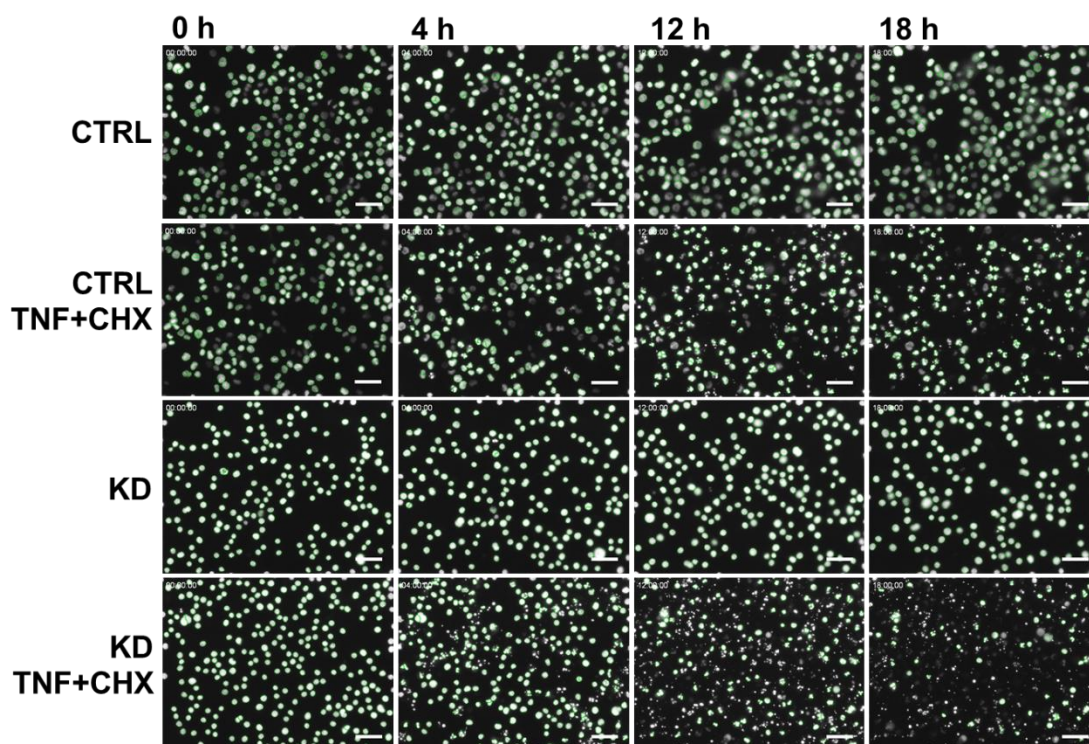

Figure 10. Representative single images each selected from fields of view from the time lapse microscopy. *The knockdown cells showing significant cell death compared to the control cells only after treatment. The similar trend is also reflected in the reduced number of tiny DNA particles and their lower distribution over the fields of view at the end of the 18 h time-lapse microscopy. Scale bar 50 µm. Figure taken from published manuscript [130].*
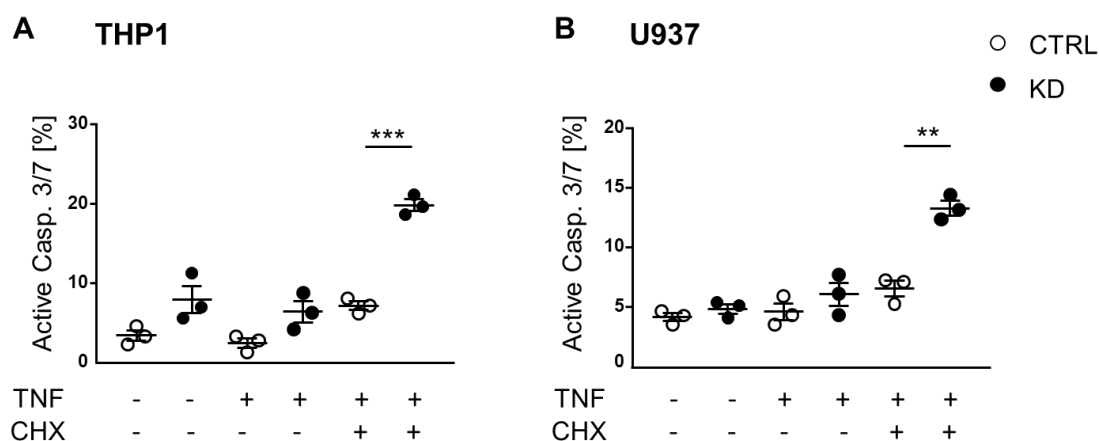
A THP1

B U937

○ CTRL
● KD

***Figure 11. Depletion of YB-1 leads to effector caspase activation**. (A-B) Flow cytometry analyses of caspase 3/7 activation in THP-1 (A) and U937 cells (B). Cells were treated with or without TNF [20 ng/ml] alone or together with cycloheximide [10 ng/ml] for 4h. Cells were harvested and stained for caspase 3/7 activation. Error bars specify the standard error mean (SEM) of at least three experiments. Statistical significance was calculated by unpaired t-test, n=3. Data represents the mean ±SEM. **p<0.01, ***p ≤ 0.001. Figure taken from published manuscript [130].*

BMDMs from WT and YB-1 KO mice were taken and confirmed the KO by western blotting (Figure 12A). A similar effect was observed in BMDMs upon TNF+CHX treatment for 6 h; alone TNF did not show any such effect on the cells (Figure 12B). We observed a significant difference in the KO cells upon TNF+CHX treatment compared to the WT cells (Figure 12C). Taken together, we observed a dramatic upregulation of cell death upon sensitization with CHX, which was augmented by the loss of YB-1.
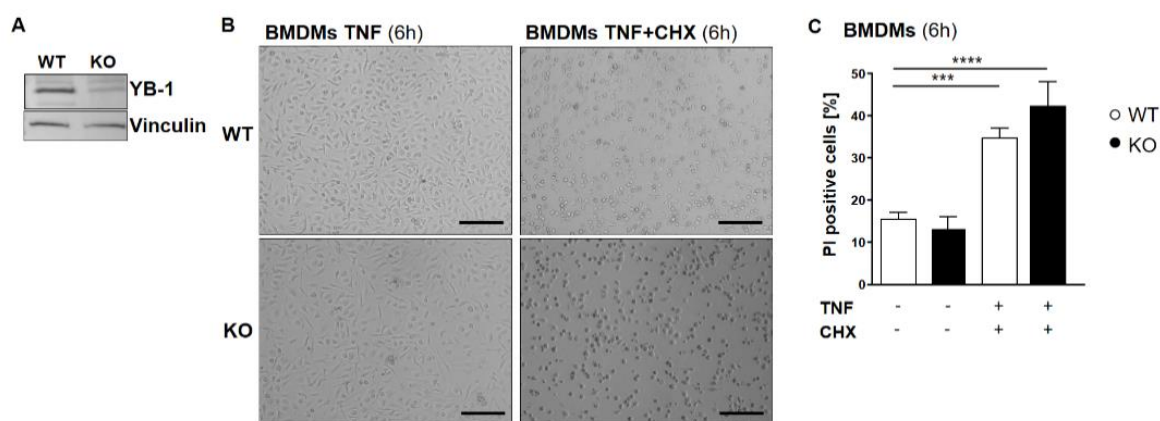
Figure 12. Enhanced apoptosis upon TNF+CHX stimulation of YB-1-deficient cells. *(A) Western blot analysis of YB-1 expression. (B) Representative single images of the BMDM cultures. (C) The graph shows enhanced cell death in murine BMDMs treated with TNF [20 ng/ml] and cycloheximide [CHX; 10 ng/ml] for 6h. Statistical significance were calculated by by one-way ANOVA with Bonferroni post-hoc test, n=3. Data represents the mean ±SEM. ***$p \leq 0.001$, ****$p \leq 0.0001$.*

## 4.4 YB-1-deficient cells die largely via apoptosis

Apoptosis is the caspase-mediated form of cell death. The extrinsic apoptotic pathway

is triggered by the binding of a ligand to its cell death-inducing surface receptors, e.g.,

TNF to TNFR1. However, TNFR1 can also initiate necroptosis. To distinguish whether

cells are dying from apoptosis or necroptosis, we pre-treated the cells with either the

pan-caspase inhibitor Z-Val-Ala-Asp-fluoromethyl ketone (Z-VAD) or the RIPK1

inhibitor necrostatin 1 (Nec-1) or their combination, and then stimulated the cells with

TNF + CHX to induce cell death. As shown in Figure 8, TNF + CHX treatment

dramatically induced cell death. Pre-treatment with zVAD completely rescued both

control and KD cells from dying (Figure 13 A), whereas cells treated with Nec-1 were

still prone to cell death. Co-treated cells (zVAD+Nec-1) were also successfully rescued

from dying (Figure 13 A). Western blotting confirmed the activation of caspase-8 and

-3, as well as the efficacy of the inhibitor (Figure 14), thus indicating that in the absence

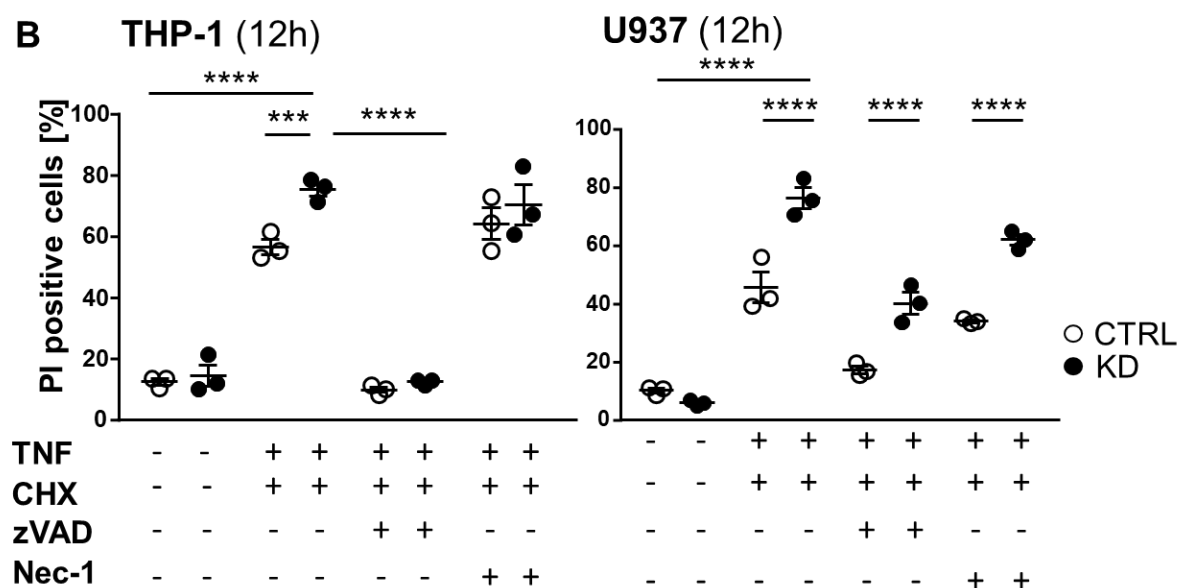of YB-1, THP-1 cells died via apoptosis and not necroptosis (Figure 13A).

A
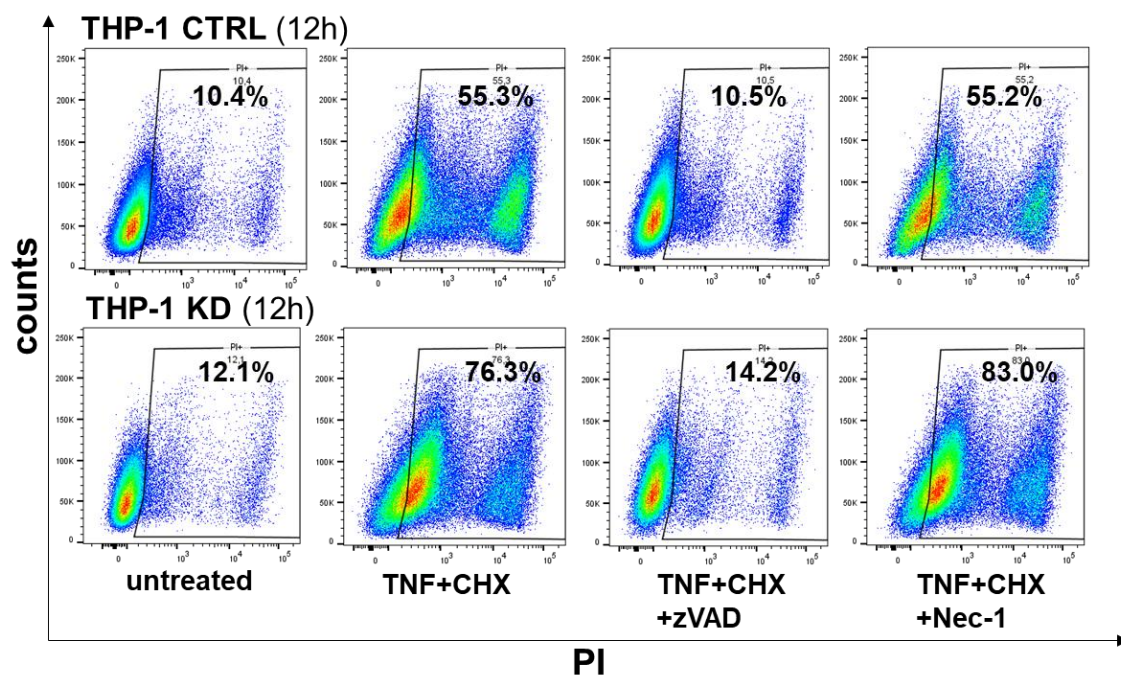
B

Figure 13. Cell death was rescued using the pan-caspase inhibitor zVAD. (A) THP-1 cells were pretreated with zVAD and/or Nec1 for 1h and treated with TNF and CHX overnight. The cells were harvested, stained with PI and analyzed by flow cytometry. The percentage of dead cells following treatment is indicated. (B-C) Graphs representing flow cytometry analysis of THP-1 (B) and U937 (C). Error bars specify the standard error mean (SEM) of at least three experiments. Statistical significance was calculated by two-way ANOVA with Bonferroni post-hoc test, n=3. Data represents the mean ±SEM. ***p ≤ 0.001, ****p ≤ 0.0001. Figure taken from published manuscript [130].

Dot plots show a significant difference upon TNF + CHX treatment in control and KD cells (Figure 13B). Surprisingly, the effect of the inhibitors was somewhat different with U937 cells. Here, zVAD offered better protection against TNF + CHX-induced death than Nec-1 compared with control cells. Although the pattern was similar to THP-1 cells, YB-1-deficient U937 cells continued to show an enhanced propensity for cell death. This indicates that in U937 cells, YB-1 is involved in a different cell death pathway other than apoptosis and necroptosis.
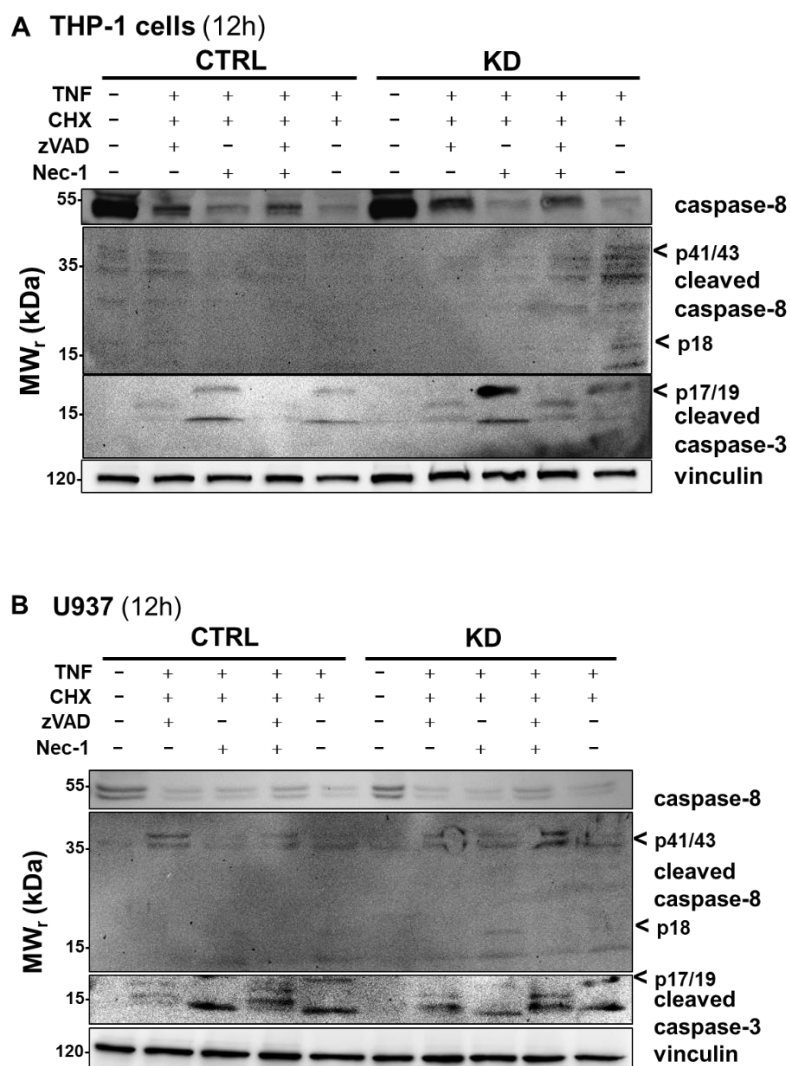
Figure 14. YB-1 knockdown enhances caspase activation. *(A-B) Western blot analyses of caspase activation in THP-1 cells (A) and U937 cells (B). Cells were pre-treated with zVAD and/or Nec1 for 1 h and treated with TNF (20 ng/ml) and CHX (10 ng/ml) for 12 h. Figure taken from published manuscript [130].*

The cellular Inhibitor of Apoptosis Proteins (cIAPs) play a key role in preventing the induction of cell death by inducing constitutive RIPK1 ubiquitination [188]. Normally, this would activate the classical pathway of NF-κB and MAP kinase [189]. Subsequently, the expression of anti-apoptotic proteins, such as FLIPL is upregulated that inhibits caspase-8 and -10 activation. Following TNF stimulation of YB-1

knockdown cells, we observed a drastic reduction in TRAF2 expression (Figure 6).

Since cIAP1 and cIAP2, both interact directly with TRAF2 [188], we suspected that

cIAPs might be dysregulated in terms of ubiquitination that results in cell death

induction. Moreover, both the cIAPs regulate TNF-induced NF-κB signaling that

modulates also their anti-apoptotic activity [190]. To test the hypothesis, U937 cells

harboring YB-1 knockdown were stimulated with TNF in the absence or presence of

BV6; an IAP antagonist that induces their proteasomal degradation, thereby

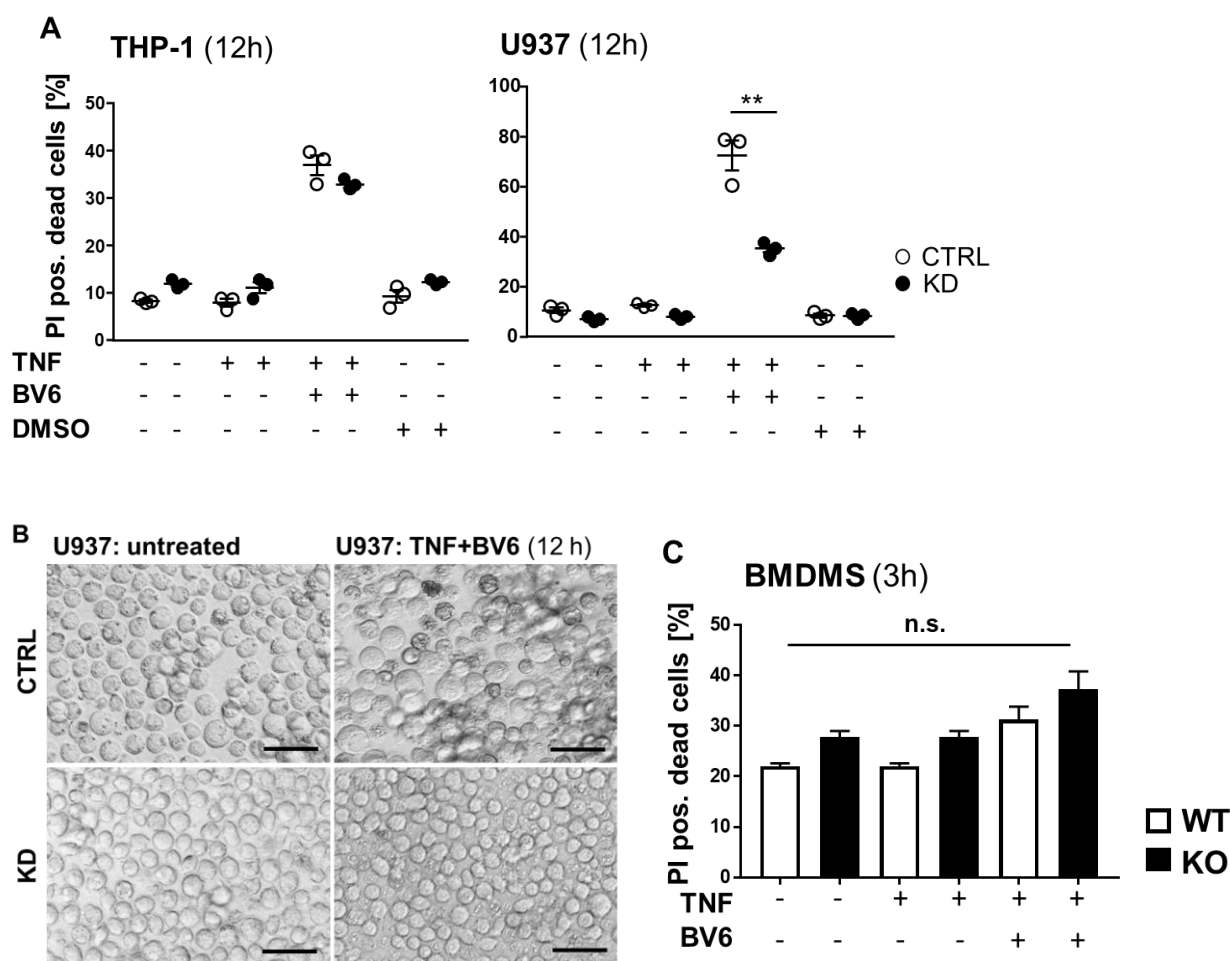sensitizing the cells to TNFα-dependent cell death [191].

Figure 15. Loss of YB-1 protects cells from BV6 and TNF-induced cell death. *(A) THP-1 cells (left) and U937 (right) were pretreated with BV6 for 1h, and treated with TNF overnight. (B) Representative images show increased cell death after 12 h of TNF+BV6 treatment in CTRL cells whereas in the KD cells, they were protected against the cell death. Scale bar 100 μm. (C) BMDMs were also treated with BV6 for 1h, and treated with TNF for 3 hours. The cells were harvested, stained with PI and analyzed by flow cytometry. The percentage of dead cells following treatment is indicated. Error bars specify the standard error mean (SEM) of at least three experiments. Statistical significance was calculated by two-way ANOVA with Bonferroni post-hoc test, n=3. Data represents the mean ±SEM. ***p ≤ 0.001, ****p ≤ 0.0001.*

BV6 would antagonize IAP (Inhibitor of apoptosis protein) i.e. cIAP1/2 interactions with

inititator caspases and thereby triggering their degradation. Treating the WT/control

cells with BV6 would gradually lead to enhanced cell death by degrading cIAP1/2. TNF

alone is not able to induce cell death. Therefore, pre-incubation with BV6 for 60 mins,

followed by TNF stimulation overnight, clearly sensitizes the control cells for TNF-induced cell death, whereas YB-1 deficient U937 cells are protected (Figure 15). This might a cell specific effect which we did not observe in the THP-1 cells. This suggests that in YB-1 deficient U937 cells, the recruitment of cIAPs to the receptor complex and/or their post-translational modification, i.e. ubiquitination after TNF stimulation, is perturbed.

4.5 The pattern of ubiquitination is altered in the absence of YB-1

Ubiquitin is a regulatory protein that targets proteins for degradation via the proteasome, alters their intracellular localization, influences their activity, and promotes or prevents protein-protein interactions. In TNF receptor signaling, ubiquitin plays a central role in regulating cell death and survival. The ability of YB-1 to act as a switch that regulates cell fate decisions points towards an influence on the ubiquitination status. Changes in the ubiquitination status involve the recruitment of E3 ligases and/or deubiquitinating enzymes (DUBs), which add or remove mono- and poly-ubiquitin chains from various components within the complex. To look for changes in ubiquitination, U937 cells were stimulated with TNF alone and in combination with MG132; a potent proteasomal inhibitor (Figure 16). Compared to resting control cells, there is an overall reduction of the ubiquitination status in the YB-1 KD cells. In addition, the labelled high molecular weight bands, which are clearly expressed in control cells, are absent in the KD cells. Upon stimulation with

TNF+MG132 for 60 mins, we observed reduced mono- and poly-ubiquitin-conjugated proteins in knockdown cells compared to control. Surprisingly, the distinct Ub bands at 50 kDa where we expect YB-1 are absent in KD, suggesting that YB-1 might be ubiquitinated. Depleting YB-1 affects the overall ubiquitination of the cells. Reduced ubiquitination upon TNF stimulation (Figure 16) will have a profound effect on complex I formation, disruption of which promotes the formation of complex II leading to the activation of cell death.
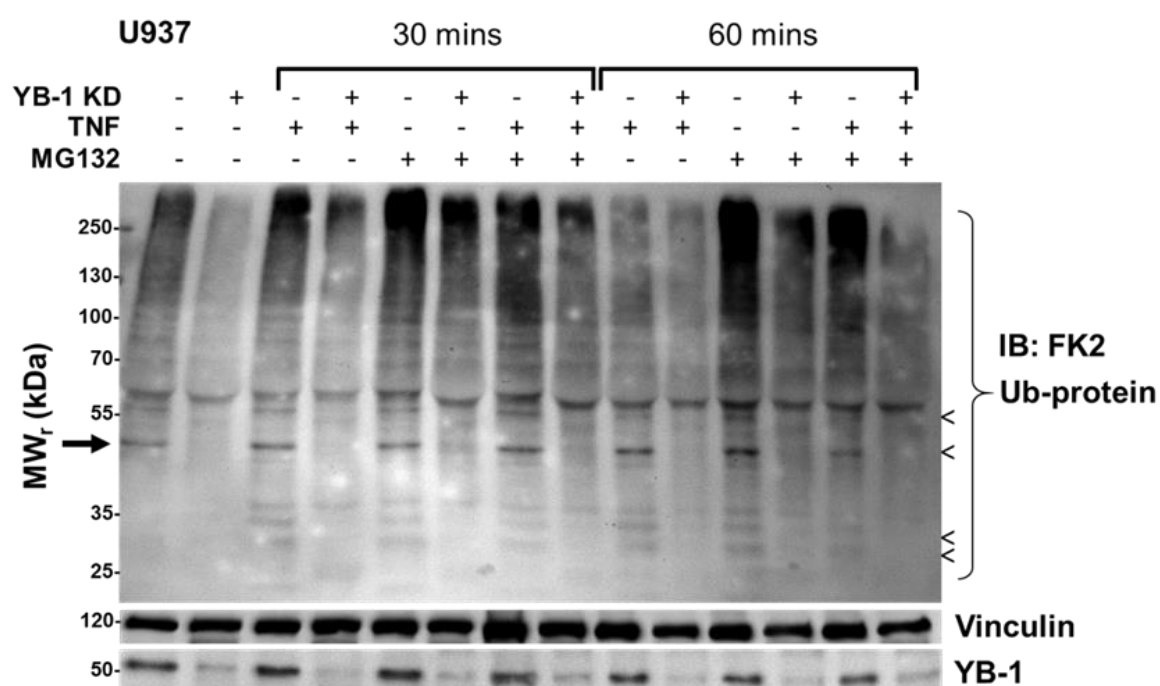

Figure 16. In U937 cells, ubiquitinated proteins are less abundant in the absence of YB-1. *U937 cells were stimulated with and without TNF and MG132 alone and both for 30 and 60 mins. After cell lysis, proteins were blotted and ubiquitinated proteins were anaylzed by FK2 antibody. Vinculin was used as loading control and YB-1 knockdown was confirmed.*

4. RESULTS

4.6 YB-1 is an integral part of TNFR signaling complex

To determine whether YB-1 is a component of the TNFR complex I, we isolated TNFR

signaling complexes (TNFR-SC) from monocytes using ligand affinity purification

[192]. The complexes were eluted from the affinity resin by peptide competition and

the resulting eluates resolved using gel electrophoresis (Figure 17A), transferred to

the membranes, and analyzed by western blotting (Figure 17B). This clearly indicate

the presence of YB-1 within the TNFR-SC. Analysis of the isolated receptor complexes

by mass spectrometry confirmed the presence of YB-1 (Figure 17B, panel 2). DbpA,

homologue of YB-1 was also detected within the TNFR-SC (Figure 17C, panel 3).
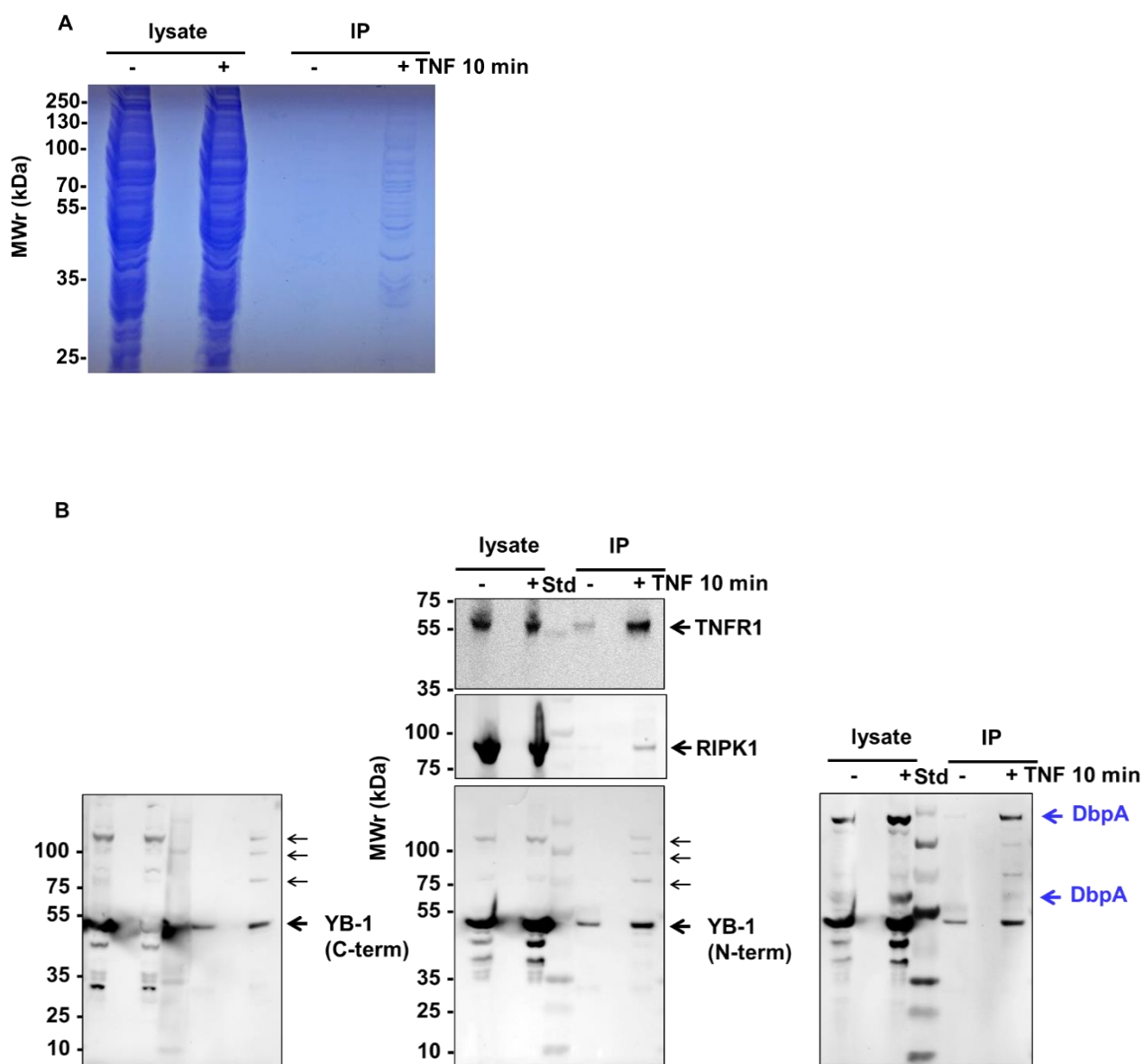
Figure 17. YB-1 is an integral part of the TNFR signaling complex. *(A) Lysates representing 10 million cells of unstimulated and 10 min TNF-stimulated cells were prepared and loaded onto SDS-PAGE. The complexes isolated by immunoaffinity purification (IP) are shown for unstimulated (-) and stimulated (+) cells. (B) The molecular weight standards (Std) were loaded between the lysates and isolated complexes (IP) from unstimulated (-) and stimulated (+) samples. The blots were probed for different antibodies as indicated. The YB-1 blot was reprobed for DbpA. Two unique bands were detected, indicated in blue.*

In addition, cells transduced with shRNA constructs (YB-1 KD) were also used to

isolate TNFR-SC. A comparison of the TNFR-SC from control versus KD cells enabled

us to determine which proteins assemble independently of YB-1 (present in the TNFR

complexes isolated from KD cells) from those factors that depend upon YB-1

expression for their recruitment (i.e., those which are absent in KO/KD cells).

Evidently, critical proteins in TNFR-SC I, RIPK1 and TRAF2 were not recruited to the

receptor complex. Expression of TRAF2 was reduced upon TNF stimulation in the

control cells, which was completely absent in the YB-1 KD cells. RIPK1 seemed to be

strongly destabilized in the absence of YB-1 (Figure 18). This could be due to active

caspase-8 which cleaves RIPK1 yielding a similar band pattern (smears). The results

point to an important role for YB-1 in TNFR signaling cascade. To investigate the

complex further, we perfromed mass spectrometric analysis with the samples in

collaboration with Prof. Ole N. Jensen, University of Southern Denmark. Typically, 463

peptides were identified from the known protein database (SwissProt). The number of

identified peptides is summarized in Table 4. Bioinformatic analysis was carried out

with the publically available STRING database (https://string-db.org/) of known and

predicted interactions, which includes both direct physical and indirect functional

protein associations. Results are elaborated as an interaction map (Figure 19), several

suspected ubiquitin binding proteins were found to be interacting with YB-1. Apart from

proteins regulating ubiquitination, proteins functions attributed to pre- and mRNA

binding, RNA binding, the mTOR complex, the endoplasmic reticulum and golgi

compartments were found interacting with YB-1 (Figure 19). Additionally, a heat map

was generated with the help of the Spearman correlation to indicate the top 50 significant proteins. Obviously we detected YBX1, which was clearly downregulated in knockdown samples (columns in blue) compared to control samples (columns in red) (Figure 20). Interestingly proteins such as UBA6 (Ubiquitin Like Activating Enzyme 6), TRAP1 (TNF Receptor Associated Protein 1), and NEDD4 (Neural Precursor Cell Expressed Developmentally Down-Regulated Protein 4); an E3 ubiquitin ligase, were downregulated in the absence of YB-1 (Figure 20). This points a potential role for YB-1 in regulating ubiquitination within complex I.
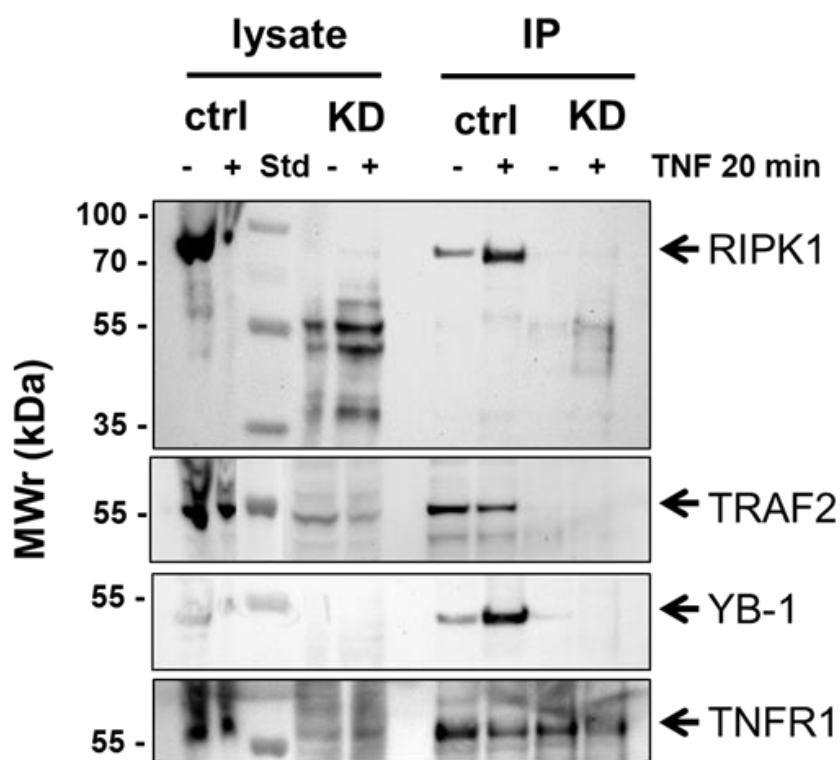

Figure 18. Comparison of the TNFR-SC from control versus KD cells. *U937 cells were treated with flag TNF for 20 mins and TNFR-SC were isolated as indicated above. Membranes were blotted for RIPK1, TNFR1, TRAF2 and YB-1 proteins. The molecular weight standard (Std.) is indicated.*
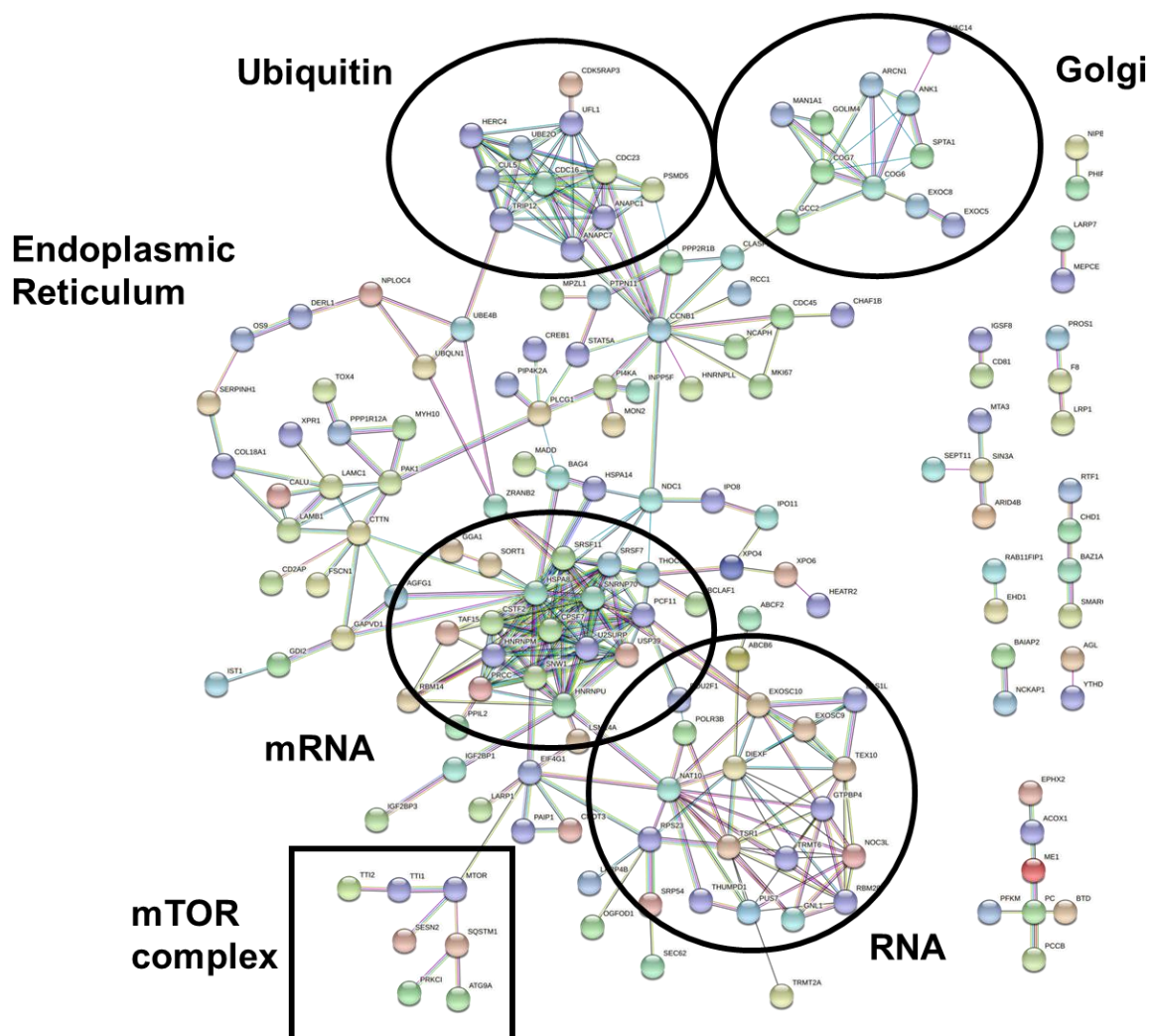
Figure 19. String analysis of control vs. YB-1 KD cells. *String analysis of the YB-1 interaction in U937 cells. Proteins were sorted with their functional attribution: ubiquitination, pre-mRNA and mRNA splicing, RNA binding, mTOR complex, endoplasmic reticulum and independent of the interaction were golgi proteins within the group of cells. Generated using STRING version 11.0.*

Overall results from PD search of the human SwissProt database (all samples)	
Peptides (ion score ≥ 15)	463
Phospho-peptides (ion score ≥ 15)	455 (so 98.3% phospho-peptides)
Peptide isoforms (ion score ≥ 15)	569
Phospho-peptide isoforms (ion score ≥ 15)	561
Peptides (ion score ≥ 25)	294
Protein groups	269

Table 4. Phosphopeptide analysis of U937 cells.

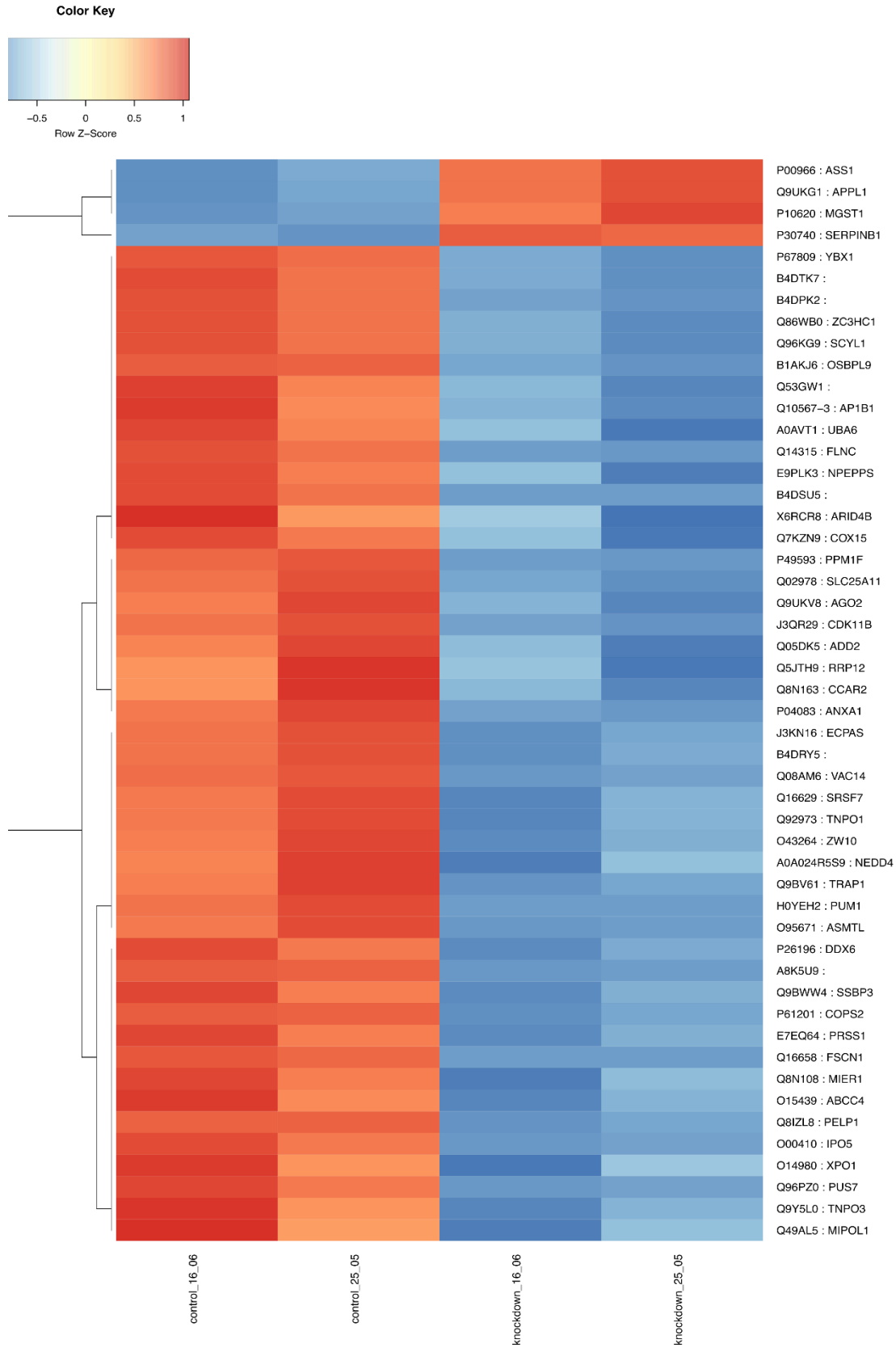**Figure 20. Heat map of top 50 significant proteins with log2 Fold Change (FC).** *Here are listed top 50 proteins with filtering criteria: p-value <= 0.05 and log2 FC >= 1.5. The clustering is based on the Spearman correlation analysis.*

5. Discussion

This part of thesis has been published in:

Shah A, Plaza-Sirvent C, Weinert S, et al. YB-1 Mediates TNF-Induced Pro-Survival Signaling by Regulating NF-κB Activation. Cancers (Basel). 2020;12(8):2188. Published 2020 Aug 5. doi:10.3390/cancers12082188.

Shah A, Lindquist JA, Rosendahl L, Schmitz I, Mertens PR. Novel Insights into YB-1 Signaling and Cell Death Decisions. Cancers (Basel). 2021;13(13):3306. Published 2021 Jul 1. doi:10.3390/cancers13133306.

YB-1 plays a central role in promoting cell survival by regulating NF-κB activation. This points to an important role of YB-1 as a switch between cell survival and death signaling. YB-1 has been shown to be both phosphorylated and ubiquitinated in response to TNF-stimulation [60]. Here our observation is supported by previous studies that have identified YB-1 as an essential element for IL-1R-induced NF-κB activation. In the absence of YB-1, TNF-stimulated monocytes and macrophages are more prone to undergo apoptosis. A recent study from Florian Heidel's group has demonstrated the importance of YB-1 in regulating mRNA splicing events in myeloproliferative neoplasia (MNP), which contribute to cell fate decisions [193]. Similar observations regarding the role of YB-1 in cell survival have been made for T cells [132]. In addition, we also observed a TNF-induced destabilization of TRAF2 upon YB-1 suppression [130]. Destabilization of TRAF2 in the absence of YB-1 resulted in defective NF-κB activation and hence promoting apoptosis.

5. DISCUSSION

TRAF2 is a common signaling component of both TNFR1 and TNFR2 that plays an important role in regulating canonical versus non-canonical NF-κB activation, as well as the balance between survival and death signaling [139]. Thus, recruitment of TRAF2 at the receptor-membrane would amplify the canonical NF-κB pathway (p50/p65). The phosphorylation of TRAF2 by protein kinase C regulates its ability to recruit and activate IKKα, which in turn phosphorylates IκBα, targeting it for degradation and thereby activating NF-κB (p50/p65). Recent reports indicates that TRAF2 mediates the recruitment of ubiquitin ligases to the TNFR1 complex I thereby promoting NF-κB activation [141]. Also, we have successfully showed that YB-1 is critical in regulating the stability of TRAF2 (Figure 5). Moreover, YB-1 has been shown to directly interact with p65 (RelA) and act as a transcriptional co-activator [142]. Numerous kinases (AKT, RSK, PKC, ERK, CKII) have been identified that phosphorylate YB-1 within its cold shock domain, thereby inducing nuclear translocation [143]. Thus, it is foreseeable that the TNF receptor oligomerization induced by ligand binding results in the recruitment and oligomerization of adaptor protein such as TRAF2, which in turn recruit kinases and ubiquitin ligases that activate both NF-κB and its *trans*-activator YB-1. However, the mechanism by which YB-1 influences TRAF2 stability is unclear.

Ubiquitination is an important post-translational modification that regulates protein stability and the activity of signaling cascades. Multiple sites of ubiquitination have

been identified within YB-1 [182]. Fascinatingly, the type of ubiquitin linkage determines the fate of the protein. For instance, the K63-linked polyubiquitinaton plays a vital role in stabilizing the receptor complex I, aiding the recruitment of the downstream adaptor proteins and activating kinases [194]. K-48 linked polyubiquitination is known to degrade the inhibitor of NF-κB (IκBα), thus releasing the NF-κB dimers into the nucleus. Met1-linked or linear polyubiquitinaton is a novel kind of ubiquitin modification in activating NF-κB pathway [195]. Interestingly, three sites within YB-1 i.e., K137, K170 and K264 were identified to be ubiquitinated upon TNF stimulation [60, 196]. Ubiquitination and deubiquitination events on RIPK1 are well known to be critical for determining cell fate. The expression of RIPK1 is comparable upon TNF stimulation in both WT and YB-1 KD cells (Figure 5). The observation that the expression of RIPK1 remained unaltered, also it was not recruited to the TNFR signaling complex I in the absence of YB-1 (Figure 20). In the absence of ubiquitination, RIPK1 dissociates from complex I and associates with FADD through binding its death domain [197]. FADD recruits pro-caspase-8 and or its catalytically inactive homolog to form complex II [198]. Therefore, we suspect that a dysregulated RIPK1 ubiquitination in our YB-1 deficient cells could explain the switch to complex II formation. However, whether this is due to changes in E3 ligase activity (cIAP and LUBAC) or deubiquitinating enzymes such as CYLD, tumor necrosis factor alpha-induced protein 3 (Tnfip3 or A20), and OUT deubiquitinase with linear linkage

specificity (OTULIN) is not clear. Hence, we suspect that an imbalance within the ubiquitination status caused by the loss of YB-1 led to the apoptotic phenotype observed.

HACE1 (HECT domain and ankryin repeat-containing E3 ligase 1) has been reported to interact with YB-1, but this involves the polyubiquitination of the YB-1 protein via K27-linked ubiquitin chains leading to protein secretion [45]. The HACE1 is a central gatekeeper for TNFR1-induced cell death. HACE1 is essential for the ubiquitination of TRAF2 and thereby the formation of the apoptotic complex [183]. In addition to HACE1, YB-1 also interacts with the deubiquitinase otubain-1 (OTUB1) [44]. OTUB1 is responsible for removing K48-linked ubiquitin chains from YB-1 and thus stabilizes YB-1 expression. Since OTUB1 also modulates the stability of cIAP1, it is a likely candidate that would explain the observed phenotype. Furthermore, RIPK1 not recruited to the complex 1 in the absence of YB-1 (Figure 18) destabilizes the signalosome complex I. We have demonstrated YB-1 within the TNFR-SC, which is supported by the data of Wagner et al., showing TNF-induced posttranslational modification of YB-1.

YB-1 is being an integral component of the TNFR signaling pathway, however its position within the network is unclear. Within YB-1, five sites of Ser phosphorylation were identified upon TNF stimulation, including Ser165 and Ser176. These sites are critical for NF-κB activation by the IL-1βR [61, 62]. However, how phosphorylation at

these sites contributes to NF-κB activation needs further investigation. Recently, role of methylated YB-1 has been identified in colorectal cancer [63]. Importantly, post-translational modification at these sites promotes tumor formation by differentially regulating the expression of different subgroups of NF-κB target genes.

Open questions

To date, there are no established therapies in clinical practice that specifically target YB-1 [193]. However, the genetic inactivation of YB-1 does not appear to perturb steady-state haematopoiesis and no functional impairment of hematopoietic stem and progenitor cells has been observed. Thus, YB-1 appears to be a safe target in cancer treatment. In cancer, higher YB-1 expression and nuclear localization are linked to its ability to induce multi-drug resistance and a poor prognosis [83, 199, 200]. Thus, drugs enforcing a cytosolic localization of YB-1 should be more effective at preventing YB-1's pro-survival signaling. Several inhibitors have been reported that influence the subcellular localization of YB-1 [201]. Molecules that induce nuclear translocation, e.g., HSc025, which disrupts YB-1's binding to poly(A)-binding protein (PABP), thereby disrupting its cytosolic retention; and molecules that promote cytoplasmic retention, e.g., fisetin, which binds to the cold-shock domain and prevents the phosphorylation of Ser102, which precludes nuclear translocation [131, 202, 203]. Recently, two new compounds have been identified. One is from black tea (theaflavin-3-gallate (TF2A)) that binds to a pocket within cold shock domain and appears to

stabilize YB-1. Xiao et al., have demonstrated that a long non-coding RNA, Hnscr was highly expressed in the hypothalamic neural stem/progenitor cell (htNSC) of young mice but not in aged mice. Knocking out Hnscr was enough to drive the senescence of htNSCs. Hnscr binds to YB-1 which prevents its degradation. The use of T2FA reduced the senescence of hypothalamic neural stem cells. This suggests that YB-1 might be a potential molecular target modulating htNSC senescence [204]. The other, SU056 was developed as a therapy for ovarian cancer [205]. Of note, determining the molecular mechanism underlying the YB-1-mediated NF-κB activation we described for TNFR1 signaling should aid in identifying new therapeutic targets for intervention in cancer. We predict that targeting YB-1 should sensitize cells to TNF-induced apoptosis, a potent strategy to attack cancer cells [83, 206, 207].

The recent findings from several studies provide novel insights into YB-1's functions, but also raise new questions. Concerning extracellular YB-1, how many post-translational modifications does it possess, which enzymes are responsible, and how do these modifications affect protein stability and receptor affinity? Could differences in post-translational modifications be responsible for the differences in the observed activities of extracellular YB-1 that have been reported? Do these modifications contribute to the development of autoantibodies against YB-1 [208-210], and what is the relationship between the presence of autoantibodies in serum and disease progression?

5. DISCUSSION

Numerous post-translational modifications are also reported for intracellular YB-1. Are specific modifications associated with distinct activities or subcellular compartments of YB-1? Here, extensive work is required to assign functions to the known modifications, and to define the mechanisms by which the known modifications exert their function. Do these modifications function independently of one another, or are they part of larger, as yet uncharted, regulatory networks? To date, only a handful of receptors are known that utilize YB-1 as a signaling component. Whether or not YB-1's role in signal transduction is independent of its mRNA splicing and translational regulatory activities is yet to be demonstrated, but clearly each of these activities alone can impact upon the life and death decisions of the cell.

Graphical summary
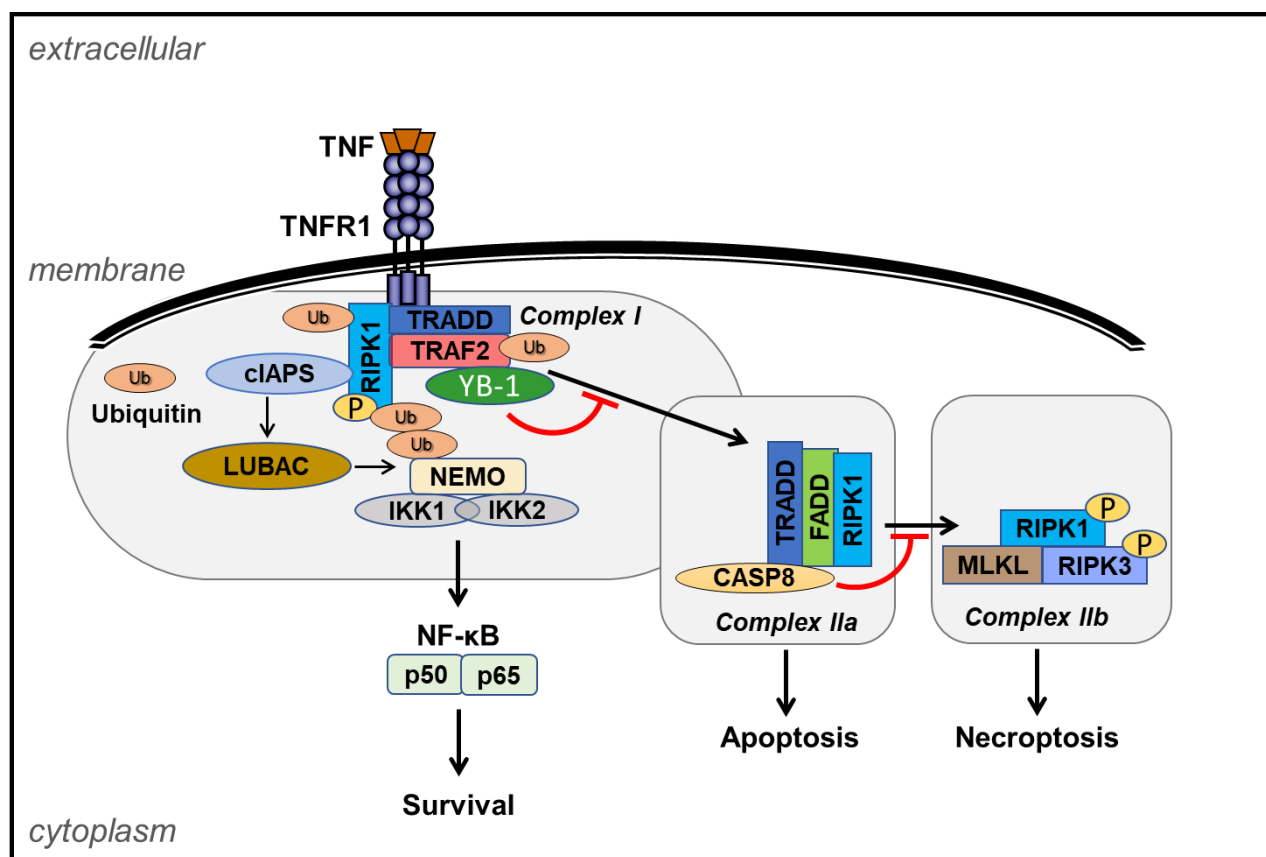
Figure 21. Graphical summary of the study. *Binding of TNF to TNFR1 induces the formation of complex I, which leads to the activation of NF-κB and the induction of pro-survival signaling. In the absence of YB-1, TNF binding results in the formation of complex IIa, which induces apoptotic cell death via caspase activation. Caspase inhibitor zVAD-fmk, rescues cells from TNF-induced cell death, whereas the RIPK1 inhibitor does not.*

References

1.   Eliseeva, I.A., et al., Y-box-binding protein 1 (YB-1) and its functions. Biochemistry (Mosc), 2011. 76(13): p. 1402-33.
2.   Wistow, G., Cold shock and DNA binding. Nature, 1990. 344(6269): p. 823-4.
3.   Manival, X., et al., RNA-binding strategies common to cold-shock domain- and RNA recognition motif-containing proteins. Nucleic Acids Res, 2001. 29(11): p. 2223-33.
4.   Horn, G., et al., Structure and function of bacterial cold shock proteins. Cell Mol Life Sci, 2007. 64(12): p. 1457-70.
5.   Landsman, D., RNP-1, an RNA-binding motif is conserved in the DNA-binding cold shock domain. Nucleic Acids Res, 1992. 20(11): p. 2861-4.
6.   Hasegawa, S.L., et al., DNA binding properties of YB-1 and dbpA: binding to double-stranded, single-stranded, and abasic site containing DNAs. Nucleic Acids Res, 1991. 19(18): p. 4915-20.
7.   Lenz, J., et al., Identification of a mammalian nuclear factor and human cDNA-encoded proteins that recognize DNA containing apurinic sites. Proc Natl Acad Sci U S A, 1990. 87(9): p. 3396-400.
8.   Gaudreault, I., D. Guay, and M. Lebel, YB-1 promotes strand separation in vitro of duplex DNA containing either mispaired bases or cisplatin modifications, exhibits endonucleolytic activities and binds several DNA repair proteins. Nucleic Acids Res, 2004. 32(1): p. 316-27.
9.   Ise, T., et al., Transcription factor Y-box binding protein 1 binds preferentially to cisplatin-modified DNA and interacts with proliferating cell nuclear antigen. Cancer Res, 1999. 59(2): p. 342-6.
10. Izumi, H., et al., Y box-binding protein-1 binds preferentially to single-stranded nucleic acids and exhibits 3'-->5' exonuclease activity. Nucleic Acids Res, 2001. 29(5): p. 1200-7.
11. Guay, D., et al., The human endonuclease III enzyme is a relevant target to potentiate cisplatin cytotoxicity in Y-box-binding protein-1 overexpressing tumor cells. Cancer Sci, 2008. 99(4): p. 762-9.
12. Bader, A.G. and P.K. Vogt, Inhibition of protein synthesis by Y box-binding protein 1 blocks oncogenic cell transformation. Mol Cell Biol, 2005. 25(6): p. 2095-106.
13. Dooley, S., et al., Y-box protein-1 is the crucial mediator of antifibrotic interferon-gamma effects. J Biol Chem, 2006. 281(3): p. 1784-95.
14. van Roeyen, C.R., et al., Cold shock Y-box protein-1 proteolysis autoregulates its transcriptional activities. Cell Commun Signal, 2013. 11: p. 63.
15. Ranjan, M., S.R. Tafuri, and A.P. Wolffe, Masking mRNA from translation in somatic cells. Genes Dev, 1993. 7(9): p. 1725-36.
16. Bouvet, P. and A.P. Wolffe, A role for transcription and FRGY2 in masking maternal mRNA within Xenopus oocytes. Cell, 1994. 77(6): p. 931-41.
17. Lyabin, D.N., et al., Interplay between Y-box-binding protein 1 (YB-1) and poly(A) binding protein (PABP) in specific regulation of YB-1 mRNA translation. RNA Biol, 2011. 8(5): p. 883-92.
18. Davydova, E.K., et al., Overexpression in COS cells of p50, the major core protein associated with mRNA, results in translation inhibition. Nucleic Acids Res, 1997. 25(14): p. 2911-6.
19. Matsumoto, K., F. Meric, and A.P. Wolffe, Translational repression dependent on the interaction of the Xenopus Y-box protein FRGY2 with mRNA. Role of the cold shock domain, tail domain, and selective RNA sequence recognition. J Biol Chem, 1996. 271(37): p. 22706-12.
20. Jurchott, K., et al., YB-1 as a cell cycle-regulated transcription factor facilitating cyclin A and cyclin B1 gene expression. J Biol Chem, 2003. 278(30): p. 27988-96.

21.	Das, S., et al., Stimulation of NEIL2-mediated oxidized base excision repair via YB-1 interaction during oxidative stress. J Biol Chem, 2007. 282(39): p. 28474-84.

22.	Fujita, T., et al., Increased nuclear localization of transcription factor Y-box binding protein 1 accompanied by up-regulation of P-glycoprotein in breast cancer pretreated with paclitaxel. Clin Cancer Res, 2005. 11(24 Pt 1): p. 8837-44.

23.	Koike, K., et al., Nuclear translocation of the Y-box binding protein by ultraviolet irradiation. FEBS Lett, 1997. 417(3): p. 390-4.

24.	Stein, U., et al., Hyperthermia-induced nuclear translocation of transcription factor YB-1 leads to enhanced expression of multidrug resistance-related ABC transporters. J Biol Chem, 2001. 276(30): p. 28562-9.

25.	Holm, P.S., et al., YB-1 relocates to the nucleus in adenovirus-infected cells and facilitates viral replication by inducing E2 gene expression through the E2 late promoter. J Biol Chem, 2002. 277(12): p. 10427-34.

26.	Basaki, Y., et al., Akt-dependent nuclear localization of Y-box-binding protein 1 in acquisition of malignant characteristics by human ovarian cancer cells. Oncogene, 2007. 26(19): p. 2736-46.

27.	Higashi, K., et al., Y-box-binding protein YB-1 mediates transcriptional repression of human alpha 2(I) collagen gene expression by interferon-gamma. J Biol Chem, 2003. 278(7): p. 5156-62.

28.	Higashi, K., et al., A novel small compound that promotes nuclear translocation of YB-1 ameliorates experimental hepatic fibrosis in mice. J Biol Chem, 2011. 286(6): p. 4485-92.

29.	Lu, Z.H., J.T. Books, and T.J. Ley, YB-1 is important for late-stage embryonic development, optimal cellular stress responses, and the prevention of premature senescence. Mol Cell Biol, 2005. 25(11): p. 4625-37.

30.	Uchiumi, T., et al., YB-1 is important for an early stage embryonic development: neural tube formation and cell proliferation. J Biol Chem, 2006. 281(52): p. 40440-9.

31.	Matsumoto, K. and B.H. Bay, Significance of the Y-box proteins in human cancers. J Mol Genet Med, 2005. 1(1): p. 11-7.

32.	Lyabin, D.N., I.A. Eliseeva, and L.P. Ovchinnikov, YB-1 protein: functions and regulation. Wiley Interdiscip Rev RNA, 2014. 5(1): p. 95-110.

33.	Kedersha, N. and P. Anderson, Mammalian stress granules and processing bodies. Methods Enzymol, 2007. 431: p. 61-81.

34.	Yang, W.H. and D.B. Bloch, Probing the mRNA processing body using protein macroarrays and "autoantigenomics". RNA, 2007. 13(5): p. 704-12.

35.	Anderson, P. and N. Kedersha, Stress granules: the Tao of RNA triage. Trends Biochem Sci, 2008. 33(3): p. 141-50.

36.	Han, T.W., et al., Cell-free formation of RNA granules: bound RNAs identify features and components of cellular assemblies. Cell, 2012. 149(4): p. 768-79.

37.	Kato, M., et al., Differential expression of genes related to drug responsiveness between sparsely and densely granulated somatotroph adenomas. Endocr J, 2012. 59(3): p. 221-8.

38.	Guryanov, S.G., et al., Formation of amyloid-like fibrils by Y-box binding protein 1 (YB-1) is mediated by its cold shock domain and modulated by disordered terminal domains. PLoS One, 2012. 7(5): p. e36969.

39.	Chernov, K.G., et al., Role of microtubules in stress granule assembly: microtubule dynamical instability favors the formation of micrometric stress granules in cells. J Biol Chem, 2009. 284(52): p. 36569-80.

40.	Frye, B.C., et al., Y-box protein-1 is actively secreted through a non-classical pathway and acts as an extracellular mitogen. EMBO Rep, 2009. 10(7): p. 783-9.

41.	Prabhu, L., et al., Role of post-translational modification of the Y box binding protein 1 in human cancers. Genes Dis, 2015. 2(3): p. 240-246.

42.	Chibi, M., et al., RBBP6 interacts with multifunctional protein YB-1 through its RING finger domain, leading to ubiquitination and proteosomal degradation of YB-1. J Mol Biol, 2008. 384(4): p. 908-16.

43. di Martino, O., et al., DeltaNp63alpha controls YB-1 protein stability: evidence on YB-1 as a new player in keratinocyte differentiation. Genes Cells, 2016. 21(6): p. 648-60.

44. Dong, W., et al., Activated Protein C Ameliorates Renal Ischemia-Reperfusion Injury by Restricting Y-Box Binding Protein-1 Ubiquitination. J Am Soc Nephrol, 2015. 26(11): p. 2789-99.

45. Palicharla, V.R. and S. Maddika, HACE1 mediated K27 ubiquitin linkage leads to YB-1 protein secretion. Cell Signal, 2015. 27(12): p. 2355-62.

46. Ferreira, A.R., et al., Serum YB-1 (Y-box binding protein 1) as a biomarker of bone disease progression in patients with breast cancer and bone metastases. J Bone Oncol, 2017. 6: p. 16-21.

47. Tacke, F., et al., High prevalence of Y-box protein-1/p18 fragment in plasma of patients with malignancies of different origin. BMC Cancer, 2014. 14: p. 33.

48. Tacke, F., et al., Y-box protein-1/p18 fragment identifies malignancies in patients with chronic liver disease. BMC Cancer, 2011. 11: p. 185.

49. Hanssen, L., et al., YB-1 is an early and central mediator of bacterial and sterile inflammation in vivo. J Immunol, 2013. 191(5): p. 2604-13.

50. Ou, Y., et al., Kindlin-2 interacts with beta-catenin and YB-1 to enhance EGFR transcription during glioma progression. Oncotarget, 2016. 7(46): p. 74872-74885.

51. Kosnopfel, C., et al., Tumour Progression Stage-Dependent Secretion of YB-1 Stimulates Melanoma Cell Migration and Invasion. Cancers (Basel), 2020. 12(8).

52. Kim, E.R., et al., The proteolytic YB-1 fragment interacts with DNA repair machinery and enhances survival during DNA damaging stress. Cell Cycle, 2013. 12(24): p. 3791-803.

53. Rauen, T., et al., YB-1 acts as a ligand for Notch-3 receptors and modulates receptor activation. J Biol Chem, 2009. 284(39): p. 26928-40.

54. Rohr, I., et al., Y-box protein-1/p18 as novel serum marker for ovarian cancer diagnosis: A study by the Tumor Bank Ovarian Cancer (TOC). Cytokine, 2016. 85: p. 157-64.

55. Sorokin, A.V., et al., Proteasome-mediated cleavage of the Y-box-binding protein 1 is linked to DNA-damage stress response. EMBO J, 2005. 24(20): p. 3602-12.

56. Stenina, O.I., E.J. Poptic, and P.E. DiCorleto, Thrombin activates a Y box-binding protein (DNA-binding protein B) in endothelial cells. J Clin Invest, 2000. 106(4): p. 579-87.

57. Djudjaj, S., et al., Notch-3 receptor activation drives inflammation and fibrosis following tubulointerstitial kidney injury. J Pathol, 2012. 228(3): p. 286-99.

58. Raffetseder, U., et al., Extracellular YB-1 blockade in experimental nephritis upregulates Notch-3 receptor expression and signaling. Nephron Exp Nephrol, 2011. 118(4): p. e100-8.

59. Hessman, C.L., et al., YB-1 Interferes with TNFalpha-TNFR Binding and Modulates Progranulin-Mediated Inhibition of TNFalpha Signaling. Int J Mol Sci, 2020. 21(19).

60. Wagner, S.A., et al., SPATA2 links CYLD to the TNF-alpha receptor signaling complex and modulates the receptor signaling outcomes. EMBO J, 2016. 35(17): p. 1868-84.

61. Martin, M., et al., Novel Serine 176 Phosphorylation of YBX1 Activates NF-kappaB in Colon Cancer. J Biol Chem, 2017. 292(8): p. 3433-3444.

62. Prabhu, L., et al., Critical role of phosphorylation of serine 165 of YBX1 on the activation of NF-kappaB in colon cancer. Oncotarget, 2015. 6(30): p. 29396-412.

63. Hartley, A.V., et al., PRMT5-mediated methylation of YBX1 regulates NF-kappaB activity in colorectal cancer. Sci Rep, 2020. 10(1): p. 15934.

64. Stratford, A.L., et al., Y-box binding protein-1 serine 102 is a downstream target of p90 ribosomal S6 kinase in basal-like breast cancer cells. Breast Cancer Res, 2008. 10(6): p. R99.

65. Shibata, T., et al., Y-box binding protein-1 contributes to both HER2/ErbB2 expression and lapatinib sensitivity in human gastric cancer cells. Mol Cancer Ther, 2013. 12(5): p. 737-46.

66. Bargou, R.C., et al., Nuclear localization and increased levels of transcription factor YB-1 in primary human breast cancers are associated with intrinsic MDR1 gene expression. Nat Med, 1997. 3(4): p. 447-50.

67. Basaki, Y., et al., Y-box binding protein-1 (YB-1) promotes cell cycle progression through CDC6-dependent pathway in human cancer cells. Eur J Cancer, 2010. 46(5): p. 954-65.

68. Kohno, K., et al., The pleiotropic functions of the Y-box-binding protein, YB-1. Bioessays, 2003. 25(7): p. 691-8.

69. FitzGerald, P.C., et al., Clustering of DNA sequences in human promoters. Genome Res, 2004. 14(8): p. 1562-74.

70. Marino-Ramirez, L., et al., Statistical analysis of over-represented words in human promoter sequences. Nucleic Acids Res, 2004. 32(3): p. 949-58.

71. Dolfini, D. and R. Mantovani, Targeting the Y/CCAAT box in cancer: YB-1 (YBX1) or NF-Y? Cell Death Differ, 2013. 20(5): p. 676-85.

72. Blum, R., et al., Molecular signatures of prostate stem cells reveal novel signaling pathways and provide insights into prostate cancer. PLoS One, 2009. 4(5): p. e5722.

73. Calvo, A., et al., Molecular characterization of the Ggamma-globin-Tag transgenic mouse model of hormone refractory prostate cancer: comparison to human prostate cancer. Prostate, 2010. 70(6): p. 630-45.

74. Forsberg, E.C., et al., Molecular signatures of quiescent, mobilized and leukemia-initiating hematopoietic stem cells. PLoS One, 2010. 5(1): p. e8785.

75. Niida, A., et al., Integrative bioinformatics analysis of transcriptional regulatory programs in breast cancer cells. BMC Bioinformatics, 2008. 9: p. 404.

76. Salvatore, G., et al., A cell proliferation and chromosomal instability signature in anaplastic thyroid carcinoma. Cancer Res, 2007. 67(21): p. 10148-58.

77. Scafoglio, C., et al., Comparative gene expression profiling reveals partially overlapping but distinct genomic actions of different antiestrogens in human breast cancer cells. J Cell Biochem, 2006. 98(5): p. 1163-84.

78. Tabach, Y., et al., The promoters of human cell cycle genes integrate signals from two tumor suppressive pathways during cellular transformation. Mol Syst Biol, 2005. 1: p. 2005 0022.

79. Thomassen, M., Q. Tan, and T.A. Kruse, Gene expression meta-analysis identifies metastatic pathways and transcription factors in breast cancer. BMC Cancer, 2008. 8: p. 394.

80. Brandt, S., et al., Cold shock Y-box protein-1 participates in signaling circuits with auto-regulatory activities. Eur J Cell Biol, 2012. 91(6-7): p. 464-71.

81. Hanahan, D. and R.A. Weinberg, The hallmarks of cancer. Cell, 2000. 100(1): p. 57-70.

82. Hanahan, D. and R.A. Weinberg, Hallmarks of cancer: the next generation. Cell, 2011. 144(5): p. 646-74.

83. Lasham, A., et al., YB-1: oncoprotein, prognostic marker and therapeutic target? Biochem J, 2013. 449(1): p. 11-23.

84. Blenkiron, C., et al., Links between the oncoprotein YB-1 and small non-coding RNAs in breast cancer. PLoS One, 2013. 8(11): p. e80171.

85. Lasham, A., et al., Regulation of the human fas promoter by YB-1, Puralpha and AP-1 transcription factors. Gene, 2000. 252(1-2): p. 1-13.

86. Lasham, A., et al., YB-1, the E2F pathway, and regulation of tumor cell growth. J Natl Cancer Inst, 2012. 104(2): p. 133-46.

87. Chatterjee, M., et al., The Y-box binding protein YB-1 is associated with progressive disease and mediates survival and drug resistance in multiple myeloma. Blood, 2008. 111(7): p. 3714-22.

88. Lasham, A., et al., The Y-box-binding protein, YB1, is a potential negative regulator of the p53 tumor suppressor. J Biol Chem, 2003. 278(37): p. 35516-23.

89. Duffy, M.J., et al., p53 as a target for the treatment of cancer. Cancer Treat Rev, 2014. 40(10): p. 1153-60.

90. Raffetseder, U., et al., Role of cold shock Y-box protein-1 in inflammation, atherosclerosis and organ transplant rejection. Eur J Cell Biol, 2012. 91(6-7): p. 567-75.

91. Hanssen, L., et al., Y-box binding protein-1 mediates profibrotic effects of calcineurin inhibitors in the kidney. J Immunol, 2011. 187(1): p. 298-308.

92. Mertens, P.R., et al., YB-1 regulation of the human and rat gelatinase A genes via similar enhancer elements. J Am Soc Nephrol, 1999. 10(12): p. 2480-7.

93. Raffetseder, U., et al., Splicing factor SRp30c interaction with Y-box protein-1 confers nuclear YB-1 shuttling and alternative splice site selection. J Biol Chem, 2003. 278(20): p. 18241-8.

94. Krohn, R., et al., Y-box binding protein-1 controls CC chemokine ligand-5 (CCL5) expression in smooth muscle cells and contributes to neointima formation in atherosclerosis-prone mice. Circulation, 2007. 116(16): p. 1812-20.

95. Raffetseder, U., et al., Differential regulation of chemokine CCL5 expression in monocytes/macrophages and renal cells by Y-box protein-1. Kidney Int, 2009. 75(2): p. 185-96.

96. Bradley, J.R., TNF-mediated inflammatory disease. J Pathol, 2008. 214(2): p. 149-60.

97. Seymour, H.E., et al., Anti-TNF agents for rheumatoid arthritis. Br J Clin Pharmacol, 2001. 51(3): p. 201-8.

98. Grivennikov, S.I., et al., Distinct and nonredundant in vivo functions of TNF produced by t cells and macrophages/neutrophils: protective and deleterious effects. Immunity, 2005. 22(1): p. 93-104.

99. Black, R.A., Tumor necrosis factor-alpha converting enzyme. Int J Biochem Cell Biol, 2002. 34(1): p. 1-5.

100. Carpentier, I., B. Coornaert, and R. Beyaert, Function and regulation of tumor necrosis factor receptor type 2. Curr Med Chem, 2004. 11(16): p. 2205-12.

101. Faustman, D. and M. Davis, TNF receptor 2 pathway: drug target for autoimmune diseases. Nat Rev Drug Discov, 2010. 9(6): p. 482-93.

102. Tartaglia, L.A., et al., The two different receptors for tumor necrosis factor mediate distinct cellular responses. Proc Natl Acad Sci U S A, 1991. 88(20): p. 9292-6.

103. Bodmer, J.L., P. Schneider, and J. Tschopp, The molecular architecture of the TNF superfamily. Trends Biochem Sci, 2002. 27(1): p. 19-26.

104. Brenner, D., H. Blaser, and T.W. Mak, Regulation of tumour necrosis factor signalling: live or let die. Nat Rev Immunol, 2015. 15(6): p. 362-74.

105. Ting, A.T. and M.J.M. Bertrand, More to Life than NF-kappaB in TNFR1 Signaling. Trends Immunol, 2016. 37(8): p. 535-545.

106. Chung, J.Y., et al., Structural revelations of TRAF2 function in TNF receptor signaling pathway. Adv Exp Med Biol, 2007. 597: p. 93-113.

107. Hsu, H., J. Xiong, and D.V. Goeddel, The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell, 1995. 81(4): p. 495-504.

108. Emmerich, C.H., et al., The linear ubiquitin chain assembly complex forms part of the TNF-R1 signalling complex and is required for effective TNF-induced gene induction and prevents TNF-induced apoptosis. Adv Exp Med Biol, 2011. 691: p. 115-26.

109. Vucic, D., TRAF2 and cellular IAPs: a critical link in TNFR family signaling. Adv Exp Med Biol, 2011. 691: p. 63-78.

110. Van Antwerp, D.J., et al., Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science, 1996. 274(5288): p. 787-9.

111. Wang, C.Y., et al., NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science, 1998. 281(5383): p. 1680-3.

112. Kreuz, S., et al., NF-kappaB inducers upregulate cFLIP, a cycloheximide-sensitive inhibitor of death receptor signaling. Mol Cell Biol, 2001. 21(12): p. 3964-73.

113. Papa, S., et al., Linking JNK signaling to NF-kappaB: a key to survival. J Cell Sci, 2004. 117(Pt 22): p. 5197-208.

114. Micheau, O. and J. Tschopp, Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell, 2003. 114(2): p. 181-90.

115. Wang, C.Y., M.W. Mayo, and A.S. Baldwin, Jr., TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. Science, 1996. 274(5288): p. 784-7.

116. Pasparakis, M. and P. Vandenabeele, Necroptosis and its role in inflammation. Nature, 2015. 517(7534): p. 311-20.

117. Sun, L., et al., Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell, 2012. 148(1-2): p. 213-27.

118. Khraishi, M., Comparative overview of safety of the biologics in rheumatoid arthritis. J Rheumatol Suppl, 2009. 82: p. 25-32.

119. Sedger, L.M. and M.F. McDermott, TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants - past, present and future. Cytokine Growth Factor Rev, 2014. 25(4): p. 453-72.

120. Kim, H.W., et al., Effect of anti-tumor necrosis factor alpha treatment of rheumatoid arthritis and chronic kidney disease. Rheumatol Int, 2015. 35(4): p. 727-34.

121. Askling, J. and T. Bongartz, Malignancy and biologic therapy in rheumatoid arthritis. Curr Opin Rheumatol, 2008. 20(3): p. 334-9.

122. Askling, J. and W. Dixon, The safety of anti-tumour necrosis factor therapy in rheumatoid arthritis. Curr Opin Rheumatol, 2008. 20(2): p. 138-44.

123. Matteson, E.L. and T. Bongartz, Tumor necrosis factor antagonists and cancer in patients with rheumatoid arthritis. Nat Clin Pract Rheumatol, 2007. 3(1): p. 14-5.

124. Ross, R., The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature, 1993. 362(6423): p. 801-9.

125. Ross, R., Atherosclerosis--an inflammatory disease. N Engl J Med, 1999. 340(2): p. 115-26.

126. Schreyer, S.A., J.J. Peschon, and R.C. LeBoeuf, Accelerated atherosclerosis in mice lacking tumor necrosis factor receptor p55. J Biol Chem, 1996. 271(42): p. 26174-8.

127. Tipping, P.G. and W.W. Hancock, Production of tumor necrosis factor and interleukin-1 by macrophages from human atheromatous plaques. Am J Pathol, 1993. 142(6): p. 1721-8.

128. Collins, T., et al., Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. FASEB J, 1995. 9(10): p. 899-909.

129. Dudeck, J., et al., Directional mast cell degranulation of tumor necrosis factor into blood vessels primes neutrophil extravasation. Immunity, 2021. 54(3): p. 468-483 e5.

130. Shah, A., et al., YB-1 Mediates TNF-Induced Pro-Survival Signaling by Regulating NF-kappaB Activation. Cancers (Basel), 2020. 12(8).

131. Gieseler-Halbach, S., et al., RSK-mediated nuclear accumulation of the cold-shock Y-box protein-1 controls proliferation of T cells and T-ALL blasts. Cell Death Differ, 2017. 24(2): p. 371-383.

132. Meltendorf, S., et al., Cell Survival Failure in Effector T Cells From Patients With Systemic Lupus Erythematosus Following Insufficient Up-Regulation of Cold-Shock Y-Box Binding Protein 1. Arthritis Rheumatol, 2020. 72(10): p. 1721-1733.

133. Capowski, E.E., et al., Y box-binding factor promotes eosinophil survival by stabilizing granulocyte-macrophage colony-stimulating factor mRNA. J Immunol, 2001. 167(10): p. 5970-6.

134. Coles, L.S., et al., Cold shock domain proteins repress transcription from the GM-CSF promoter. Nucleic Acids Res, 1996. 24(12): p. 2311-7.

135. Zhang, T., et al., AU-rich element-mediated translational control: complexity and multiple activities of trans-activating factors. Biochem Soc Trans, 2002. 30(Pt 6): p. 952-8.

136. Lyons, S.M., et al., YB-1 regulates tiRNA-induced Stress Granule formation but not translational repression. Nucleic Acids Res, 2016. 44(14): p. 6949-60.

137. Somasekharan, S.P., et al., YB-1 regulates stress granule formation and tumor progression by translationally activating G3BP1. J Cell Biol, 2015. 208(7): p. 913-29.

138. Stamou, P. and D.L. Kontoyiannis, Posttranscriptional regulation of TNF mRNA: a paradigm of signal-dependent mRNA utilization and its relevance to pathology. Curr Dir Autoimmun, 2010. 11: p. 61-79.

139. Borghi, A., L. Verstrepen, and R. Beyaert, TRAF2 multitasking in TNF receptor-induced signaling to NF-kappaB, MAP kinases and cell death. Biochem Pharmacol, 2016. 116: p. 1-10.

140. Hayden, M.S. and S. Ghosh, Regulation of NF-kappaB by TNF family cytokines. Semin Immunol, 2014. 26(3): p. 253-66.

141. Peltzer, N., M. Darding, and H. Walczak, Holding RIPK1 on the Ubiquitin Leash in TNFR1 Signaling. Trends Cell Biol, 2016. 26(6): p. 445-461.

142. Raj, G.V., et al., Transcriptional regulation of human polyomavirus JC: evidence for a functional interaction between RelA (p65) and the Y-box-binding protein, YB-1. J Virol, 1996. 70(9): p. 5944-53.

143. Sutherland, B.W., et al., Akt phosphorylates the Y-box binding protein 1 at Ser102 located in the cold shock domain and affects the anchorage-independent growth of breast cancer cells. Oncogene, 2005. 24(26): p. 4281-92.

144. Sen, R. and D. Baltimore, Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. Cell, 1986. 47(6): p. 921-8.

145. Reinhard, K., et al., The role of NF-kappaB activation during protection against Leishmania infection. Int J Med Microbiol, 2012. 302(4-5): p. 230-5.

146. Sun, S.C., Non-canonical NF-kappaB signaling pathway. Cell Res, 2011. 21(1): p. 71-85.

147. Vallabhapurapu, S. and M. Karin, Regulation and function of NF-kappaB transcription factors in the immune system. Annu Rev Immunol, 2009. 27: p. 693-733.

148. Bonizzi, G. and M. Karin, The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol, 2004. 25(6): p. 280-8.

149. Hoesel, B. and J.A. Schmid, The complexity of NF-kappaB signaling in inflammation and cancer. Mol Cancer, 2013. 12: p. 86.

150. Moynagh, P.N., The NF-kappaB pathway. J Cell Sci, 2005. 118(Pt 20): p. 4589-92.

151. Beinke, S., et al., NF-kappaB1 p105 negatively regulates TPL-2 MEK kinase activity. Mol Cell Biol, 2003. 23(14): p. 4739-52.

152. Hayden, M.S. and S. Ghosh, Signaling to NF-kappaB. Genes Dev, 2004. 18(18): p. 2195-224.

153. Karin, M. and M. Delhase, The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. Semin Immunol, 2000. 12(1): p. 85-98.

154. Li, Q. and I.M. Verma, NF-kappaB regulation in the immune system. Nat Rev Immunol, 2002. 2(10): p. 725-34.

155. Mitchell, S., J. Vargas, and A. Hoffmann, Signaling via the NFkappaB system. Wiley Interdiscip Rev Syst Biol Med, 2016. 8(3): p. 227-41.

156. Sun, S.C., The non-canonical NF-kappaB pathway in immunity and inflammation. Nat Rev Immunol, 2017. 17(9): p. 545-558.

157. Zinngrebe, J., et al., Ubiquitin in the immune system. EMBO Rep, 2014. 15(1): p. 28-45.

158. Huang, X. and V.M. Dixit, Drugging the undruggables: exploring the ubiquitin system for drug development. Cell Res, 2016. 26(4): p. 484-98.

159. Davis, M.E., et al., MMP inhibition as a potential method to augment the healing of skeletal muscle and tendon extracellular matrix. J Appl Physiol (1985), 2013. 115(6): p. 884-91.

160. George, A.J., et al., A Comprehensive Atlas of E3 Ubiquitin Ligase Mutations in Neurological Disorders. Front Genet, 2018. 9: p. 29.

161. Akutsu, M., I. Dikic, and A. Bremm, Ubiquitin chain diversity at a glance. J Cell Sci, 2016. 129(5): p. 875-80.

162. Stolz, A. and I. Dikic, Heterotypic Ubiquitin Chains: Seeing is Believing. Trends Cell Biol, 2018. 28(1): p. 1-3.

163. Yau, R. and M. Rape, The increasing complexity of the ubiquitin code. Nat Cell Biol, 2016. 18(6): p. 579-86.
164. Elia, A.E., et al., Quantitative Proteomic Atlas of Ubiquitination and Acetylation in the DNA Damage Response. Mol Cell, 2015. 59(5): p. 867-81.
165. Kim, W., et al., Systematic and quantitative assessment of the ubiquitin-modified proteome. Mol Cell, 2011. 44(2): p. 325-40.
166. Kulathu, Y. and D. Komander, Atypical ubiquitylation - the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages. Nat Rev Mol Cell Biol, 2012. 13(8): p. 508-23.
167. Dynek, J.N., et al., c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signalling. EMBO J, 2010. 29(24): p. 4198-209.
168. Goto, E., et al., Contribution of lysine 11-linked ubiquitination to MIR2-mediated major histocompatibility complex class I internalization. J Biol Chem, 2010. 285(46): p. 35311-9.
169. Wickliffe, K.E., et al., K11-linked ubiquitin chains as novel regulators of cell division. Trends Cell Biol, 2011. 21(11): p. 656-63.
170. Fei, C., et al., Smurf1-mediated Lys29-linked nonproteolytic polyubiquitination of axin negatively regulates Wnt/beta-catenin signaling. Mol Cell Biol, 2013. 33(20): p. 4095-105.
171. Huang, H., et al., K33-linked polyubiquitination of T cell receptor-zeta regulates proteolysis-independent T cell signaling. Immunity, 2010. 33(1): p. 60-70.
172. Yuan, W.C., et al., K33-Linked Polyubiquitination of Coronin 7 by Cul3-KLHL20 Ubiquitin E3 Ligase Regulates Protein Trafficking. Mol Cell, 2014. 54(4): p. 586-600.
173. Xu, P., et al., Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. Cell, 2009. 137(1): p. 133-45.
174. Zhou, W. and J. Yuan, Necroptosis in health and diseases. Semin Cell Dev Biol, 2014. 35: p. 14-23.
175. Lee, B.L., et al., Molecular Basis for K63-Linked Ubiquitination Processes in Double-Strand DNA Break Repair: A Focus on Kinetics and Dynamics. J Mol Biol, 2017. 429(22): p. 3409-3429.
176. Liu, P., et al., K63-linked polyubiquitin chains bind to DNA to facilitate DNA damage repair. Sci Signal, 2018. 11(533).
177. Meng, J., et al., ARRDC4 regulates enterovirus 71-induced innate immune response by promoting K63 polyubiquitination of MDA5 through TRIM65. Cell Death Dis, 2017. 8(6): p. e2866.
178. Pugh, D.J., et al., DWNN, a novel ubiquitin-like domain, implicates RBBP6 in mRNA processing and ubiquitin-like pathways. BMC Struct Biol, 2006. 6: p. 1.
179. Belgnaoui, S.M., et al., Linear ubiquitination of NEMO negatively regulates the interferon antiviral response through disruption of the MAVS-TRAF3 complex. Cell Host Microbe, 2012. 12(2): p. 211-22.
180. Ikeda, F. and I. Dikic, Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. EMBO Rep, 2008. 9(6): p. 536-42.
181. Miranda, M. and A. Sorkin, Regulation of receptors and transporters by ubiquitination: new insights into surprisingly similar mechanisms. Mol Interv, 2007. 7(3): p. 157-67.
182. Hornbeck, P.V., et al., PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. Nucleic Acids Res, 2015. 43(Database issue): p. D512-20.
183. Tortola, L., et al., The Tumor Suppressor Hace1 Is a Critical Regulator of TNFR1-Mediated Cell Fate. Cell Rep, 2016. 15(7): p. 1481-1492.
184. Bernhardt, A., et al., Inflammatory cell infiltration and resolution of kidney inflammation is orchestrated by the cold-shock protein Y-box binding protein-1. Kidney Int, 2017. 92(5): p. 1157-1177.
185. Weischenfeldt, J. and B. Porse, Bone Marrow-Derived Macrophages (BMM): Isolation and Applications. CSH Protoc, 2008. 2008: p. pdb prot5080.

186. Hohlfeld, R., et al., Crosstalk between Akt signaling and cold shock proteins in mediating invasive cell phenotypes. Oncotarget, 2018. 9(27): p. 19039-19049.

187. Vucic, D., V.M. Dixit, and I.E. Wertz, Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death. Nat Rev Mol Cell Biol, 2011. 12(7): p. 439-52.

188. Rothe, M., et al., TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. Science, 1995. 269(5229): p. 1424-7.

189. Wajant, H. and P. Scheurich, TNFR1-induced activation of the classical NF-kappaB pathway. FEBS J, 2011. 278(6): p. 862-76.

190. Mahoney, D.J., et al., Both cIAP1 and cIAP2 regulate TNFalpha-mediated NF-kappaB activation. Proc Natl Acad Sci U S A, 2008. 105(33): p. 11778-83.

191. Varfolomeev, E., et al., IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. Cell, 2007. 131(4): p. 669-81.

192. Reichert, M., et al., Characterization of the TNFR1-SC Using "Modified Tandem Affinity Purification" in Conjunction with Liquid Chromatography-Mass Spectrometry (LC-MS). Methods Mol Biol, 2018. 1857: p. 161-169.

193. Jayavelu, A.K., et al., Splicing factor YBX1 mediates persistence of JAK2-mutated neoplasms. Nature, 2020. 588(7836): p. 157-163.

194. Chen, J. and Z.J. Chen, Regulation of NF-kappaB by ubiquitination. Curr Opin Immunol, 2013. 25(1): p. 4-12.

195. Iwai, K., Linear Polyubiquitination: A Crucial Regulator of NF-kappaB Activation, in Innovative Medicine: Basic Research and Development, K. Nakao, N. Minato, and S. Uemoto, Editors. 2015: Tokyo. p. 51-59.

196. Tanzer, M.C., et al., Quantitative and Dynamic Catalogs of Proteins Released during Apoptotic and Necroptotic Cell Death. Cell Rep, 2020. 30(4): p. 1260-1270 e5.

197. Dillon, C.P., et al., RIPK1 blocks early postnatal lethality mediated by caspase-8 and RIPK3. Cell, 2014. 157(5): p. 1189-202.

198. Majkut, J., et al., Differential affinity of FLIP and procaspase 8 for FADD's DED binding surfaces regulates DISC assembly. Nat Commun, 2014. 5: p. 3350.

199. Johnson, T.G., et al., Why Be One Protein When You Can Affect Many? The Multiple Roles of YB-1 in Lung Cancer and Mesothelioma. Front Cell Dev Biol, 2019. 7: p. 221.

200. Maurya, P.K., et al., Role of Y Box Protein-1 in cancer: As potential biomarker and novel therapeutic target. J Cancer, 2017. 8(10): p. 1900-1907.

201. Brandt, S. and P.R. Mertens, A remedy for kidney disease successfully alters the cold shock protein response during inflammation. Kidney Int, 2016. 90(6): p. 1148-1150.

202. Khan, M.I., et al., YB-1 expression promotes epithelial-to-mesenchymal transition in prostate cancer that is inhibited by a small molecule fisetin. Oncotarget, 2014. 5(9): p. 2462-74.

203. Sechi, M., et al., Fisetin targets YB-1/RSK axis independent of its effect on ERK signaling: insights from in vitro and in vivo melanoma models. Sci Rep, 2018. 8(1): p. 15726.

204. Xiao, Y.Z., et al., Reducing Hypothalamic Stem Cell Senescence Protects against Aging-Associated Physiological Decline. Cell Metab, 2020. 31(3): p. 534-548 e5.

205. Tailor, D., et al., Y box binding protein 1 inhibition as a targeted therapy for ovarian cancer. Cell Chem Biol, 2021.

206. Sangermano, F., A. Delicato, and V. Calabro, Y box binding protein 1 (YB-1) oncoprotein at the hub of DNA proliferation, damage and cancer progression. Biochimie, 2020. 179: p. 205-216.

207. Kuwano, M., et al., Oncogenic Y-box binding protein-1 as an effective therapeutic target in drug-resistant cancer. Cancer Sci, 2019. 110(5): p. 1536-1543.

208. Morgenroth, R., et al., Autoantibody Formation and Mapping of Immunogenic Epitopes against Cold-Shock-Protein YB-1 in Cancer Patients and Healthy Controls. Cancers (Basel), 2020. 12(12).

209. Nguyen, H.H., et al., Evaluation of classical and novel autoantibodies for the diagnosis of Primary Biliary Cholangitis-Autoimmune Hepatitis Overlap Syndrome (PBC-AIH OS). PLoS One, 2018. 13(3): p. e0193960.
210. Bloch, D.B., R.A. Nobre, and W.H. Yang, GW/P-bodies and autoimmune disease. Adv Exp Med Biol, 2013. 768: p. 61-70.

Declaration of Honour

"I hereby declare that I prepared this thesis without the impermissible help of third parties and that none other that the aids indicated have been used; all sources of information are clearly marked, including my own publications.

In particular I have not consciously:
- fabricated data or rejected undesirable results,
- misused statistical methods with the aim of drawing other conclusions than those warranted by the available data,
- plagiarized external data or publications,
- presented the results of other researchers in a distorted way.

I am aware that violations of copyright may lead to injunction and damage claims by the author and also to prosecution by law enforcement authorities.
I hereby agree that the thesis may be electronically reviewed with the aim of identifying plagiarism.

This work has not been submitted as a doctoral thesis in the same or a similar form in Germany, nor in any other country. It has not yet been published as a whole."
Magdeburg,
(Place, date)
07.04.2022

(Signature)