The Role of ANKIB1 In Regulating Cell Death Signaling Versus Gene Activation Following TLR3 Activation

by

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Koç University

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ABSTRACT

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Biochemical reactions in cells are necessary to fulfill underlying cellular processes and signal transduction from both, the extracellular and intracellular environment. Their dysregulation can lead to an increase of morbidity and mortality in organisms. Because of that, they are strictly regulated to hamper adverse outcomes. One of the most common regulatory mechanisms are post-translational modifications (PTMs) of proteins, including by ubiquitination. Different pairs of E2 ubiquitin conjugating enzymes and E3 ubiquitin ligases can generate different types of ubiquitination that lead to diverse outcomes for the substrate protein. Nonetheless, their implication in the control of the different immune and inflammatory signaling pathways needs to be further studied. In the current thesis, I dissected the role of a new E3 ubiquitin ligase, Ankyrin repeat and IBR domain-containing protein 1 (ANKIB1), in the Toll-like Receptor 3 (TLR3) signaling pathway and in the antiviral response.

The Walczak group previously showed that ANKIB1 has an active catalytic domain and interacts with the TLR3 signaling complex (TLR3-SC) upon stimulation. I have demonstrated that ANKIB1, especially its catalytic activity, is required for the activation of TANK-Binding Kinase 1 (TBK1) and, consequently, type I interferon (IFN) production following TLR3 activation. Oppositely, the deletion of ANKIB1 does not have any consequences on Tumor Necrosis Factor (TNF) signaling. I have shown that, upon TLR3 stimulation, ANKIB1 does not only interact with activated TBK1 but also with optineurin (OPTN) and the NF-kappa-B Essential Modulator (NEMO), the primordial protein for TBK1 activation.

To further elucidate the regulation of this process, I have performed an unbiased proteomic analysis of the ubiquitinated proteins upon TLR3 stimulation and found that TBK1, Interferon regulatory factor 3 (IRF3), and TNF Induced Protein 3 (A20) contain ubiquitinated sites that are dependent on the presence of ANKIB1. Interestingly, I have also seen the same consequence on Gasdermin E suggesting a potential role of this new E3 ligase in the regulation of cell death. Therefore, I dissected the potential role of ANKIB1 on TLR3-induced cell death in vitro using WT and ANKIB1 KO cells. This analysis revealed that ANKIB1 deficiency in HT29 cells results in higher susceptibility to TLR3-induced cell death.

Overall, this study highlights the requirement of ANKIB1, a previously uncharacterized E3 ligase, for TBK1 activation and, consequently, type I IFN production in TLR3 signaling. Several components of this signaling complex are impacted by the absence of ANKIB1 in terms of ubiquitination, implying an important contribution of ANKIB1 to TLR3-induced gene activation and regulation of cell death, primordial to the induction of an efficient immune response against pathogens.

ÖZETÇE

ANKIB1'in TLR3 Tarafından Uyarılmış Hücre Ölüm Sinyalini ve Gen Aktivasyonu Düzenlemedeki Rolü

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Moleküler Biyoloji ve Genetik, Yüksek Lisans

4 Ağustos, 2022

Hücrelerdeki biyokimyasal reaksiyonlar, temel hücresel süreçleri ve hem hücre dışı hem de hücre içi ortamdan gelen sinyal iletimini yerine getirmek için gereklidir. Bunların düzensizliği organizmalarda morbidite ve mortalitenin artmasına yol açabilir. Bu nedenle, olumsuz sonuçları engellemek için sıkı bir şekilde düzenlenirler. En yaygın düzenleyici mekanizmalardan biri, proteinlerin ubikitinasyon gibi post translasyonel modifikasyonlarıdır (PTM'ler). Farklı E2 ubikitin konjugasyon enzimi ve E3 ubikitin ligaz çiftleri, substrat protein için farklı sonuçlara yol açan farklı ubikitinasyon türleri oluşturabilir. Bununla birlikte, farklı bağışıklık ve inflamatuar sinyal yollarının kontrolündeki etkilerinin daha fazla incelenmesi gerekmektedir. Bu tezde, yeni bir E3 ubikitin ligaz olan Ankyrin repeat and IBR domain-containing protein 1'in (ANKIB1) Toll-like Receptor 3 (TLR3) sinyal yolağı ve antiviral yanıttaki rolünü inceledik.

Walczak grubu daha önce ANKIB1'in aktif bir katalitik aktiviteye sahip olduğunu ve uyarım üzerine TLR3 sinyal kompleksi (TLR3-SC) ile etkileşime girdiğini göstermiştir. ANKIB1'in, özellikle katalitik aktivitesinin, TANK Bağlayıcı Kinaz 1 aktivasyonu ve sonuç olarak TLR3 aktivasyonunu takiben tip I interferon (IFN) üretimi için gerekli olduğunu belirledik. Tersine, ANKIB1'in silinmesi Tümör Nekroz Faktörü (TNF) yolu üzerinde herhangi bir sonuç doğurmamaktadır. ANKIB1'in aktive edilmiş TBK1'in yanı sıra optineurin (OPTN) ve TBK1 aktivasyonu için ilk protein olan NF-kappa-B Temel Modülatörü (NEMO) ile etkileşime girdiğini gösterdik.

Bu sürecin düzenlenmesini daha da aydınlatmak için, TLR3 uyarımı üzerine ubikuitinlenmiş proteinlerin tarafsız bir proteomik analizini gerçekleştirdik ve TBK1, İnterferon düzenleyici faktör 3 (IRF3) ve Tümör Nekroz Faktörü İndüklenmiş Protein 3'ün (A20) ANKIB1'in varlığına bağlı olarak ubikuitinlenmeyen ubikuitin bölgesi içerdiğini bulduk. İlginç bir şekilde, aynı sonucu Gasdermin E'de de gördük ve bu yeni E3 ligazın hücre ölümünün düzenlenmesinde potansiyel bir rolü olduğunu düşündük. Bu nedenle, WT ve ANKIB1 KO hücrelerini kullanarak ANKIB1'in TLR3 ile uyarılmış hücre ölümü üzerindeki potansiyel rolünü in vitro olarak inceledik. HT29 hücrelerindeki ANKIB1 eksikliğinin hücre ölümüne karşı daha yüksek duyarlılıkla sonuçlandığını ortaya koymuştur.

Genel olarak, çalışmamız TBK1 aktivasyonu ve dolayısıyla TLR3 sinyalizasyonunda tip I IFN üretimi için daha önce karakterize edilmemiş bir E3 ligaz olan ANKIB1'in gerekliliğini vurgulamaktadır. Bu sinyal kompleksinin çeşitli bileşenleri, ubikitinasyon profili açısından ANKIB1'in yokluğundan etkilenmektedir; bu da ANKIB1'in patojenlere karşı etkili bir bağışıklık tepkisi oluşturmak için öncelikli olan gen aktivasyonu ve hücre ölümü düzenlemesi için önemli bir katkısı olduğunu düşündürmektedir.

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This study cannot belong to only an individual. It could reveal with efforts of all scientists that contribute to improvement of science, especially biology from past to today.

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Last, but not the least, I would like to state that I am thankful to my family for enabling me to get to where I am today and for their endless support and faith in me and that, in the end, I shall be successful in what I aim to achieve in my life.

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ABBREVIATIONS

ATP	Adenosine Triphosphate
ANKIB1	Ankyrin Repeat and IBR Domain-Containing Protein 1
APAF1	Apoptotic Protease Activating Factor 1
BID	BH3-Interacting Domain Death Agonist
tBID	Truncated BID
cGAS	Cyclic GMP-AMP Synthase
c-FLIP	Cellular FLICE Inhibitory Protein
cIAP 1/2	Cellular Inhibitor of The Apoptosis Proteins 1/2
CYLD	Cylindromatosis
DAMP	Damage Associated Molecular Pattern
DD	Death Domain
DED	Death Effector Domain
DR	Death Receptor
DISC	Death-Inducing Signaling Complex
DUB	Deubiquitinase
DAI	DNA-Induced Activator of the IFN
DMEM	Dulbecco's Modified Eagle's Medium
ESCRT	Endosomal Sorting Complexes Required for Transport
EDTA	Etilendiamine Tetraacetic Acid
FADD	Fas-Associated DD
FBS	Fetal Bovine Serum
HOIL-1	Heme-Oxidized IRP2 Ubiquitin Ligase-1
HOIP	HOIL-1–Interacting Protein,
IFNAR	IFN α/β Receptor
IRF 3/7	IFN Regulatory Factor 3/7
ISG	IFN Stimulatory Gene
ISRE	IFN-Stimulated Response Element
IkB	Inhibitor of The Nuclear Factor kB
IKKα/β/ε	IkB Kinase Subunit α/β/ε
IFN	Interferon
LUBAC	Linear Ubiquitin Chain-Assembly Complex

MK2	MAPK-Activated Protein Kinase 2
MDA-5	Melanoma Differentiation-Associated Protein 5
MOMP	Mitochondrial Outer Membrane Permeabilization
MAPK	Mitogen-Activated Protein Kinase
MLKL	Mixed Lineage Kinase Domain-Like
MYD88	Myeloid Differentiation Primary Response 88
NEMO	NF-kB Essential Modulator
NF-kB	Nuclear Factor k Light-Chain Enhancer of The Activated B Cells
OPTN	Optineurin
OTULIN	OTU Deubiquitinase With Linear Linkage Specificity
PAMP	Pathogen Associated Molecular Pattern
PRR	Pattern Recognition Receptor
PEI	Polyethylenimine
PTM	Post Translational Modification
RING	Really Interesting New Gene
RIPK1/3	Receptor-Interacting Protein Kinase 1/3
RIG-I	Retinoic Acid-Inducible Gene I
RHIM	RIPK Homotypic Interaction Motif,
RPMI	Roswell Park Memorial Institute
SMAC/DIABLO	Second Mitochondria-Derived Activator of the Caspase/Direct Iap-Binding Protein with Low pI
SHARPIN	SHANK-Associated RH-Domain–Interacting Protein
STAT1/2	Signal Transducer and Activator of the Transcription 1/2
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel
STING	Stimulator of IFN Genes
TAB 2/3	TAK1-Binding Proteins 2/3
TBK1	TANK-Binding Kinase 1
HECT	Homologous To The E6-AP Carboxyl Terminus
RBR	The Ring Between Ring Fingers
TRIF	TIR-Domain-Containing Adapter-Inducing Interferon-B
TRAF 2/3/5/6	TNF Receptor-Associated Factor 2/3/5/6
TRADD	TNFR1-Associated Via Death Domain

TRAIL1/2	TNF-Related Apoptosis-Inducing Ligand Receptor 1/2
A20	TNF-A Induced Protein 3
TLR3/4	Toll-Like Receptor 3/4
TANK	TRAF Family Member Associated NF-kB Activator
NAP1	Nucleosome Assembly Protein 1
TAK1	Transforming Growth Factor-B-Activated Kinase 1
TNF	Tumor Necrosis Factor
TNFR	TNF Receptor
UB	Ubiquitin
UBD	Ubiquitin Binding Domain
UIM	Ubiquitin Interacting Motif
E3	Ubiquitin Protein Ligase
E1	Ubiquitin-Activating Enzyme
E2	Ubiquitin-Conjugating Enzyme
XIAP	X-Linked Inhibitor of the Apoptosis

Chapter 1:INTRODUCTION

Together with novel discoveries and accumulating data associated with biochemical circumstances in the cell, relationships among pathological state of the cells, dysfunctions and defects in biochemical reactions have gained a great importance. Biochemical responses upon stimuli, such as signaling molecules, infections leading to numerous consequences like cell fate determination, cellular growth, cell behavior, and defense against pathogens. Furthermore, these biochemical circumstances have various check points and are strictly regulated by physical or chemical alteration of their main modules, which is primarily carried out through the activity of regulatory proteins and PTMs, which involve covalent attachment of functional molecules to critical components of the biochemical pathways like other crucial proteins. Dysfunctions of the regulatory proteins or mutations on PTM sites of the essential proteins may cause disruption of the on-off switch mechanisms in a variety of the signaling pathways and metabolism resulting in increased lethality or morbidity. Main characteristics of the regulatory proteins are their scaffolding and/or enzymatic activity. Their scaffolding activity changes conformations and functionalities of proteins within the pathways whereas their enzymatic activity is mostly responsible for PTMs like phosphorylation, glycosylation, ubiquitination and others [1, 2]. While one of the most common PTM is protein phosphorylation, the covalent attachment of a ubiquitin, which is called ubiquitination, also happens quite abundantly and occurs in almost every cellular process. Since decades, researchers have been proving that ubiquitination plays essential roles in signaling pathways, cellular processes, inflammation, and cell death. It has been extensively shown that dysfunction of the ubiquitination processes can lead to the emergence of the cancer, autoimmune diseases, and neurodegenerative diseases etc. [3].

1.1 Ubiquitin and Ubiquitination System

Ubiquitin is a small, globular, highly compact protein and ubiquitously present in cells. It consists of 76 conserved amino acids and is encoded by 4 different genes in mammals, i.e. UBB, UBC, RPS27, and UBA52. Ubiquitination is one of the most common PTMs and it plays a crucial role in probably all signaling pathways regarding their activity, persistence, and localization. Early discovery of the ubiquitin system and

its functionality are based on studies performed by Avram Hershko, Aaron Ciechanover, and Irwin Rose between 1978 and 1980. They have investigated in vitro proteolysis, using lysate of the rabbit reticulocytes that lack lysosomes, and several intriguing circumstances were observed during these studies. First of all, degradation of the substrates was associated with ATP hydrolysis. Secondly, requirement of the tiny, heat stable proteinaceous molecules' presence was noticed, and thirdly substrates got larger before their degradation [4,5,6]. Even though this tiny proteinaceous molecule was named as ATP dependent proteolysis factor 1(APF-1), it had been identified before as "ubiquitin" that had features for promotion of the lymphocyte differentiation [7,8]. Further studies showed that heat stable properties of the ubiquitin stems from its comprehensive intramolecular interactions and larger forms of the substrates arise from covalently attached minimally four ubiquitin moieties to the substrate preceding degradation in ATP hydrolysis dependent manner [5, 6, 9]. Even if the function of the ubiquitination was firstly described to promote protein degradation via proteasome, it was further shown that ubiquitination also harbors a non-proteolytic function on proteins. Basically, ubiquitinated proteins comprise varied fates even if they share same ubiquitination mechanism in principle, which results from some structural differences of the ubiquitination system components and complexity of the ubiquitination based on the monoubiquitination or polyubiquitination chains [10]. In a general manner, ubiquitination is mediated by the formation of a covalent bond between α-COOH group of the ubiquitin in its C terminus and the ε-NH2 group of the substrate's lysine. It is usually an isopeptide bond [11]. Targeted proteins can be monoubiquitinated, described as one ubiquitin attachment. They can also be multi-monoubiquitinated depicted as one ubiquitin addition to different lysine residues within the same protein. Both types of ubiquitination usually play similar roles in various phenomena like DNA repair, viral budding, and transcription. Finally, polyubiquitination also occurs and is defined as the addition of multiple ubiquitin molecules on one single lysine residue of a protein. Several ubiquitin molecules are linked to each other with an isopeptide bond as shown in Figure 1.1. A ubiquitin molecule harbors 8 NH2 groups involving its amino terminus (M1) and 7 internal lysine residues (K6, K11, K27, K29, K33, K48, K63). Each lysine residue of the ubiquitin as well as the N-terminal NH2 group may serve as substrate for the following ubiquitin. This leads to formation of a specific type of ubiquitin chain also referred to as linkage, such as K48 or K63 ubiquitin chain or linkage, respectively [12,13]. Depending on the type of chain

formed, poly-ubiquitination exerts different roles as described in Figure 1.2 [14,15]. For instance, K48 ubiquitin chains are affiliated with proteasomal degradation while K63-linked ubiquitin chains display either scaffold activity for further protein-protein interaction or trigger conformational changes of the target protein. Also, M1 ubiquitin chains serve as a platform to recruit different functional proteins like kinases and thus induce initiation of the signal transduction. Despite some studies regarding their function, several types of chains are still not fully understood yet [12,13,15].

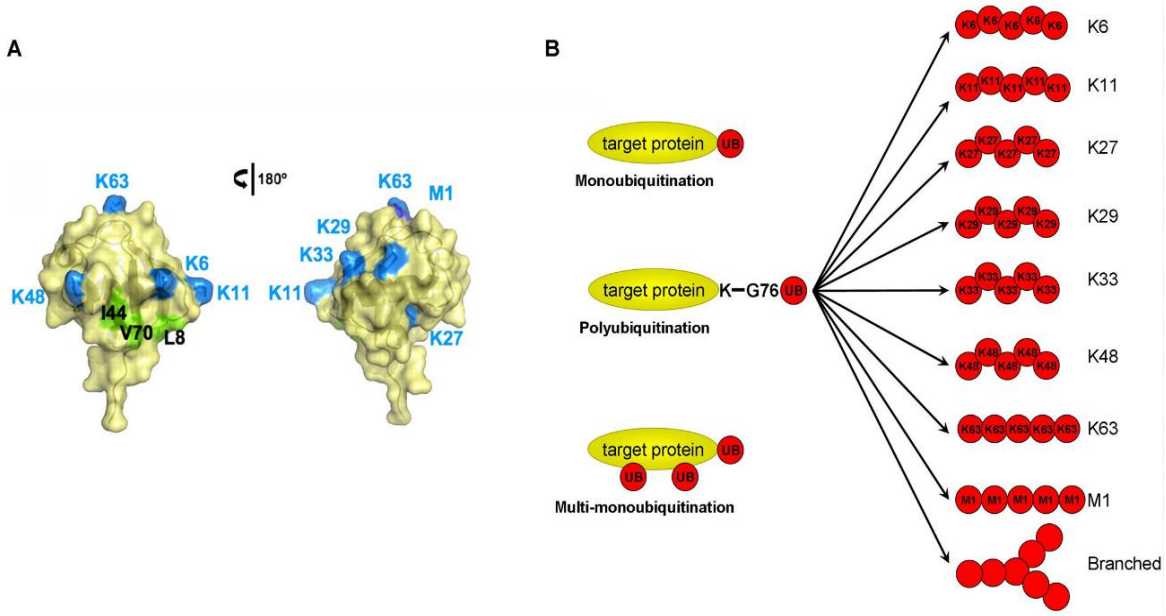


Figure 1.1: Structure of Ubiquitin And Ubiquitination Types. A) Depiction of the ubiquitin's structure and positions of the lysine residues. **B)** Ubiquitination types monoubiquitination (above), polyubiquitination (middle), and multi-monoubiquitination (below). Adapted from [12].

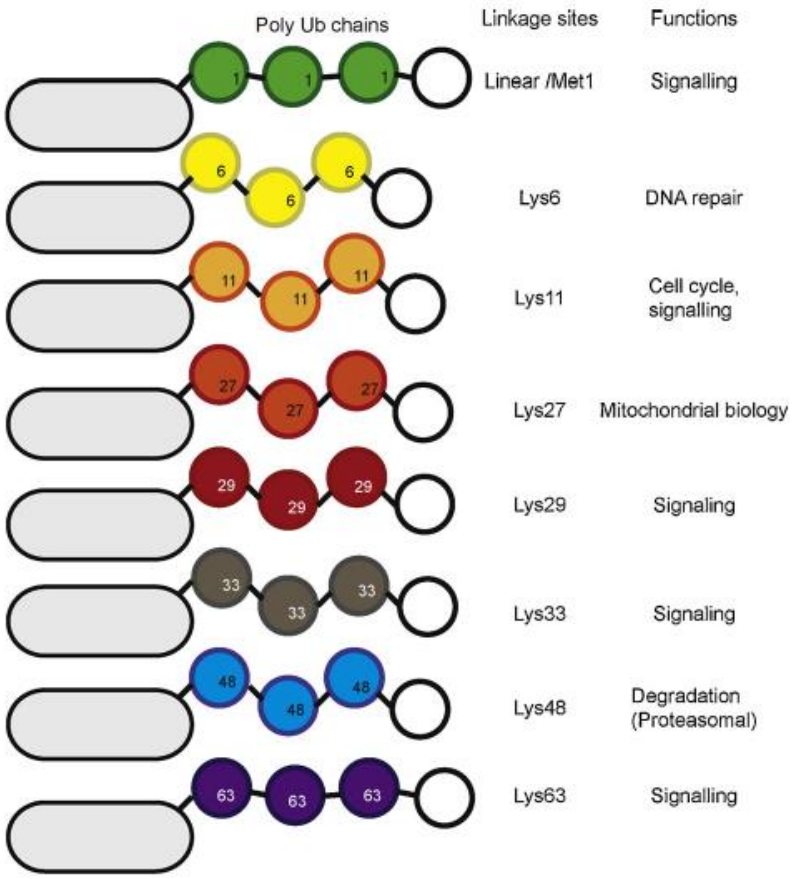


Figure 1.2: Different Types of Polyubiquitin Chains and Their Biological Significances known so far. Redrawn and adapted from [13].

The ubiquitination process needs 3 different enzymes, which are (i) the ubiquitin-activating enzyme (E1), (ii) the ubiquitin-conjugating enzyme (E2), and (iii) the ubiquitin protein ligase (E3). As illustrated in **Figure 1.3**, E1 initially catalyzes formation of the thioester bond between the C-terminal carboxyl group of the ubiquitin and a cysteine residue of itself in an ATP-dependent manner. Then, ubiquitin is transferred from E1 to a cysteine residue on E2 by thioester transfer reaction. Lastly, the E3 ubiquitin ligase fulfils ubiquitination of the substrate by facilitating a direct transfer of the ubiquitin moiety either from E2 to the substrate or from an E3-ubiquitin intermediate to the substrate, which depends on the type of the E3 ligase. In humans, two E1s, roughly 30 E2s and more than 600 E3s have been identified so far. Therefore, specificity of the substrate mainly results from E3 ligases. Depending on the E3 ligase, different ubiquitin linkages are generated in terms of the length and composition. Due to their differential and substrate specific activities, E3 ligases and their associated type of ubiquitin modification need to be characterized in depth in order to reveal their specific and

individual functions and in a more general aspect to gain a deeper mechanistic insight of the ubiquitination machinery [16-18].

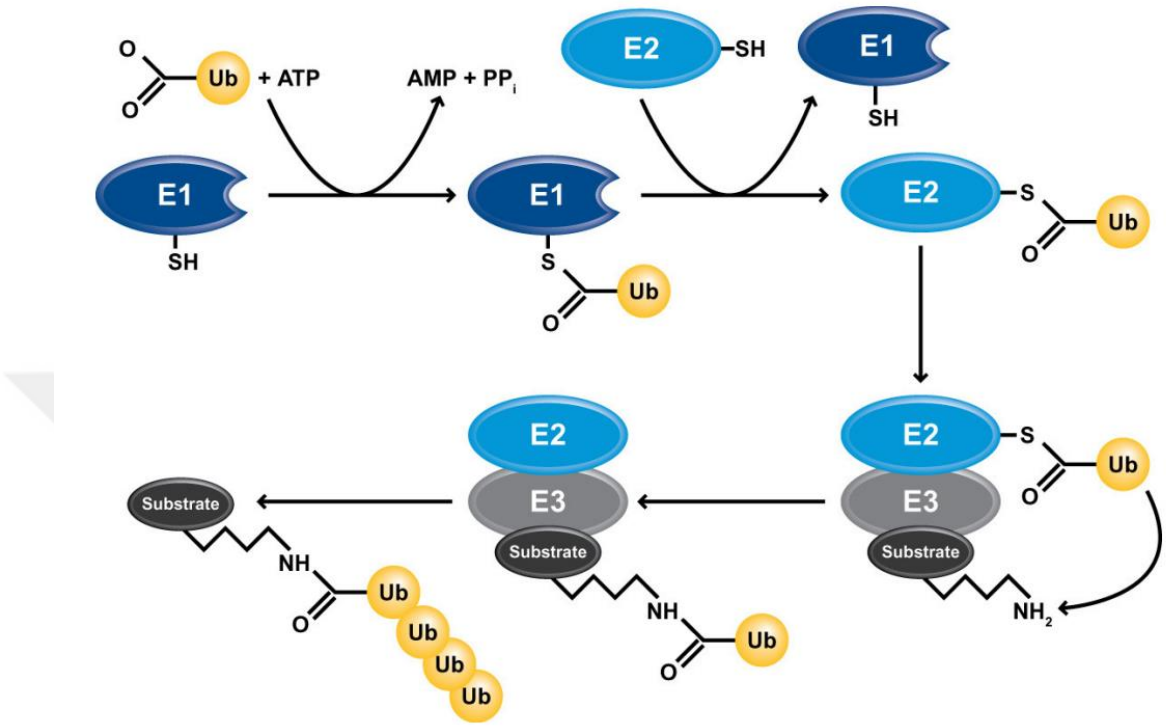


Figure 1.3: General Overview of Ubiquitination Mechanism. Ubiquitin (Ub), ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin protein ligase (E3). Adapted from [19].

Like other PTMs, ubiquitination can also be removed from a targeted protein. It is essential to prevent an uncontrolled signaling that could be detrimental for the cell homeostasis which is why deubiquitination is as important as ubiquitination. The process of controlled ubiquitin removal is mediated by a family of enzymes called deubiquitinases (DUBs) and approximately 100 distinct DUBs are known in humans. Similar to the E3 ligases, DUBs have specific activities depending on the substrate and type of ubiquitin chain that needs to be degraded [20].

1.2 Literature Review

1.2.1 Discovery of E3 Ubiquitin Protein Ligases

After identification of the ubiquitin molecule, the first deep characterization of the ubiquitin system was performed in the early 1980s by Hershko and his colleagues. They were the first to identify three groups of enzymes, namely the E1, E2 and E3 enzymes which were identified in a protein degradation dependent manner [21,22]. Nonetheless, the first study of the specific E3 ubiquitin ligase, UBR1, was carried out in Saccharomyces cerevisiae to assess biochemical significance of the E3s in 1990. Later, multiple E3 ubiquitin ligases were identified in various species and classified depending on their structural similarities [23, 24].

1.2.2 Types of E3 Ubiquitin Ligases

All E3 ubiquitin ligases comprise an E2 ubiquitin binding domain and they are divided into 3 groups, regarding the structure of this domain and their difference in catalyzing the ubiquitination: (i) really interesting new gene (RING) class, (ii) the homologous to the E6-AP carboxyl terminus (HECT) class, and (iii) the ring between ring fingers (RBR) class; and each class shares some conserved domains or residues within themselves [25]. RING type E3 ligases are defined by either their RING or their U-box catalytic domain, enabling direct ubiquitin transfer from E2 to the target protein. Regarding 270 identified genes of the RING type E3 ligases, it is the largest E3 group in human. Some of them may directly bind to an E2 and function on the target protein as a monomer, whereas others require the recruitment of different proteins, pursuing multimeric structure to work properly. Also, they have some conserved cysteines and histidines in the RING domain that enable the binding to Zn^{2+} ions thus stabilizing their conformation, which is necessary for their proper activity [26, 27]. Another category of the E3 ligases is HECT ubiquitin ligases that own binary structure involving an E2 binding domain in their N-terminal region and a catalytic cysteine in their C- terminal region. Furthermore, the most prominent feature of this group is the formation of a ubiquitin-HECT intermediate during the ubiquitination process, which is performed by transfer of the ubiquitin from E2 to its catalytic cysteine followed by formation of an isopeptide bond between ubiquitin and the substrate. Different to the RING E3s, only 28

genes encoding HECT ubiquitin ligases have been identified so far [28, 29]. The third class of the E3s comprises the RBR E3 ligases which are different from both RING E3s and HECT E3s in terms of their catalyzing mechanism. RBRs include RING1, in-between RING (IBR) and really interesting new gene 2 (RING2). RING1 shares similar features with RING type E3s in terms of the conserved cysteine and histidine residues. It also allows binding to E2. The RING2 has a catalytic cysteine, facilitating the catalysis of the ubiquitination after the transfer of the activated ubiquitin molecule. The formation of the ubiquitin-RING2 intermediate happens in a similar way to the HECT E3s during this process as depicted in **Figure 1.4**. In comparison to HECT and RING type E3, only 14 genes encoding RBR E3s have been defined to date and the biological significance of the very few numbers of the members was revealed, such as heme-oxidized IRP2 ubiquitin ligase-1 (HOIL-1), and HOIL-1–interacting protein (HOIP) in immune response signaling [30, 31]. Further studies are crucial to understand and dissect functions and biochemical importance of the other RBR E3 ligases members.

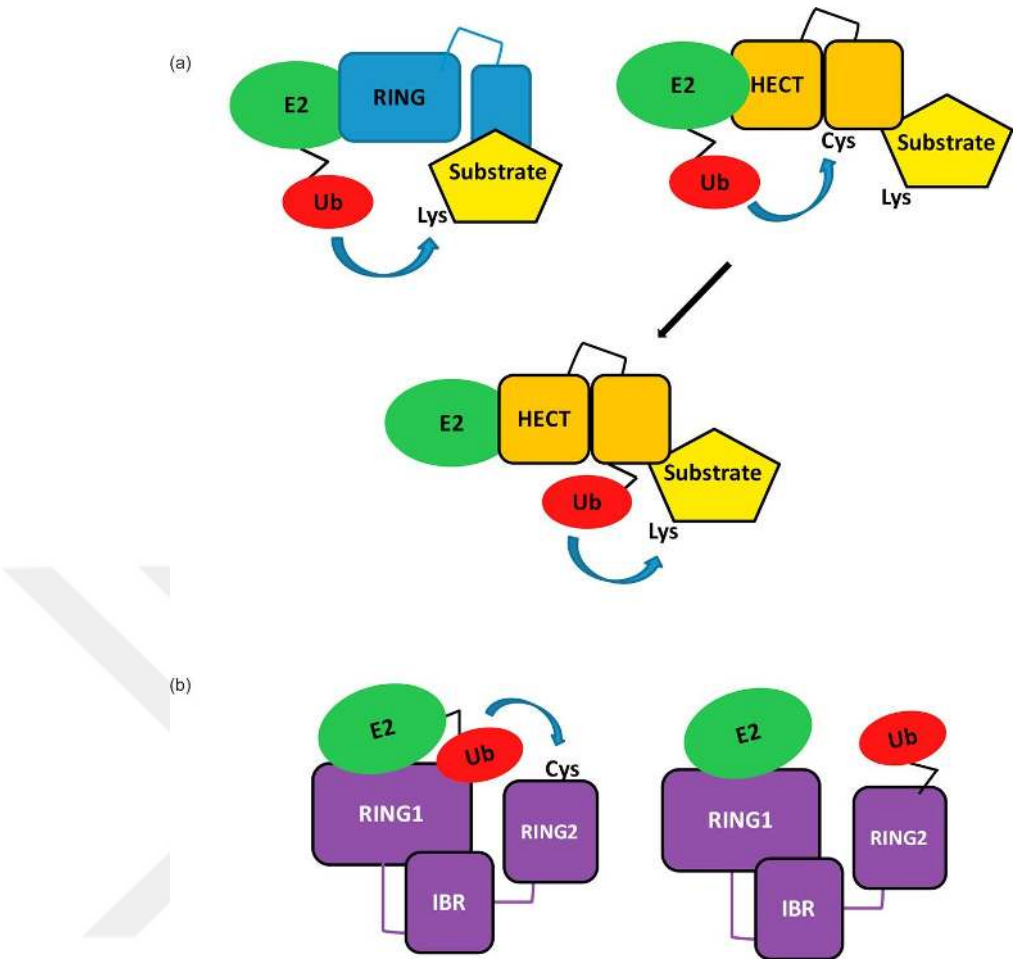


Figure 1.4: General overview of E3 ubiquitin ligase classes. Ubiquitin (Ub), ubiquitin-conjugating enzyme (E2), the homologous to the E6-AP carboxyl terminus (HECT), really interesting new gene (RING), and RING1-IBR-RING2 (RBR). a) Illustration of the ubiquitination mechanism for RING type and HECT type E3s. **b)** Illustration of the ubiquitination mechanism for RBR type E3s. Adapted from [25].

1.2.3 Roles of E3 Ubiquitin Ligases in Gene Activation and Cell Death

To benefit from the different functionalities of the various protein ubiquitination linkages and mechanisms, eukaryotic cells express hundreds of E3 ubiquitin ligases, which provide a broad spectrum of substrate recognition modules, and regulatory components. The unique characteristics of each E3 ligase affect stability and conformation of their target proteins thus enabling diverse cellular activities. One of their roles is the regulation of gene activation by directly ubiquitinating proteins which are

implicated in the activation of some gene activation pathways. It can also act indirectly upon stimulus by triggering proteasomal degradation of either the transcription factor or the inhibitor protein of the transcription factor by K48 ubiquitin chain, which serves as switch of the gene expression [32]. In addition, some of the E3 ubiquitin ligases can execute their functions by either directly or indirectly regulating cell death pathways like programmed cell death (apoptosis) and regulated necrosis (necroptosis). For instance, they can enhance stability of the receptor complexes upon stimulus thereby preventing cell death signaling and promoting gene activation instead. On the other hand, some members of the E3 ubiquitin ligases can promote cell death signaling and cellular disruption [33]. These double-edged sword features of the E3 ubiquitin ligases are often displayed in immune signaling in terms of the regulation of both gene activation and cell death, such as in TNF and TLR3 signaling pathway [34].

1.2.4 TNF Signaling Pathway

Following an infection or a pathogenic income, cells are able to produce and release different cytokines and chemokines. These factors mediate the recruitment and the activation of immune cells, necessary to trigger a robust inflammation and an immune response. One of the most important families implicated in this process is the TNF receptor superfamily (TNFR-SF) [35,36] which comprises 26 different receptors, including the well-known TNFR1, CD95 (Fas), TNF-related apoptosis-inducing ligand receptor 1 and 2 (TRAIL-R1 and TRAIL-R2). These receptors, called death receptors (DRs), can trigger inflammation and control pro-death signals via their cytoplasmic dead domain (DD), causing either apoptotic or necroptotic cell death, which may make a great contribution for cell defense and further immune response. Nonetheless, transition between these two signaling outcomes is tightly regulated [37]. It is therefore essential to understand in depth how the signaling mediated by those receptors is driven and controlled. One of the well characterized systems of this superfamily is TNFR1 signaling and its complex (TNFR1-SC). The first step in this system is the binding of TNF ligand to its receptor TNFR1, which leads to trimerization of the latter. This event is followed by the concerted recruitment of several proteins which leads to the formation of TNFR1-SC or complex I of TNFR1. In more detail, TNFR-associated via death domain (TRADD) binds to the receptor and allows the recruitment of the receptor interacting

serine/threonine protein kinase 1 (RIPK1), TNF receptor-associated factor 2/5 (TRAF2 and/or TRAF5), and the E3 ubiquitin ligases cellular inhibitor of the apoptosis (cIAP) proteins 1 and 2 (cIAP1/2) as shown in **Figure 1.5** [37,38,39]. Once recruited to the complex, cIAP1/2 start to generate K63 ubiquitin chains on various proteins of the TNFR1-SC, including RIPK1 and itself. This auto-ubiquitination allows the recruitment of another E3 ubiquitin ligase complex, called linear ubiquitin chain assembly complex (LUBAC) [40,41]. LUBAC consists of the three different proteins (HOIP, HOIL-1, and SHANK-associated RH-domain–interacting protein (SHARPIN) and it is a unique E3 ligase in that regard that it can generate M1 linked ubiquitin chains (linear chain) on its substrates comprising TNFR1, TRADD, and RIPK1 in TNF signaling [42]. RIPK1 is a central protein in the TNFR1-SC due to its scaffolding activity and it is crucial for the recruitment of other functional proteins and promotes both, the mitogen-activated protein kinase (MAPK) and the nuclear Factor k Light-chain enhancer of the activated B cells (NF-kB) pathways. Once activated, MAPK pathway drives enhancement of the cellular growth through activation of the transcription factors that are necessary for expression of pro-survival genes. On the other hand, NF-kB is one of the main transcription factors that induces production of inflammatory cytokines and it is vital for a proper immune response. In an early phase of this signaling, K63 ubiquitin chain modification on RIPK1 is important to recruit transforming growth factor-β-activated kinase 1(TAK1) and TAK1-binding proteins 2 and 3 (TAB2/TAB3). TAK1 not only induces MAPK pathway, but also contributes to the activation of inhibitor of the nuclear factor kB (IkB) kinase (IKK) complex [43,44]. Meanwhile IKK complex is formed on complex I. It is composed by IKKα, IKKβ, and NF-kB essential modulator (NEMO). The latter is preferentially recruited through M1 chains on RIPK1 as it contains a M1-specific binding domain called Ub-binding domain in ABIN proteins and NEMO (UBAN) domain. Once recruited, TAK1 phosphorylates IKKβ on S177, which enables autophosphorylation of the IKKβ on S181 and activation of the IKK complex. Once activated, IKKβ phosphorylates IkB, leading to its K48 ubiquitination and consequently its proteasomal degradation. This is required for release of the NF-kB complex (p50-p65) and its translocation to the nucleus [45, 46]. Collectively, both MAPK and NF-kB pathways promote expression of pro survival and pro-inflammatory genes. Additionally, M1 chains on RIPK1 are crucial for the recruitment of TRAF Family Member Associated NF-kB Activator (TANK) and/or nucleosome assembly protein 1 (NAP1) through NEMO. They are required to recruit non-

canonical IKKs, TBK1, and IKKε to the TNFR1-SC as well as autophosphorylation and/or transphosphorylation of the TBK1 and IKKε, respectively. These two kinases play an important role in the control of RIPK1 activity and thereby also impact the TNF-induced cell death detailed below [47]. Thus, post-translational modifications that happen in the TNFR-SC are essential to promote the gene activation pathway and prevent the TNF-induced cell death.

In the absence of appropriate ubiquitinations and/or phosphorylations of RIPK1, TNFR1-SC can trigger apoptotic or necroptotic cell death. This requires the formation of a special cytosolic complex with death-inducing capacity, generally named complex II. It includes new components that are able to induce cell death but also other proteins from complex I such as RIPK1 and/or TRADD. They interact with adaptor protein Fas-associated DD (FADD). FADD contains a Death Effector Domain (DED) that promotes the binding to the DED domain of a procaspase 8 homodimer. The latter induces its auto-processing. During auto-processing, procaspase 8 homodimers firstly create p43, p10 fragments and then creation of p30, p26, p18, and p10 fragments are executed due to their self-cleavage activity. This is followed by tetramer complex formation of two p18 and two p10 fragments, called active caspase 8. After its detachment from complex II, active caspase 8 can trigger further activation of the executioner caspases (caspase 3/7) leading to apoptosis [48, 49]. In addition, RIPK1 in complex II can interact with RIPK3 through its RIPK homotypic interaction motif (RHIM) and may lead to its phosphorylation and activation, but phosphorylation of RIPK3 is still controversial. Active RIPK3 executes phosphorylation and activation of the mixed lineage kinase like (MLKL), which is the effector kinase of necroptosis [50-53]. Independently of interaction with RIPK1 and in the course of other signaling pathways, RIPK3 can be activated by binding to other RHIM containing proteins like DNA-induced activator of the IFN (DAI or ZBP1) and TIR-domain-containing adapter-inducing interferon-β (TRIF) and may induce necroptosis [54,55]. However, how interaction partners of RIPK3 are determined for necroptosis initiation is still unclear. Besides, cellular FLICE-inhibitory protein (c-FLIP) contributes to regulation of cell death and all isoforms can form heterodimer with caspase 8, which blocks complete auto-processing and activation of the procaspase 8, thereby preventing apoptosis [56-58]. As mentioned before, RIPK1 is the central protein and main player in TNFR1-SC as it has a decisive role in regulation of the TNFR1-SC. The modification patterns on RIPK1 determine the fate of the TNF signaling pathway. Even though

scaffolding function of RIPK1 protects cells from dying, its kinase activity creates pro-death signals. Thus, restriction of RIPK1's kinase activity and enhancement of its scaffolding function are vital for the cell, which is accomplished by ubiquitination and phosphorylation of its specific residues [38]. For instance, upon TNFα stimulation, cIAP1/2 generates K63 ubiquitin chain on K376/377 of RIPK1, resulting in recruitment of TAK1 complex. TAK1 then initiates a signaling cascade for activation of the MAP kinase-activated protein kinase 2 (MK2) which is normally not recruited to TNFR1-SC yet causes pro-survival phosphorylation of the RIPK1 on S320 in humans [59, 60]. Similarly, IKK complex and non-canonical IKKs are recruited to M1 ubiquitin chains on different sites of the RIPK1 thereby mediating pro-survival phosphorylation of the RIPK1. Moreover, they block autophosphorylation of RIPK1 on S166, a site which if phosphorylated leads to complex II formation and initiation of cell death [61, 62]. Overall, these checkpoints determine TNFα induced signaling output.

In addition to kinases and E3 ubiquitin ligases, DUBs play a crucial role for TNFα-induced inflammation and cell death, such as cylindromatosis (CYLD) and A20, which are present in TNFR1-SC [63,64]. Through its catalytic activity, CYLD limits TNFα-induced gene activation by cleaving K63 ubiquitin chains on RIPK1, leading to initiation of the cell death signal. On the other hand, A20 binds and protects M1 ubiquitin chains, independently of its DUB function, conferring maintenance of the stability of the complex I, thereby persistence of the signal [65,66]. Another regulatory DUB is OTU deubiquitinase with linear linkage specificity (OTULIN) which specifically cleaves M1 chains to either prevents M1 linkage dependent recruitment of canonical and non-canonical IKKs to the complex or switch off the inflammation-related gene activation signaling. After that, cells also render more prone to cell death signaling. OTULIN may be recruited to the complex I by direct interaction with HOIP as they possibly function as a bimolecular editing couple, albeit this is still not well defined [67].

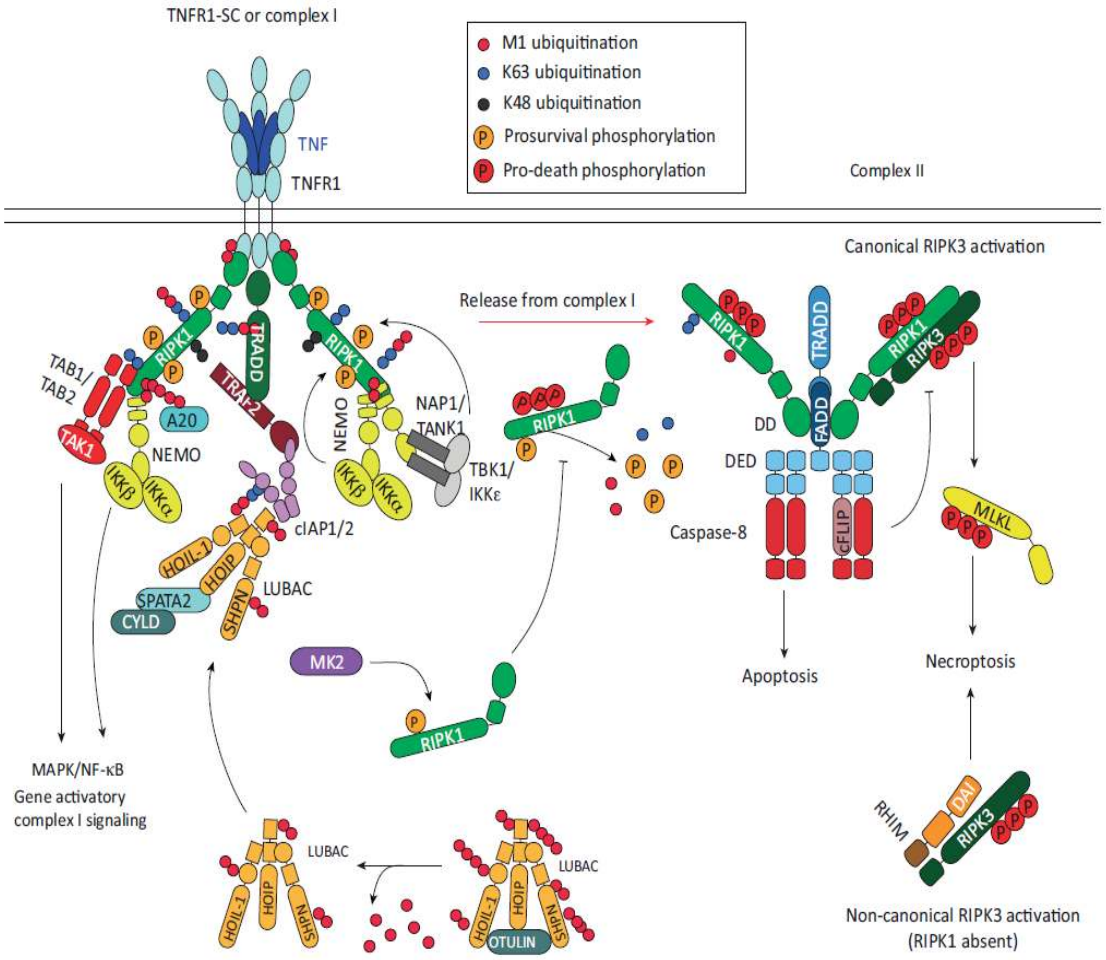


Figure 1.5: General overview of Tumor Necrosis Factor (TNF) Receptor 1 (TNFR1) Signaling Complexes (TNFR1-SCs) and Their Regulation. Mitogen-activated protein kinase (MAPK), nuclear factor k light-chain enhancer of the activated B cells (NF-kB), TNFR1-associated via death domain (TRADD), TNF receptor-associated factor 2 (TRAF2), and cellular inhibitor of the apoptosis proteins 1 and 2 (cIAP1/2) ,receptor-interacting protein kinase 1/3 (RIPK1/3), transforming growth factor-β-activated kinase 1 (TAK1), TAK1-binding proteins 2 and 3 (TAB2/TAB3), inhibitor of the nuclear factor kB kinase subunit α and β (IKKα/IKKβ), NF-kB essential modulator (NEMO), TANK-binding kinase 1 (TBK1), linear ubiquitin chain-assembly complex (LUBAC), SHANK-associated RH-domain–interacting protein (SHARPIN), heme-oxidized IRP2 ubiquitin ligase-1 (HOIL-1), and HOIL-1–interacting protein (HOIP), MAPK-activated protein kinase 2 (MK2) CYLD (cylindromatosis), death domain (DD), death effector domain (DED) Fas-associated DD (FADD), RIPK homotypic interaction motif (RHIM), Cellular

FLICE inhibitory protein (c-FLIP) DNA-induced activator of the IFN (DAI, ZBP1), mixed lineage kinase domain-like (MLKL). Adapted from [37].

1.2.5 TLR3 Signaling Pathway

In vertebrates the immune system can be classified into two subgroups, the adaptive immunity and the innate immunity. While adaptive immunity functions on basis of antigen specific receptors and is a more sophisticated defense system, innate immunity, which is the first line of defense in the body, relies on recognition of similar molecules associated with pathogens or host-cell stress. These molecules are evaluated in two categories; pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). DAMPs may originate from host cells involving tumor cells, dead cells, such as RNA or DNA and they are relevant to tissue damage and cellular stress. PAMPs on the other hand consist of molecules that are of pathogen origin, e.g. bacterial lipopolysaccharide or double strand RNA (dsRNA) typical for some viruses [35]. Thus, both immune and non-immune cells express those receptors able to recognize such molecules, called pattern recognition receptors (PRRs) and take an action [35, 36]. One of the largest class of the PRRs is the class of the TLRs that have the ability to recognize a broad spectrum of the PAMPs. Each subtype of the TLRs is specialized to one sort of molecule [68]. For example, TLR3 is a crucial receptor for recognition of viral dsRNA and initiation of anti-viral response. It is widely expressed among cells and is firstly glycosylated in the Golgi apparatus and then directed to the endosomal membrane with the help of the chaperon UNC93B. Later, it has to be cleaved on its extracellular domain by some unknown cathepsins in order to form homodimers upon binding of the ligand [69]. Once dimerized, TLR3 recruits TRIF to relay signals to different downstream effectors as illustrated in **Figure 1.6** [70]. TRIF interacts with TRADD, RIPK1, but also three RING type E3 ubiquitin ligases, Pellino-1, TNF receptor associated factor 6 (TRAF6) and TRAF3, which initiates formation of the TLR3 signaling complex (TLR3-SC) [71, 72]. TRAF6 and RIPK1 are associated with MAPK and NF-kB pathways whereas TRAF3 induces type I interferon production (IFNα/β). Furthermore, RIPK1 interaction with TRIF is based on RHIM-RHIM binding of the two proteins whereas TRAF6 binds to the N-terminus of TRIF, collectively relaying the signal. Yet, both proteins require K63-linked polyubiquitination for the binding of the TAB2/3, facilitating

the recruitment of TAK1 and initiation of MAPK pathway and activation of IKKβ activation. Although TRAF6 can ubiquitinate RIPK1, RIPK1 requires the contribution of additional E3 ubiquitin ligases for an enhanced stability and stimulation of the several outcomes [73-76]. Similar to TNFR-SC, LUBAC is also recruited to TLR3-SC and generates M1-linked ubiquitin chains on components of the TLR3-SC, following recruitment and activation of the IKK complex that triggers phosphorylation and degradation of the IkB and nuclear translocation of the NF-kB. Yet, which TLR3 complex components serve as LUBAC substrates and are linearly ubiquitinated requires further clarification [77]. On the other hand, TRAF3 generates K63 polyubiquitin chains on itself, which presents a platform to recruit consecutively NEMO, adaptor proteins TANK and/or NAP1, following TBK1 and IKKε similarly as in TNFR-SC; nonetheless it has been suggested that K63 ubiquitin chains are generated on TBK1 in favor of its activation by mind bomb (MIB) and RING finger 128 (RNF128) prior to its recruitment to the complex. However, this is not confirmed with in vivo studies yet [78-80]. Recruitment of TBK1 and IKKε leads to their auto- or transphosphorylation and activation. Active TBK1 firstly phosphorylates serine of the TRIF's LxIS IRF3 binding motif, which is important for recruitment of IRF3. Then TBK1 phosphorylates recruited IRF3, leading to its homodimerization and dissociation from the complex. Subsequently, IRF3 homodimer is translocated to the nucleus in order to bind IFN-stimulated response element (ISRE) and induces IFNα/β expression. Furthermore, TBK1 can phosphorylate IRF7 and induce heterodimer formation between IRF3 and IRF7. However, expression of IRF7 is usually much lower than of IRF3, which is why IRF3 serves as the main regulator for initiation of the IFNα/β transcription [81-83]. In addition to IRF3 and TRIF, TBK1 can phosphorylate and activate Pellino-1, leading to RIPK1 ubiquitination and NF-kB activation [84, 85]. Like in TNF signaling, regulation of the TLR3 signaling mostly relies on the E3 ubiquitin ligases and DUBs. For example, distinctive E3 ubiquitin ligases encompass proteasomal degradation of the different components of the complex by adding K48-linked polyubiquitin chains such as modulation of TBK1 turnover through activity of TRAF-interacting protein (TRIP), while DUBs like CYLD and A20 remove K63-linked polyubiquitin chains on the modules of the TLR3-SC, such as TRAF6, thereby destabilizing the complex, consequently turning off signal transduction [68,86-90]. Additionally, some regulatory proteins compete with TLR3 for TRIF binding in order to block its recruitment to the complex [91]. Overall, the TLR3 pathway is less well

characterized than the different pathways of the TNF superfamily and further investigations are required in order to fully understand this essential pathway. For instance, activation and regulation of the TBK1 and IKKε are not well characterized and need further studies to be elicited.

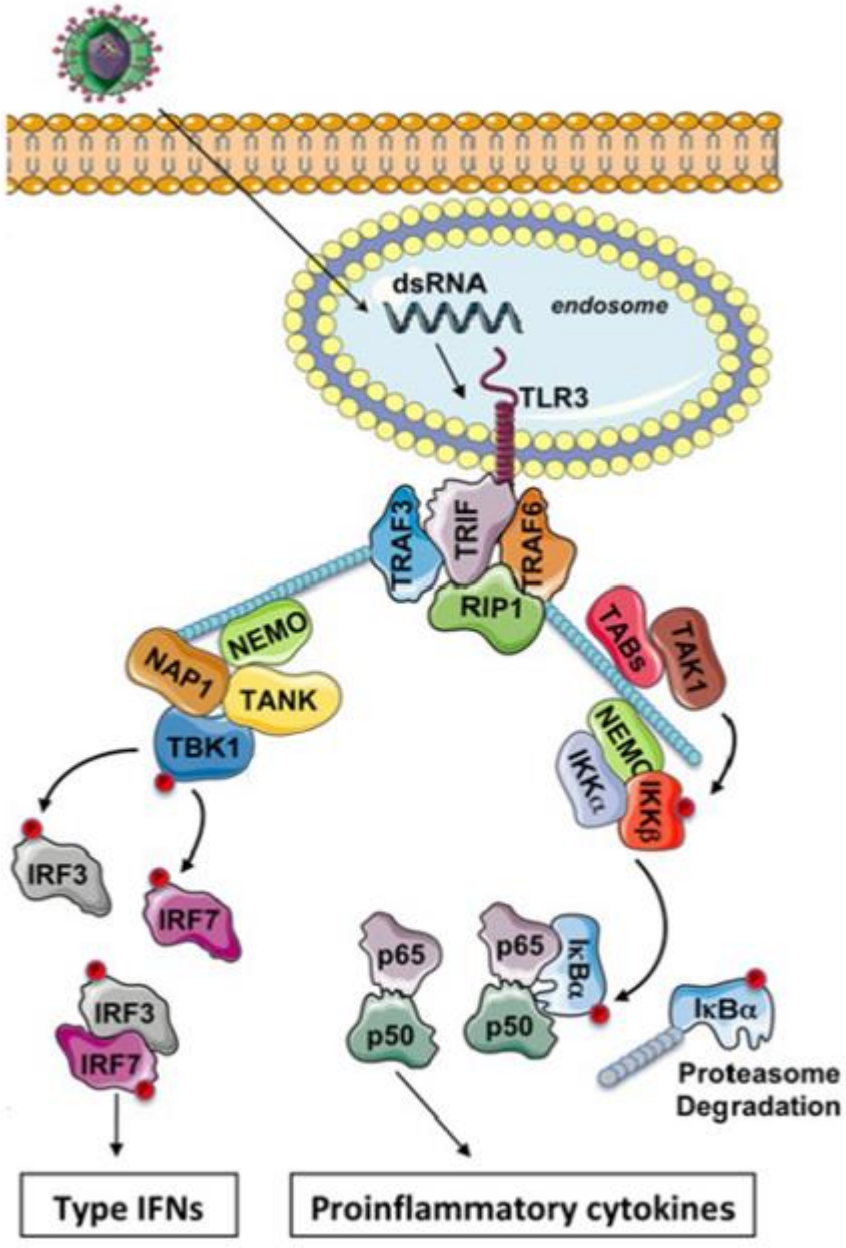


Figure 1.6: Overview of TLR3 pathway. Toll-like Receptor 3 (TLR3), TIR-domain-containing adapter-inducing interferon-β (TRIF), TNF receptor-associated factor 3/6 (TRAF3/6), receptor-interacting protein kinase 1 (RIPK1), transforming growth factor-β-activated kinase 1 (TAK1), TAK1-binding proteins (TABs), inhibitor of the nuclear factor kB (IkB) kinase subunit α and β (IKKα/IKKβ), NF-kB essential modulator

(NEMO), TRAF Family Member Associated NF-kB Activator (TANK), nucleosome assembly protein 1 (NAP1), TANK-binding kinase 1 (TBK1), NF-kB proteins (p50-p65), IFN regulatory factor 3/7 (IRF3/7), interferon (IFN). Adapted from [70].

In addition to gene activation, TLR3 can induce cell death. Upon viral infection, viruses try to block IFN and proinflammatory cytokine production by either inhibiting functional proteins within TLR3-SC or their downstream effectors in order to replicate themselves efficiently. As another host cell defense mechanism, infected cells may trigger cell death pathways to reduce viral replication and initiate inflammation [92]. Studies showed that similar to complex II formation in TNF signaling, TLR3- induced Death-Inducing Signaling Complex (DISC) that leads to cell death originates from membrane bound TLR3-SC and dissociate over time. Yet, data generated by the host lab and other laboratories working on that signaling pathway suggest that there is also the possibility that the TLR3-SC and the TLR3- induced DISC may be formed sequentially and independently from each other and further elucidation is necessary to understand the actual mechanism. Interestingly, towards inhibition of the signal from TLR3 and growing viral replication in the cell, TLR3 can induce initiation of the TLR3-induced DISC along with lacking of the proper PTM and some of the other crucial proteins on TLR3-SC. First of all, TLR3 recruits TRIF and RIPK1 consecutively, facilitating RIPK1- FADD interaction and further procaspase 8 recruitment in a similar context to TNF-induced complex II formation. [73, 77, 92, 93]. Subsequently, self-cleavage and activation of the procaspase 8 is triggered, which also causes activation of the executioner caspases and eventually the cell death through apoptosis. As a regulatory mechanism cIAPs may be found in TLR3- induced DISC complex and thwart cell death by K48-linked ubiquitination of RIPK1 and its degradation. Moreover, LUBAC indirectly blocks TLR3-induced cell death by stabilizing TLR3-SC for gene activation pathway which can simultaneously increase c-FLIP expression that is able to inhibit cell death. In case of the inhibition of any essential protein like caspases in apoptosis pathway, cells may die via alternative necroptotic pathway. In this pathway, RIPK1 binds to RIPK3 via the RHIM domains and may directly phosphorylate it or provide its autophosphorylation, which is however not well defined yet. Active RIPK3 then induces the conformational change of MLKL as a consequence of its phosphorylation, followed by pore formation in plasma membrane and leakage of the cellular content [92]. In certain scenario, particularly in

TLR3 and TLR4 pathways, necroptosis may be accomplished via the RIPK3-TRIF interaction axis, and thereby independently of RIPK1 [94].

1.2.6 Apoptosis

Apoptosis is a unique version of programmed cell death. It is a crucial process that is implicated in not only developmental stages but also homeostasis in the cells. Once initiated, cells systematically display particular features including chromosome condensation, DNA fragmentation and cleavage of structural proteins, pursuing morphological changes like blebbing of the plasma membrane, eventually cellular disintegration. So far, two main pathways have been described for apoptosis; firstly, the extrinsic pathway associated with DRs and secondly the intrinsic pathway mediated by mitochondria after cellular stress or damage. The hallmark protein family implicated in apoptosis are the cysteine-aspartic acid proteases (caspases) [95].

Even though some immune receptors like TLRs can initiate apoptosis signaling and employ similar mechanisms to the "real" DRs, the extrinsic pathway is mostly induced by one of the three major receptor/ligand coupling systems which all belong to the DD-containing TNRFRSF-TNFSF and are: (i) TNF-TNFR1, (ii) Fas-FasL and (iii) TRAIL-TRAIL-R1/2. As illustrated in **Figure 1.7**, even though TNF-TNFR1 system propagates on previously explained complex II, Fas-FasL and TRAIL-TRAIL-R1/2 systems differ from it to induce cell death. After binding their respective ligands to Fas and TRAIL-R1/2, they can recruit procaspase 8 to membrane-bound -ligand-receptor complex via FADD. There is no TRADD engagement in the pathway unlike TNF-TNFR1 system. Juxta-positioning of procaspase 8s leads to their self-cleavage and p18-p10 tetramer formation (active caspase 8) consecutively. Once activated, caspase 8 is released from the complex and activate executioner or effector procaspases (caspase 3/6/7) by cleaving them; hence, effector caspases can dismantle many cellular substrates, such as cytoskeletal proteins, pro-survival proteins, inhibitor of the endonucleases etc., which consequently leads to apoptosis and the death of the cell. Furthermore, caspase 3 can activate caspase 6/7 and accelerate cellular disintegration [96]. However, signaling of Fas-FasL and TRAIL-TRAIL-R1/2 systems can always not result in cell death outcome thanks to some regulatory mechanism, similarly to TNF-TNFR1 signaling. For instance, large amount of the c-FLIP can block the initiation of the apoptosis in the cell by

interfering activation of the caspase 8. The caspase can still do the first processing but not the other one. Nevertheless, there is growing evidence, concerning the primordial role of cFLIP to constitute the "filament "of caspase 8 in the DISC and there are still many ongoing studies about role of cFLIP [97]. Also, one of the caspase 8 homologs, caspase 10, can negatively regulate caspase 8 mediated cell death via interference to its association with DISC [98]. Besides, TRAF2 may generate K48 ubiquitin chain on active form of the caspase 8, giving rise to its proteasomal degradation [99]. Moreover, DR mediated apoptosis pathway is sometimes not sufficient to accomplish apoptosis and active caspase 8 can cross-signal to intrinsic apoptosis pathway by severing BH3 interacting domain death agonist (BID). This facilitates its translocation to the outer mitochondrial membrane and results in an amplification of the apoptosis signaling [100]. In addition, intrinsic apoptosis is linked to mitochondria and release of proteins from its intermembrane space along with mitochondrial outer membrane permeabilization (MOMP) [101]. MOMP is regulated by members of the Bcl-2 family which shares up to 4 Bcl-2 homology domains (from BH1 to BH4) and are categorized as three different classes according to their function in apoptosis and their mutual BHs; one anti-apoptotic and two pro-apoptotic classes. Anti- apoptotic proteins like Bcl-2, Bcl-xL and Mcl-1 own either three or four BH domains, while the first pro-apoptotic class containing Bad and Bax, shares two or three BH domains. Another pro-apoptotic class has only BH3, such as Bid [102]. In the intrinsic apoptosis mechanism, Bak and Bax are mandatory components to create a pore together on the outer mitochondria membrane. In a normal situation, anti-apoptotic Bcl-2 proteins either segregate Bak and Bax proteins or inhibit apoptosis-activating BH3-only proteins thereby preventing apoptosis. These constraints may be shifted by BH3-only proteins as well [102, 103]. Upon apoptotic stimuli, BH3- only proteins like Bid (previously cleaved by caspase 8) can block anti-apoptotic Bcl-2 proteins and allow Bak-Bax activation and MOMP [104]. After formation of the pores, cytochrome C is released to the cytosol, triggering the formation of the so-called "apoptosome" which forms upon oligomerization of the apoptotic protease activating factor 1 (APAF-1) followed by clustering of procaspase 9. After self-cleavage and activation of procaspase 9, it can activate further effector caspases, enforcing apoptosis [105, 106]. In addition to cytochrome C, second mitochondria-derived activator of the caspase/direct IAP-binding protein with low pI (SMAC/DIABLO) are released to both, bind and induce the proteasomal degradation of X-linked inhibitor of apoptosis (XIAP)

and cIAP1/2 via their auto-ubiquitination. XIAP is able to directly inhibit active caspase 3 by inducing its proteasomal degradation [107, 108]. In addition to both intrinsic and extrinsic pathways, cytotoxic T cells and natural killer cells may sometimes use perforin-granzyme systems to stimulate apoptosis in the targeted cell. In this scenario, they convey perforin and granzymes via exocytosis to the targeted cell. Perforin enables pore formation on the plasma membrane of the cell whereas granzymes activate downstream caspases like procaspase 8 and procaspase 3 and trigger apoptosis [102, 109,110]. Besides, apoptosis does not usually give rise to a strong inflammatory phenotype but still leads to the activation of the immune system via the engulfment of the apoptotic cells. For a long time, it has been claimed that apoptosis is an immuno-silent modality of cell death but evidence are accumulating that shows the opposite [111].

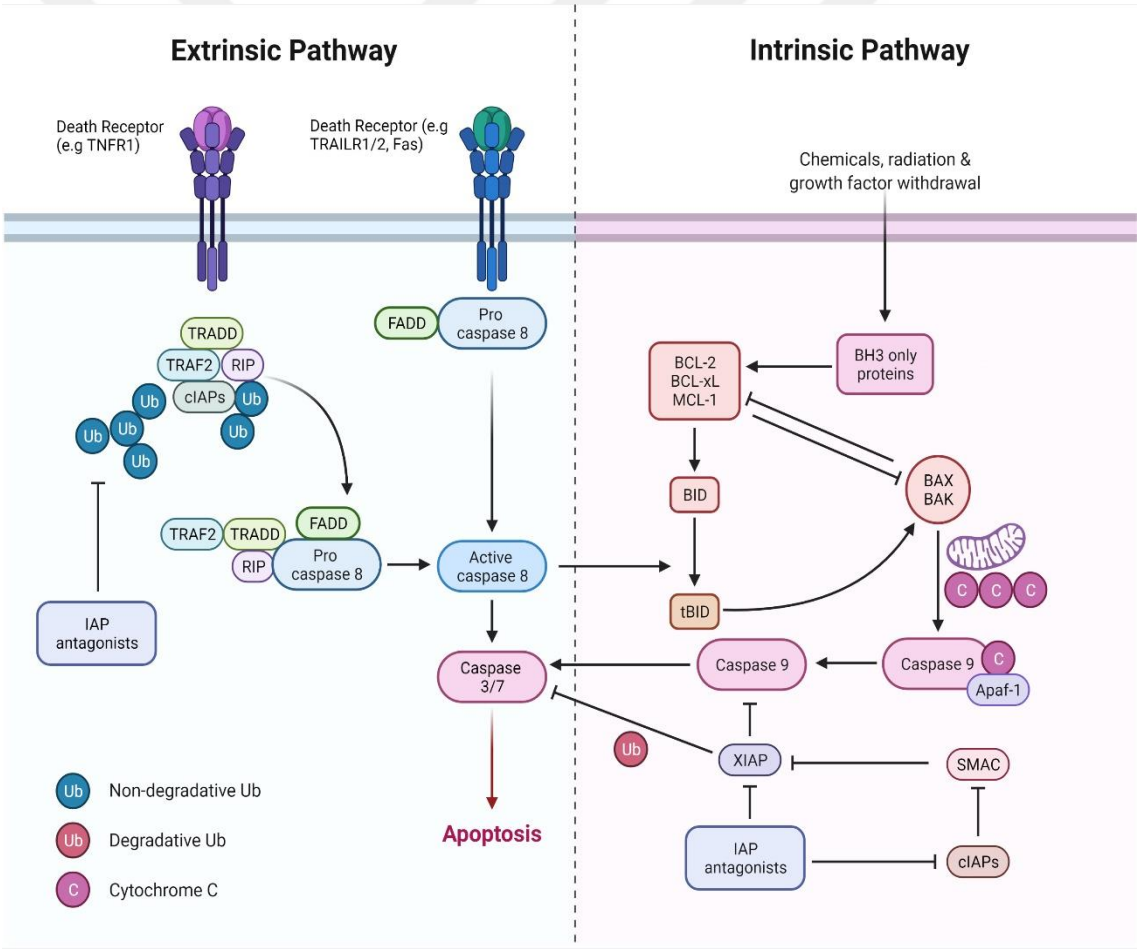


Figure 1.7: General overview of the extrinsic and intrinsic apoptosis pathways. Tumor Necrosis Factor (TNF) Receptor 1 (TNFR1), TNF-Related Apoptosis-Inducing Ligand Receptor 1/2, TNFR1-associated via death domain (TRADD), TNF receptor-associated factor 2 (TRAF2), and cellular inhibitor of the apoptosis

proteins(cIAPs),receptor-interacting protein kinase (RIP), Fas-associated DD (FADD) apoptotic protease activating factor 1 (APAF-1), X-linked inhibitor of the apoptosis (XIAP), second mitochondria-derived activator of caspase/direct IAP-binding protein with low pI (SMAC/DIABLO), BH3 interacting domain death agonist (BID),truncated BID (tBID), ubiquitin(Ub). Adapted from [112].

1.2.7 Necroptosis

Though necrosis has been thought as an accidental cell death for a long time, the fact that necrosis can be regulated was discovered in 2005. However, this regulated necrosis displays some characteristics of both necrosis and apoptosis, which is then named as necroptosis [113]. Necroptosis is morphologically described as cell swelling and disruption of the plasma membrane. Moreover, it is an alternative cell death pathway when apoptosis is inhibited by either some agents or by pathogens, which cause both, the release of increased inflammatory cytokines and DAMPs that will consequently lead to the increase of the immune cell infiltration to the inflammatory site. Necroptosis can be induced by DRs and PRRs. In context of the DRs, DISC formation is firstly exhibited in the apoptosis pathway, but if the apoptotic signal can't proceed as a result of the pharmacological inhibition or elimination of caspase 8, RIPK1 interacts with RIPK3 along with their RHIM domain, which promotes either directly or indirectly the phosphorylation and activation of RIPK3. Active RIPK3 can phosphorylate the pseudokinase MLKL, followed by its oligomerization and translocation to the plasma membrane [114, 115]. MLKL oligomers create a pore on plasma membrane, resulting in especially Ca^{2+} and Na^{+} influx. It disrupts the osmotic balance of cells and gives rise to cell swelling as well as rupture of the plasma membrane. This is known as canonical necroptosis pathway [116]. Nevertheless, the contribution of ion channels in necroptotic pathways is still not fully understood and requires further investigations. In spite of the fact that PRRs like TLR3 can induce necroptosis pathway in a very similar manner, it still differs in certain ways and thus it is clear that there are more ways to induce necroptosis. TRIF in TLR3/4 pathway and DAI proteins have also RHIM domains and they can associate with RIPK3 and result in its activation, followed by initiation of the necroptosis signal, which is known as non-canonical necroptosis. However, whether RIPK1 has a role in this pathway and RIPK3 can phosphorylate itself are still unclear [117,118]. In

regulation of the necroptosis pathway, some of E3 ubiquitin ligases play a role by either stabilizing DR signaling complexes, such as cIAP1/2 or controlling turnover of modules of necroptosis pathway. For instance, TRIM25 can induce proteasomal degradation of RIPK3 via generation of K48 ubiquitin chain [119]. In addition, some E3 ubiquitin ligases can ubiquitinate MLKL oligomers and lead to their degradation in proteasomal or lysosomal dependent manner, thereby preventing necroptosis [120]. Besides, the endosomal sorting complexes required for transport (ESCRT] machinery can compete with MLKL by repairing plasma membrane and bring on either complete blockage of the necroptosis or delayed necroptosis [121]. In addition to this, heterodimers formed of c-FLIP/procaspase 8 in the DISC complex can still cleave both RIPK1 and RIPK3, thereby blocking necroptosis [58].

1.2.8 Aim of the Project

The immune system has a sophisticated architecture consisting of both, special immune cells like macrophages as well as of molecules such as immunoglobulins and cytokines. Both are essential to clear pathogens during an infection. They are also important to prevent cancer by inducing the death of cancer cells that is why very often tumors very often aim to bypass this control by inhibiting the immune system. Any deficiency, or abnormality in the immune system may cause increased lethality, fatal infection, loss of the body homeostasis or cancer. Detection of the reasons behind these dilemmas is very critical and it is important to understand how immune responses are operated especially to develop appropriate targeted therapies to overcome these health problems. As a perspective, the most of these issues result from impairment of the biochemical circumstances and signaling both in each individual cell and among cells. Because of that, proteins which are responsible for these biochemical pathways are the most interesting candidates for therapies and their three-dimensional structures present potential various binding sites for small molecules. Nonetheless, non-immune cells like epithelial cells may contribute to immune response function and regulation by producing self-protective molecules, cytokines, leading to recruitment of the immune cells to the inflammation site and they may sometimes cause this kind of the deterioration if the inflammation persists. As a consequence of that, dissection of the immune response

associated pathways and proteins has gained great importance for development of the efficient targeted therapies.

Because TNFα is one of the major driving cytokines produced by non-immune cells that has a primordial role, researchers are extensively working on this pathway in order to fully understand the mechanism of action but also its regulation. As detailed previously, E3 ligases have been reported to play a key role of regulation in TNF, but also other immune/inflammatory signaling. The Walczak lab has notably and extensively contributed to the increase of this knowledge. Indeed, they have been one of the first labs to report the implication of the unique and essential E3 ubiquitin ligase LUBAC in TNF and TRAIL signaling but also in TLR3 signaling. LUBAC adds M1 ubiquitin chains on components of different signaling complex components, notably RIPK1[122]. Its activity is crucial for the proper cytokine production, cellular growth and survival but also to enhance stability of the signaling complexes, hampering induction of cell death [123]. Moreover, the addition of the linear ubiquitin chain on RIPK1 functions as scaffold for recruitment of TBK1 and IKKε via adaptor proteins such as NEMO and TANK/NAP1 to the TNFR signaling complex. In contrast to other signaling complexes such as TLR3, the recruitment of these non-canonical IKKs does not induce the type I interferon production. Yet, they phosphorylate RIPK1, consequently preventing its autophosphorylation and the activation, thus contributing to inhibit the cell death [47]. Intriguingly, despite the same general role for LUBAC in TLR3 in terms of cell death inhibition and MAPK/NF-kB pathway, the host lab has shown that in absence of HOIP, TBK1 recruitment and activation to the signaling complex was barely impacted. Because of its essential role for the interferon production, I aimed to decipher how TBK1 is recruited and activated in TLR3 pathway. In addition to previous explanation about how TBK1 serves in the production of IFNα/β, it was previously reported that optineurin (OPTN) can dimerize to ubiquitinated TBK1 through its ubiquitin binding domain (UBD) and triggers auto phosphorylation of TBK1 thereby enhancing the production of IFNα/β in TLR3 induction dependent manner. Any interference to this process causes impairment of IFNα/β [124]. Unlike TLR3 signaling, OPTN does not play a role in TNFα signaling according to current data of the host lab. Albeit, ubiquitination of TBK1 and interaction partners of the OPTN need to be determined for the deep understanding TLR3 induced IFNα/β production. Overall, after reviewing the reported data and several observations, I proposed that activity of a new E3 ubiquitin ligase may be essential in TLR3 pathway for

TBK1 recruitment and activation, succeeding IFN I production in an OPTN-dependent manner. Considering an immense effect of the RBR involving E3 ligases in immune response like HOIP, I decided to focus on the RBR family of the E3 ligases and as a result of the bioinformatics analysis, NEMO and OPTN were determined as putative substrates for ANKIB1 E3 ubiquitin ligase.

ANKIB1 is defined as a putative RBR type E3 ligase to date, because it has not been demonstrated whether this protein harbors a catalytic activity or not, also very few is known about ANKIB1.

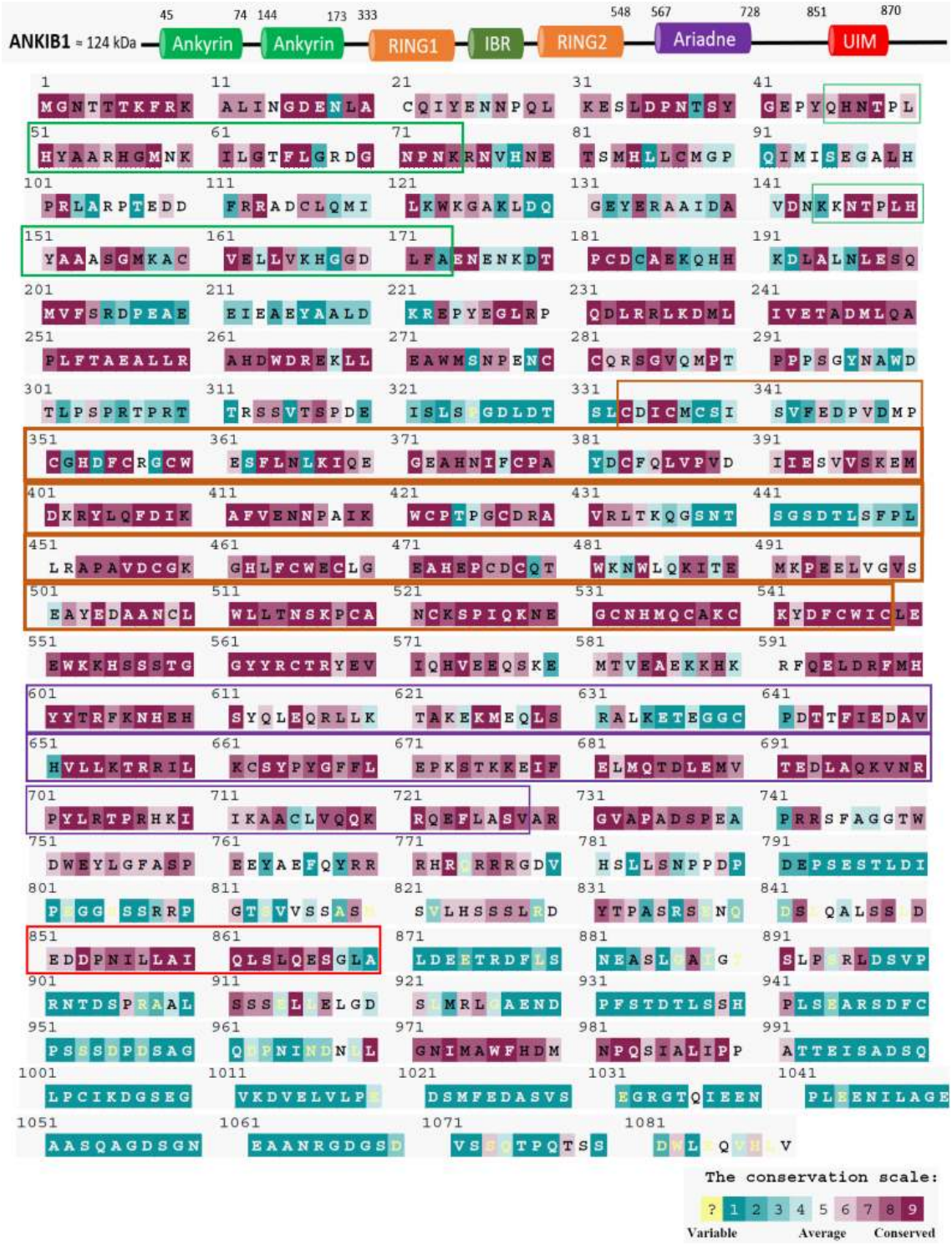


Figure 1.8: Predicted Domains and Conserved Residues of the ANKIB1. Conservation scores of residues of ANKIB1 among species were obtained from ConSurf Server.

However, it has several domains strongly conserved within the different species. ANKIB1 comprises a long Ankyrin repeat domain in its N terminus, which may be crucial

for substrate protein interaction, and the RBR domain which has intact RING1, IBR and RING2 as illustrated in **Figure 1.8**. Moreover, combination of the several reports and structural prediction by AlphaFold software revealed that ANKIB1 may have functional Ariadne domain that owns four successive alpha helices and can sterically hinder the binding site of the RING2 to ubiquitin moiety in the native conformation [25]. Consequently, I can expect that ANKIB1 requires either interaction with a substrate protein or posttranslational modifications, such as phosphorylation and ubiquitination to be activated [125, 126]. Additionally, ANKIB1 contains ubiquitin interacting motif (UIM) facilitating its binding to polyubiquitin chain preferentially. Furthermore, upregulation of the ANKIB1 was reported upon bacterial infection suggesting that ANKIB1 may have a role in the regulation of the immune signaling, especially TLRs pathways [127].

Regarding what is known about ANKIB1, the host lab recently obtained results suggesting that ANKIB1 is indeed able to generate ubiquitin chains and that it is a part of TLR3-SC. For further investigation and insight of the ANKIB1 biology, the aim of the current master thesis project is to address the role of the ANKIB1 in regulating cell death signaling versus gene activation following TLR3 activation. To do so, cell death induction and the status of the different gene activation pathways was monitored following TLR3 stimulation in cells that express ANKIB1 as compared to cells in which ANKIB1 has been deleted by CRISPR-Cas9. These cellular models were also employed to study the impact of the ANKIB1 on the production of the cytokines known to be required downstream of the TLR3 activation for mounting an effective immune response.

Chapter 2: MATERIALS AND METHODS

2.1 Mutagenesis of ANKIB1

On the purpose of the inserting flexible linker motif between ANKIB1 and C terminally located moTAP tag (1x Flag and 2x Strep tags), PCR was performed by primers #1, #2, and previously cloned ANKIB1 in pBABE-puro vector which was a gift from Hartmut Land & Jay Morgenstern & Bob Weinberg (Addgene plasmid # 1764 ; http://n2t.net/addgene:1764 ; RRID:Addgene_1764) as a template, using NEB Q5® Site-Directed Mutagenesis Kit E0554S, regarding mixture and condition of the reaction as shown in **Table 2-1** and **Table 2-2**, respectively[128].

Table 2-1: Reaction mixture for addition of linker motif to ANKIB1 in pBABE vector

Ingredients	Stock Concentration	Amount	Final Concentration
Q5 Hot Start High Fidelity 2x Master Mix	2x	12.5 µl	1x
Forward Primer	10µM	1.25 µl	0.5 µM
Reverse Primer	10µM	1.25 µl	0.5 µM
Template DNA	5 ng/µl	1 µl	-
Nuclease-free dH_2O	-	9 µl	-
Total Volume	-	25 µl	-

Table 2-2: Reaction Conditions for addition of linker motif to ANKIB1 in pBABE vector

Steps	Temperature	Time	
Initial Denaturation	98°C	30 seconds	
Denaturation	98 °C	10 seconds	25 cycles
Annealing	60 °C	30 seconds	
Extension	72 °C	3 min 15 seconds	
Final Extension	72 °C	2 minutes	

After the PCR, process was continued with KLD reaction and transformation was subsequently performed as manufacturer instructions. Bacteria were spreaded ton LB-Agar plate containing 100 μg/ml ampicillin. The following day, several bacterial colonies were picked and grown in LB containing 100 μg/ml ampicillin for overnight. Finally, plasmid isolation from bacteria was executed with QIAprep Spin Miniprep Kit Qiagen 27104, followed to sequencing of the plasmids by primers #5, #6, #7, #8 in Table C-1.

Same protocol was performed to create catalytically inactive RBR mutant of the ANKIB1-moTAP by using primers #3, #4 and WT ANKIB1-moTAP with linker motif as a template.

After validation of the plasmid, they were amplified and isolated with MN Nucleobond Xtra Midi kit (Takara Bio 740410.100).

2.2 Stable Cell Line Generation and Subcloning

In order to generate viral particles and stably reconstitute ANKIB1 expression in cells, 5 million Platinum Ampho cells were seeded to three 10 cm dishes in Dulbecco's Modified Eagle's Medium (DMEM) supplemented by only 10% Fetal Bovine Serum (FBS). Next day, 15 μg retroviral pBABE vector including either WT or RBR mutant ANKIB1-moTAP were mixed 45 μl at 1mg/ml polyethylenimine (PEI) (Sigma-Aldrich 764965) in Opti-MEM (Gibco 31985062) and they were incubated at room temperature for 20 minutes as well as it was performed for only pBABE vector (empty vector). Then, cells in each dish were transfected with one of these mixtures. After 2 days, supernatant of the cells were collected and filtered by 0.45 micron filter Later, these supernatant comprising viral particle with related vector were mixed with fresh RPMI supplemented by 5%FBS and 1% penicillin-streptomycin added to previously seeded ANKIB1 KO HeLa cells (300,000) and HT29 cells (450,000) in 6 wells plate to transduce different versions of the ANKIB1 or empty vector. Meanwhile, these cells were treated with 8 μg/ml polybrene (Sigma-Aldrich TR-1003) to increase efficiency of the transduction. Following days, HeLa and HT29 cells were selected with 3 μg/ml or 4.5 μg/ml puromycin (Sigma-Aldrich P8833-25MG), respectively. Subsequently, single cell dilution was prepared for HeLa reconstituted WT ANKIB1-moTAP pool and RBR mutant ANKIB1-moTAP cells separately before seeding them 96 wells plates. After allowed them to grow

adequately, they were transferred to 24 wells plates and 6 wells plates in time, respectively. After obtained enough amount of the cells, ANKIB1 expression was evaluated for each of them by western blotting as described below without any treatment.

2.3 Western Blot for Gene Activation and ANKIB1

300,000 WT and ANKIB1 KO HeLa (or HaCaT (human aneuploid immortal keratinocyte cell line)) as well as 450,000 WT and ANKIB1 KO HT29 (human colon cancer cell line) cells were seeded to 6 wells plates for respective conditions (0, 15 minutes, 1hour 200 ng/ml TNF and 0, 30 minutes, 1 hour 10 µg/ml poly(I:C) (Invivogen 31852-29-6) treatments. Following day, cells were treated as specified above and washed with ice chilled PBS. Subsequently, they were lysed in lysis buffer including (30 mM Tris-Cl pH 7.5, 40 mM NaCl, 1% Triton-X-100, 2 mM KCl, 2 mM EDTA, 10% Glycerol) phosSTOP (Roche 04906837001), EDTA free protease inhibitor cocktail (Roche 5056489001) and tumbled at 4 ºC. for 20 minutes before centrifugation at 13,600 RPM at 4 ºC for 30 minutes and removal of the pellet. Then, Pierce™ BCA Protein Assay (Thermo Scientific™ 23225) was performed as manufacturer's instructions. After calibrating samples to each other, they were boiled with 4x Laemmli buffer involving 100 mM DTT at 95 ºC for 5 minutes.

After loading equal volume of the samples to gel (Bio-Rad 4–15% Mini-PROTEAN, 4568086), proteins were separated in 210 V for 50 minutes by SDS-PAGE. Later, they were transferred to nitrocellulose membrane in 1.3A-25V for 10 minutes by trans-blot machine and the membrane was blocked with 2.5% milk solution in PBS-Tween 20(PBS-T) for 1h. After that, proteins were blotted by respective antibody in 2.5% bovine serum albumin (BSA) and 0.025% NaN_3 in PBS-T as shown in **Table 2-3** for overnight before the membrane was washed with PBS-T three times for 10 minutes and incubated with respective HRP conjugated- secondary antibody in **Table 2-3**. Next, the membrane was washed with PBS-T three times for 10 minutes and develop by ECL solution (Western Lightning Plus ECL 0RT265).

Same protocol was carried out for HeLa cells either reconstituted WT moTAP ANKIB1 or RBR mutant moTAP ANKIB1 as well as it was followed without TNF or poly(I:C) treatment for determination of the ANKIB1 expression after subcloning.

Table 2-3: List of the Antibodies for Immunoblotting of Gene Activation-Associated Proteins and ANKIB1

Antibody	Source	Dilution
ANKIB1	Rabbit Atlas Antibody HPA021780	1:1000
GAPDH	Rabbit Sigma G9545	1:1000
Phospho IKKα/β	Rabbit Cell Signaling Technology 2697P	1:1000
Phospho ERK 1/2	Rabbit Cell Signaling Technology 4695	1:1000
Phospho IRF3 (S386)	Abcam EPR2346	1:1000
Phospho-JNK	Mouse IgG1 Cell Signaling Technology 9255S	1:1000
Phospho-P38	Rabbit Cell Signaling Technology 9215S	1:1000
Phospho-P65	Rabbit Cell Signaling Technology 3033	1:1000
Phospho-STAT1	Rabbit Cell Signaling Technology 58D6	1:1000
Phospho-TBK1 (S172)	Rabbit Cell Signaling 5483P	1:1000
STAT1	Rabbit Cell Signaling Technology 14994S	1:1000
Total IkBα	Rabbit Cell Signaling Technology 9242S	1:1000
Total TBK1	Rabbit Cell Signaling Technology 3013	1:1000
Anti-Mouse IgG1 HRP linked Secondary Antibody	Goat Southern Biotech 1070-05	1:10000
Anti-Rabbit HRP linked Secondary Antibody	Goat Southern Biotech 4050-05	1:10000

2.4 moTAP ANKIB1 Pulldown

5 million WT and ANKIB1 KO HeLa were seeded to 15 cm dishes as well as 6 million WT and ANKIB1 KO HT29 cells were seeded to 15 cm dishes in Roswell Park Memorial Institute (RPMI) 1640 media supplemented by 5% FBS and 1% Penicillin-Streptomycin for respective conditions (0h, 30 minutes, 1 hour 10 µg/ml poly (I:C) (Invivogen 31852-29-6 (TLRL-PIC)) treatments). Following day, cells were treated as specified above and washed with ice chilled PBS. Subsequently, they were lysed in lysis buffer including (30 mM Tris-Cl pH 7.5, 40 mM NaCl, 1% Triton-X-100, 2 mM KCl, 2 mM EDTA, 10% Glycerol) phosSTOP (Roche 04906837001), EDTA free protease inhibitor cocktail (Roche 5056489001) and tumbled at 4 ºC. for 20 minutes before centrifugation at 13,600 RPM at 4 ºC for 30 minutes. Then, Pierce™ BCA Protein Assay (Thermo Scientific™ 23225) was performed as manufacturer's instructions. After calibrating samples to each other, 50 µl from each sample were kept as whole cell lysate and boiled with 4x Laemmli buffer involving 100 mM DTT at 95 ºC for 5 minutes while rest of them were incubated by 15 µl Flag antibody conjugated beads (Sigma Aldrich M8823) per conditions for overnight, after pre-washed beads with lysis buffer. Following day, beads were washed with lysis buffer 4 times and boiled with 2x Laemmli buffer involving 50 mM DTT at 95 ºC for 5 minutes. Samples were analyzed by previously described western blotting, regarding primary and secondary antibodies in **Table 2-4**.

Table 2-4: List of the Antibodies for Immunoblotting After ANKIB1-moTAP Pulldown

Antibody	Source	Dilution
ANKIB1	Rabbit Atlas Antibody HPA021780	1:1000
GAPDH	Rabbit Sigma G9545	1:1000
NAP1	Rabbit Abcam Ab192253	1:1000
NEMO	Rabbit Cell Signaling Technology 2685S	1:500
Optineurin	Rabbit Cell Signaling Technology 58981S	1:1000
Phospho IRF3 (S386)	Abcam EPR2346	1:1000
Phospho-TBK1 (S172)	Rabbit Cell Signaling 5483P	1:1000

Total TBK1	Rabbit Cell Signaling Technology 3013	1:1000
Anti-Rabbit HRP linked Secondary Antibody	Goat Southern Biotech 4050-05	1:10000

2.5 Di-Gly Proteomics Analysis in WT vs ANKIB1 KO Cells

5 million WT and ANKIB1 KO HeLa cells were seeded separately to 15 cm dishes for 0 and 1 hour 10 µg/ml poly (I:C) conditions. Before stimulating the cells as stated, they were pre-treated with 10 µM proteasomal inhibitor MG132 (Selleck Chemicals S2619). Then, they were washed with ice cold PBS before lysed them and followed manufacturer's instructions in PTMScan® HS Ubiquitin/SUMO Remnant Motif (K-ε-GG) Kit #59322. As a note, manufacturer protocol was modified by being used 40 mM chloroacetamide to alkylate sulphur groups. Also, 1% formic acid was employed instead of the 0.1% trifluoracetic acid.

2.6 Cell Death Incucyte Experiment and Viability Assay

10,000 WT and ANKIB1 KO HeLa per well were seeded to 96 wells plate well as 15,000 WT and ANKIB1 KO HT29 cells were seeded to 96 wells plate separately in RPMI 1640 media supplemented by 5% FBS and 1% Penicillin-Streptomycin as duplicate. Following day, cells were pre-treated with 1 µM BV6 (Selleck Chemicals S7597), 20 µM Z-VAD (ApexBio A 1902-25), 10 µM Necrostatin-1 (Biomol Cay11658) for 30 minutes according to their experimental conditions before addition of the 100 ng/ml TNF and 10 µg/ml poly(I:C). Percentage of the cell death were evaluated by propidium iodide (PI; sigma) staining and Cell Incucyte Software after 24 hours. Additionally, Cell Titer Glow (CTG) (Promega) cell viability assay was performed as manufacturer's instructions.

2.7 Statistical Analysis

Data from Di-Gly Proteomics Analysis were analyzed by ANOVA, Student t-test, and Fisher exact test. Data from cell viability assay were assessed by GraphPad Prism 8.2.1 software. Statistical significance between groups was determined by unpaired two tailed

Student's t-test. Significant P-values were denoted as *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Chapter 3:RESULTS

3.1 TNF or Poly(I:C) induced Gene Activation Pathways in WT and ANKIB1 KO cells

According to the already published work from Walczak lab, KO of HOIP impairs recruitment and activation of TBK1-IKKε in TNF signaling while it is barely influenced in the TLR3-pathway. After demonstrating that ANKIB1 seems to be recruited to the TLR3-SC following poly (I:C) treatment, which is the synthetic ligand for TLR3, I aimed at testing our initial research question concerning ANKIB1 potential implication for TBK1 recruitment to the complex and activation. To address this question, HeLa, HT29 cancer cell lines and HaCaT immortalized keratinocyte cell line have been used to knock-out ANKIB1. Then, I investigated the kinetic of activation of the NF-kB, MAPK pathways and TBK1 activation by Western Blotting comparing WT and ANKIB1 KO HeLa, HaCaT, HT29 cell lines upon TNF and poly (I:C) treatment. I checked phospho-IKKα/β and phospho-p65 (P-p65), upstream and downstream proteins of the NF-kB pathway activation as well as phospho-p38 (p-p38), phospho-JNK (P-JNK), and phospho ERK 1/2 (P-ERK 1/2) for MAPK pathway in addition to phospho TBK1 (active form of the TBK1). I did not observe dramatic changes in the activation of the gene activatory pathway-related proteins and TBK1 in both WT and ANKIB1 KO cells after TNF stimulation as shown in **Figure 3.1**. However, activation of TBK1 was strongly impaired after poly (I:C) treatment in the ANKIB1 KO cells compared to WT cells as displayed in **Figure 3.2**. Concerning the proteins implicated in NF-kB and MAPK pathways, they were not influenced markedly. The effect on p-p38 was different, but not consistent in every cell line, so I decided to not focus on this effect. Further studies would be necessary to define the reason why phosphorylation pattern of p38 has changed differently upon perturbation of ANKIB1 expression and stimulation of TNF or poly(I:C) in distinct cell lines. Hence, I suggested that ANKIB1 might be one of the factors that are responsible for TBK1 activation, particularly in TLR3 signaling.

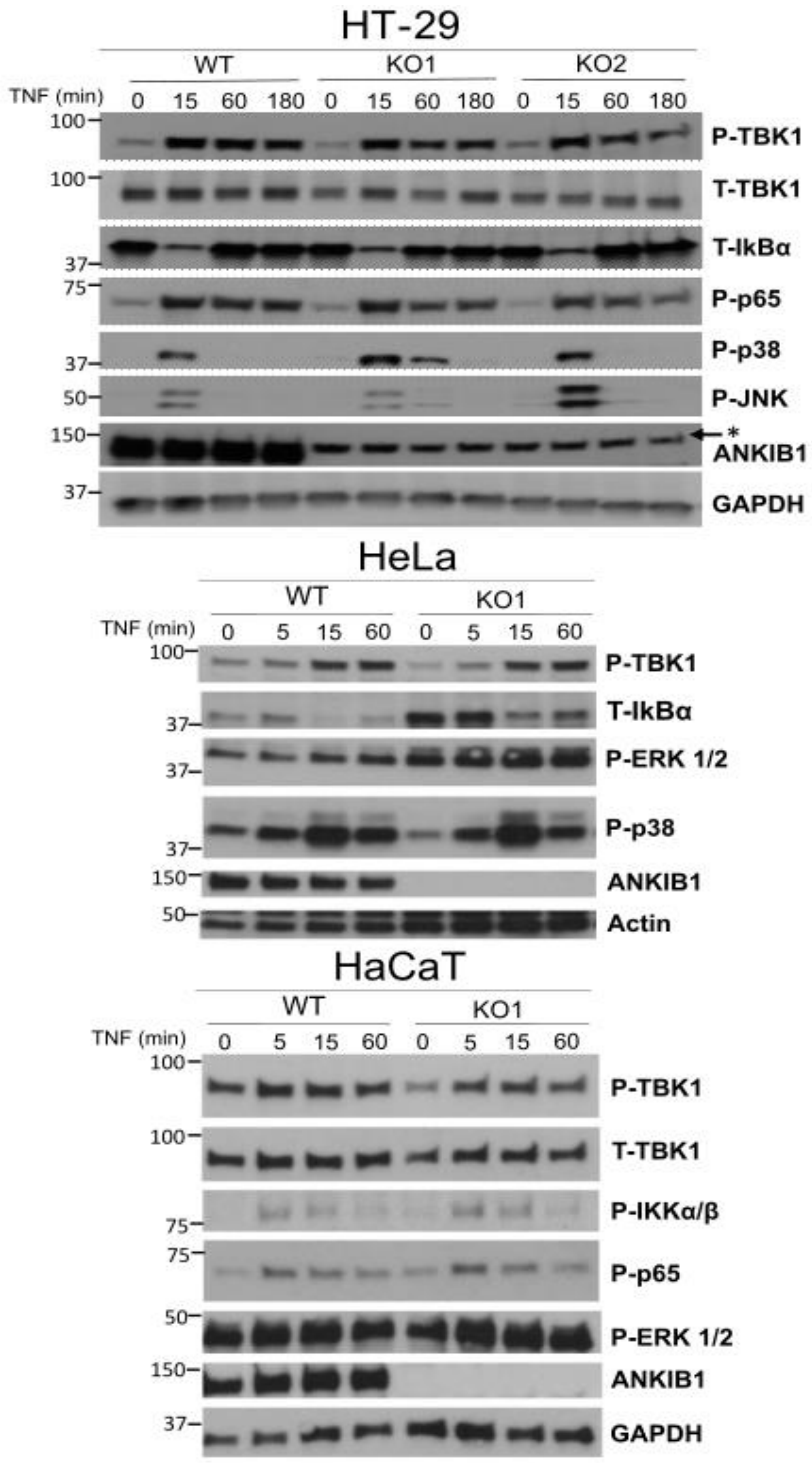


Figure 3.1: Assessment of TNF induced gene activation pathways in WT and ANKIB1 KO cells. Cells were treated with 200 ng/mL TNF in a time dependent manner and TNF induced gene activation pathways were evaluated. *: non-specific bands. I would like to acknowledge Alexis Betrancourt for ANKIB1 KO cell lines.

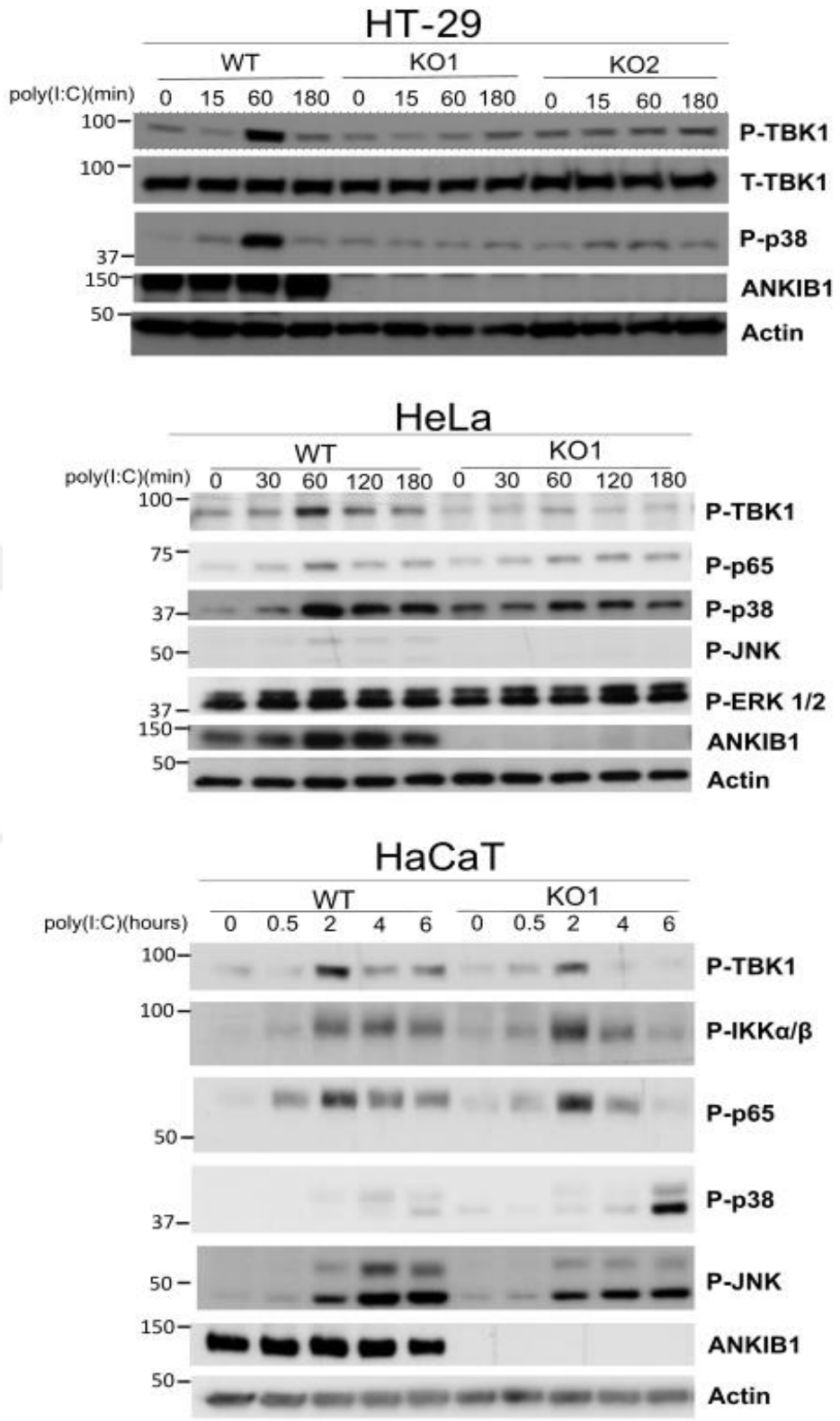


Figure 3.2: Assessment of poly (I:C) induced gene activation pathways in WT and ANKIB1 KO cells. Cells were treated with 10 μg/mL poly(I:C) in a time dependent manner and TLR3-induced gene activation pathways were evaluated by western blot.

Next, I questioned whether ANKIB1 contributes to TBK1 activation through either its catalytic activity or indirectly by its scaffolding activity. For this purpose, I firstly generated a C terminally tagged moTAP (1x Flag and 2x Strep tag) ANKIB1 with a flexible linker motif in between. In addition to this, I generated a construct where all cysteine residues are mutated for serine in RING2 of the ANKIB1 in order to obtain a catalytically inactive mutant (RBR mutant). Later, expression of WT and RBR mutant ANKIB1-moTAP genes were reconstituted by retroviral transduction in ANKIB1 KO HT29 and HeLa cells. Subcloning was necessary to acquire a similar expression level between the reconstituted WT and RBR mutant ANKIB1-moTAP expressing cells (rec. WT ANKIB1 and rec. RBR mut. ANKIB1, respectively (**Figure 3.3**). Second clone of the rec. WT ANKIB1 and first clone of the rec. RBR mut ANKIB1 were chosen for further experiments, regarding their similar level of ANKIB1 expression. First, I wanted to confirm our previous observation with the reconstituted WT HeLa in comparison to the empty vector transduced cells (KO for ANKIB1). Concerning the TNF pathway, all proteins were similarly activated, suggesting that ANKIB1 does not play a role in this signaling. However, the re-expression of ANKIB1 rescued the activation of TBK1 but also activation of the transcription factor downstream IRF3 following TLR3 activation (**Figure 3.4a**). Therefore, these data suggest that ANKIB1 may play a prominent role in the activation of TBK1 in TLR3 signaling. Then, I addressed the question whether the rescue for phosphorylation of TBK1 in HeLa rec. WT ANKIB1 cells strictly depends on the ubiquitin ligase activity of ANKIB1. The kinetic of the gene activatory pathways by poly(I:C) were assessed by western blot in HeLa rec. WT and rec. RBR mut. ANKIB1 cells. NF-kB and MAPK pathways were activated to a similar extend in both rec. WT and RBR mut. ANKIB1 cells upon TLR3 activation as visualized by p-p38 and p-p65 as indicated in **Figure 3.4b**. In addition, TBK1 phosphorylation still occurred in the rec. RBR-mut. ANKIB1 HeLa cells but seems decreased in comparison to the rec. WT ANKIB1 cells. Downstream of TBK1, IRF3 phosphorylation was also impaired in the RBR mutated cells. Regarding this observation, I expected that the production of type I interferon (IFNα/β) could also be impaired. I checked the phosphorylation of signal transducer and activator of the transcription 1 (STAT1) to determine this because STAT1 is a transcription factor that is phosphorylated after the binding of the IFNα/β molecules to the IFNα/β Receptor (IFNAR). Once activated, the secondary loop of the antiviral response occurs and triggers further expression of IFN stimulatory genes (ISGs) in the

cells [129]. In line with the phosphorylation of IRF3, the level of active STAT1 (phospho-STAT1) was strongly reduced in rec. RBR mut. ANKIB1 cells in comparison to the rec. WT ANKIB1 cells. Taken together, I implied that the catalytic activity of ANKIB1 is essential for the proper activation of TBK1-IRF3-STAT1 extent following TLR3 activation. However, it seems that rec. RBR mut ANKIB1 cells are able to mediate a stronger activation of the IFN pathway than the ANKIB1 KO cells, which could hint at scaffolding activity to a certain extent.

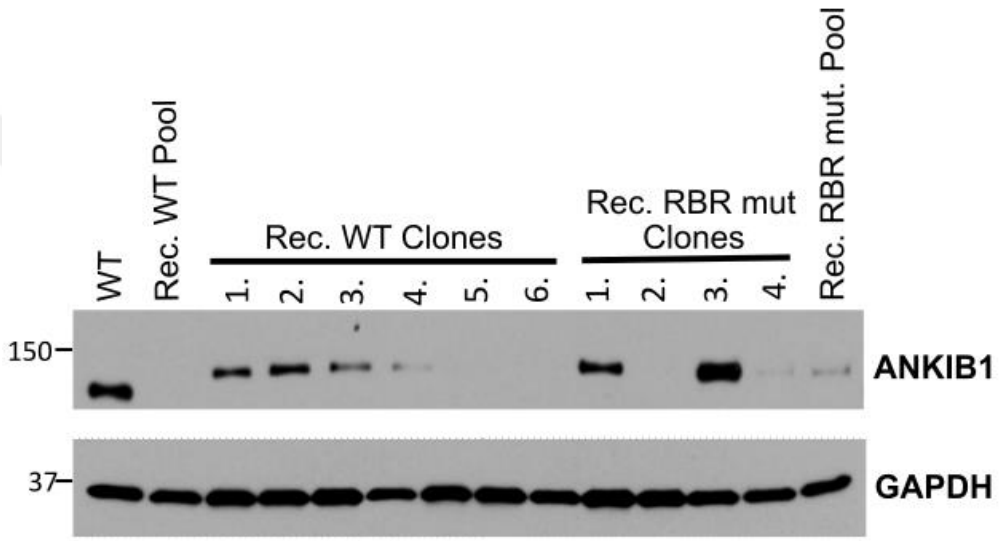


Figure 3.3: Screening of the clones obtained after subcloning of the reconstituted WT and RBR mutant ANKIB1-moTAP HeLa cells. Each clone was grown till obtaining enough number for sample preparation. Then, ANKIB1 expression of each clone, their original pool and original WT HeLa cell was compared by western blot.

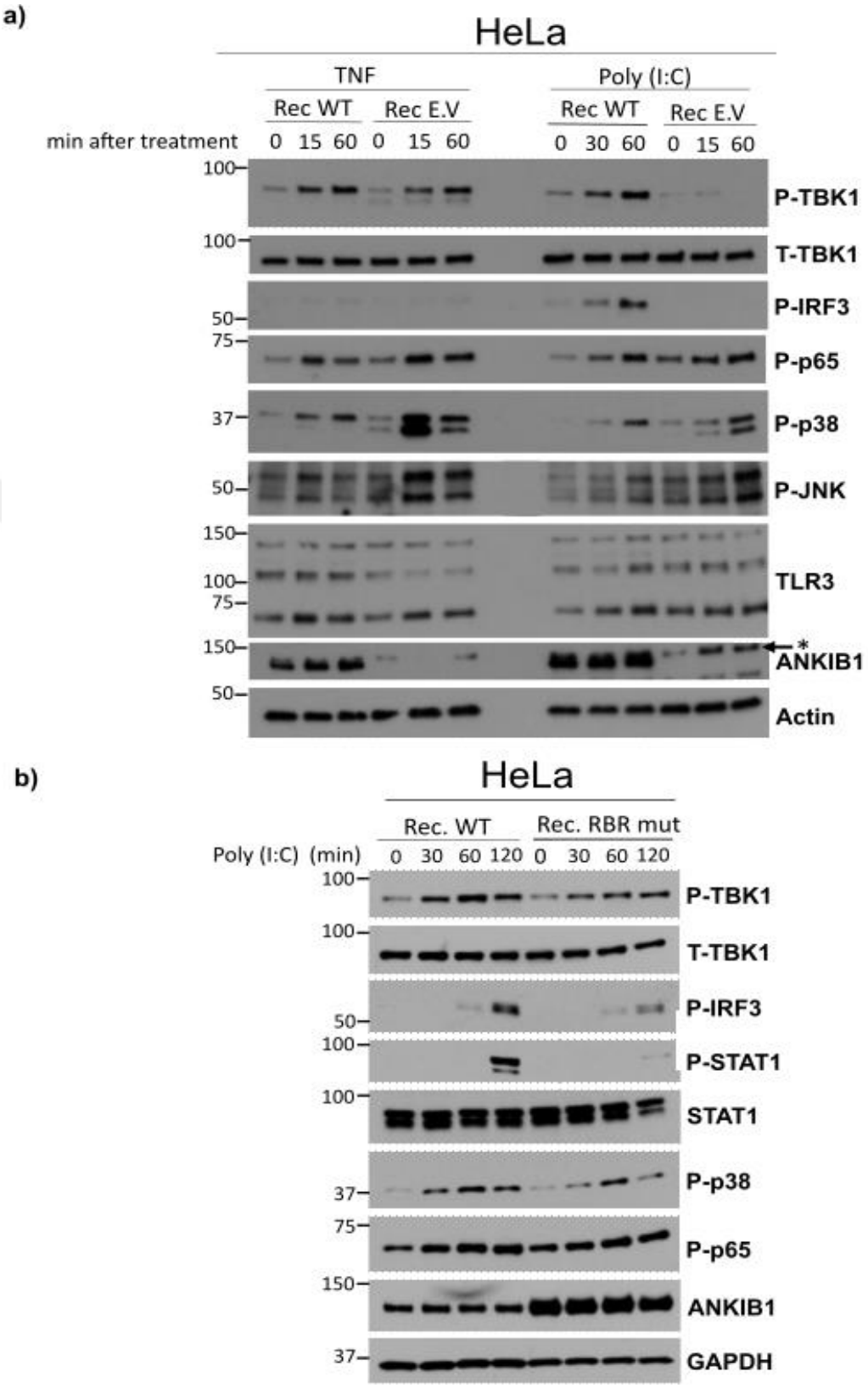


Figure 3.4: Evaluation of TLR3 induced gene activation in reconstituted WT, RBR mutant ANKIB1-moTAP and empty vector Hela cells. a) HeLa ANKIB1 KO cells (Rec. Empty Vector) and Reconstituted WT ANKIB1-moTAP (Rec WT) were treated

either with 200 ng/mL TNFα or 10 μg/mL and the activation of the different pathways were assessed by Western Blot. **b)** HeLa reconstituted rec. WT or rec. RBR mut ANKIB1 were treated either with 200 ng/mL TNFα or 10 μg/mL poly (I:C) and the activation of the different pathways were assessed by western blot. .*: non-specific bands.

3.2 Discovery of ANKIB1 Interaction Partners in TLR3-SC

I next aimed at understanding whether ANKIB1 might interact with TBK1 and other components of the TLR3-SC or if ANKIB1 is indirectly involved in TBK1 activation. To address this question, I used the Flag-tagged ANKIB1 in the reconstituted cell lines that allows us to pull it down following poly (I:C) treatment. Empty vector reconstituted cell lines were used as negative control for the specificity of the pull down. As displayed in **Figure 3.5**, in both reconstituted cell lines, ANKIB1 interacts with phospho-TBK1. However, non-phosphorylated TBK1 seems also to interact with ANKIB1. In addition, NEMO and OPTN, which are suggested substrates for ANKIB1, were also detected as interaction partners following the treatment. All these interactions are treatment dependent and do not occur basally owing to requirement of close proximity of indicated proteins and functional outcome like IFN production. Interestingly, despite its contribution to phosphorylation of IRF3, ANKIB1 does not interact with phospho-IRF3 as previously described when detached from the TLR3-SC. Consequently, I stated that ANKIB1 may interact with OPTN, NEMO and TBK1 directly or indirectly in TLR3-SC and it may contribute phosphorylation of TBK1 in this extent.

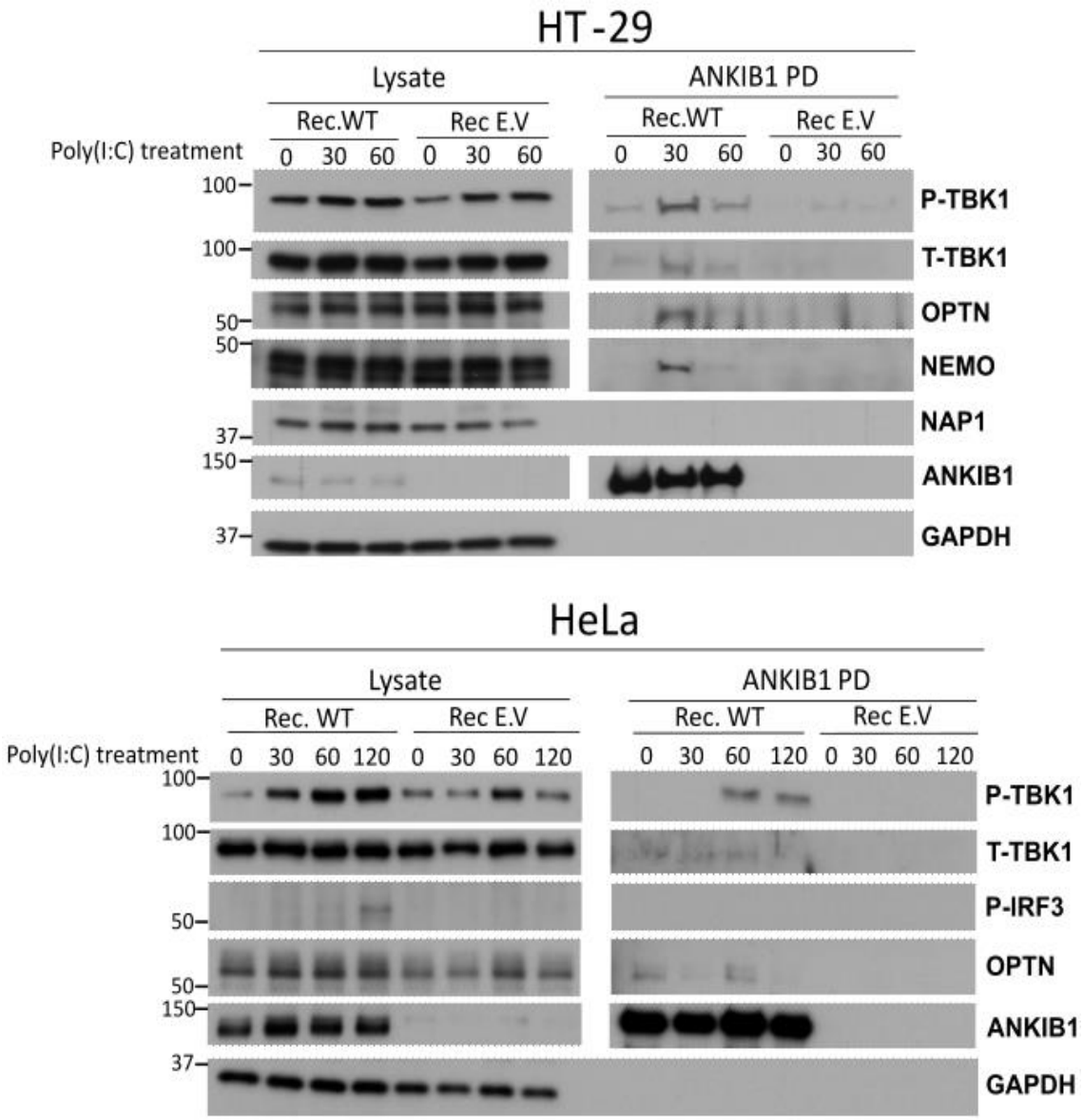


Figure 3.5: Determination of the interaction partner of ANKIB1 in poly (I:C) treatment dependent manner. Rec. WT and Rec. E.V. of both HT29 and HeLa cells were treated with 10 μg/mL poly (I:C) in time dependent manner and subsequently ANKIB1-moTAP was pulled down by Flag antibody-coupled M2 beads and analyzed by western blot.

3.3 Analysis of TLR3 Signaling Dependent Ubiquitination of Proteins in WT and ANKIB1 KO HeLa Cells

Next, I wanted to determine how ANKIB1 directly or indirectly influences the ubiquitination status of the proteome and to potentially find direct substrates for ANKIB1 upon TLR3 induction. To answer this question, I followed unbiased quantitative

proteomics approach. First, WT and ANKIB1 KO HeLa cells were treated with proteasome inhibitor MG132 to prevent the degradation of any ubiquitin chains implicated in proteasomal degradation of proteins. Cells were then stimulated with poly (I:C) for 1 h and samples were prepared. The work flow, as described in **Figure 3.6a**, has been performed in order to generate and enrich the K-GG (di-Gly) motif. This motif is obtained after trypsin digestion of the ubiquitinated proteins, and it allows to pull down only ubiquitinated proteins and to know the exact site of ubiquitination. In parallel, I additionally followed a whole proteomic analysis in order to evaluate quantitatively the overall protein amount. It is used as a control to verify that potential ubiquitin differences found between WT and ANKIB1 KO cells are not due to a difference in the total amount of protein. Subsequently, the samples were analyzed by LC-MS/MS. From the analysis of the samples, I obtained a list of nearly 8,000 different peptides. Therefore, I decided to focus at first on the group of proteins found to be ubiquitinated in stimulated either WT or ANKIB1 KO cells in order to find potential pathways impacted by the deletion of ANKIB1. For that purpose, I used the gene ontology biology process (GOBP) database to classify the genes regarding function of their respective proteins. Interestingly, in comparison with the ANKIB1 KO cells, WT cells specific-ubiquitinated proteins were enriched in “TLR3 pathway”, “apoptotic process” and “negative regulation of apoptotic process” as shown in **Figure 3.6b**, confirming potential role of ANKIB1 in TLR3 signaling. Because of our interest to decipher the role of ANKIB1 in TLR3 pathway I decided to focus on the ubiquitinated peptides implicated in TLR3 signaling and cell death induction that I found to be present only in WT cells induced following poly(I:C) treatment. First, the most upstream component of TLR3 signaling found, TRIF was ubiquitinated in both WT and KO as described in **Figure 3.7a**. This might suggest that ANKIB1 is not targeting TRIF during TLR3 activation. Moreover, NEMO was significantly ubiquitinated in poly (I:C) treated WT cells, yet its ubiquitination may not be treatment dependent compared to 2 NEMO peptides in the untreated WT cells. Intriguingly, I also found that IRF3 and A20 had profound ubiquitination only in poly (I:C) treated WT cells. Interestingly, TBK1 was found to be ubiquitinated on Lysine (K) 137, which is an already described site and shown positive contribution for IFNα/β production, [130] only in the presence of ANKIB1 after poly(I:C) stimulation. However, this hit was not significant due to a low overall intensity. I therefore need more repetition

or different time points. To conclude, the di-Gly enrichment revealed candidates as the putative substrate for ANKIB1 that needs evaluation with further experiments.

In parallel, due to the general decisive roles of E3 ubiquitin ligases on the regulation of the cell death and our first observation after GOBP analysis, I decided to also focus on peptides enriched implicated in cell death specifically after poly(I:C) treatment. As displayed in **Figure 3.7b**, I detected ubiquitinated peptides for caspase 8, c-FLIP in just WT cells but not in KO cells, yet these findings were not statistically significant because of overall low intensity. Interestingly, I also determined Gasdermin E ubiquitination only in WT cells. Additionally, Gasdermin E seems to be upregulated in ANKIB1 KO cells, regardless of the treatment. Gasdermin E is involved in the induction of pyroptosis. Pyroptosis is a form of cell death and once activated, Gasdermin D/E is cleaved caspase dependent manner. Then, N terminal domain of Gasdermin D/E is oligomerized and translocated to the plasma membrane, which facilitates the disruption of the cellular integrity via the formation of pores and release of proinflammatory cytokines like IL1β. Several alternative ways also exist to initiate pyroptosis by involvement of various caspases. Thus, this requires strict regulation and crosstalk between different immune signaling pathway [131]. Oppositely, I found that APAF-1 was only ubiquitinated in ANKIB1 KO cells, but this was also not significant on account of its low intensity. Hence, I implied that ANKIB1 might directly or indirectly be implicated in the control of cell death in context of an infection, especially pyroptosis. It is an important aspect that need to be studied.

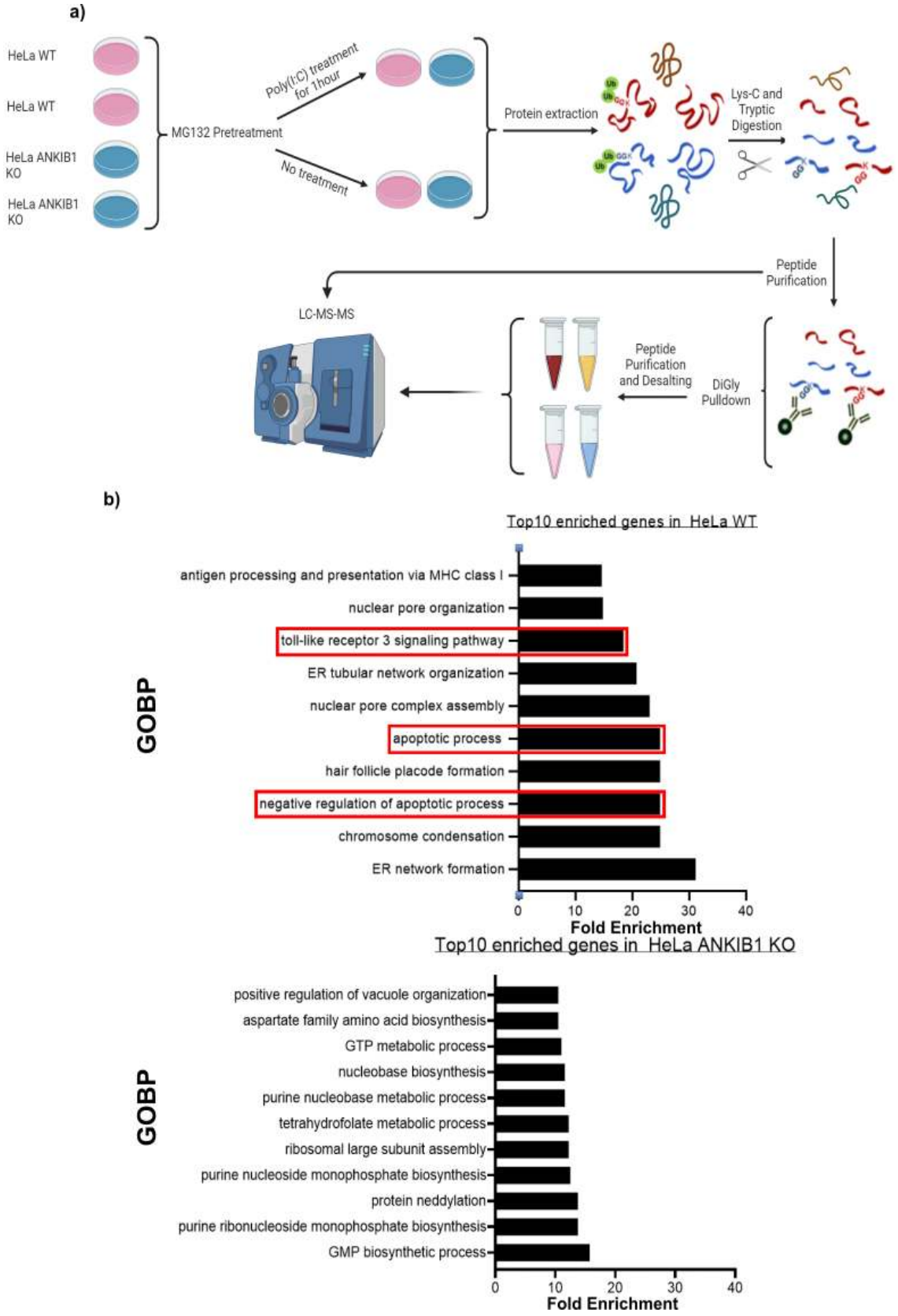


Figure 3.6: Analysis of gene enrichments and their respective pathways after di-Gly peptide enrichment. WT and ANKIB1 KO HeLa cells were pre-treated with 10 μM

MG132 (proteasome inhibitor) for an hour and induced by poly (I:C) for an hour. Proteins were digested by Lys-C and trypsin respectively before di-Gly pulldown. Whole proteome was analyzed by LC-MS/MS before and after di-Gly pulldown. **a)** General workflow of the sample preparation and di-Gly peptide enrichment. Redrawn from [137]. **b)** Upon the treatment, impacted ubiquitinated proteome and signaling pathways were associated by using Gene Ontology Biology Process (GOBP) Database.

a)

	Number of Peptides found (/4)				Ub Site	Amount of Protein	Significance
Protein Names	WT		ANKIB1 KO				
	UT	1h PIC	UT	1h PIC			
TBK1/IKKε	0	3	0	0	137	Same/NA	Non- significant
NEMO	2	4	0	0	344	Same	Significant
IRF3	0	4	0	0	193	Same	Significant
TRIF	0	4	0	3	65	NA	Non- significant
A20	0	3	0	0	81	Same	Significant
A20_2	0	3	0	0	82	Same	Significant

b)

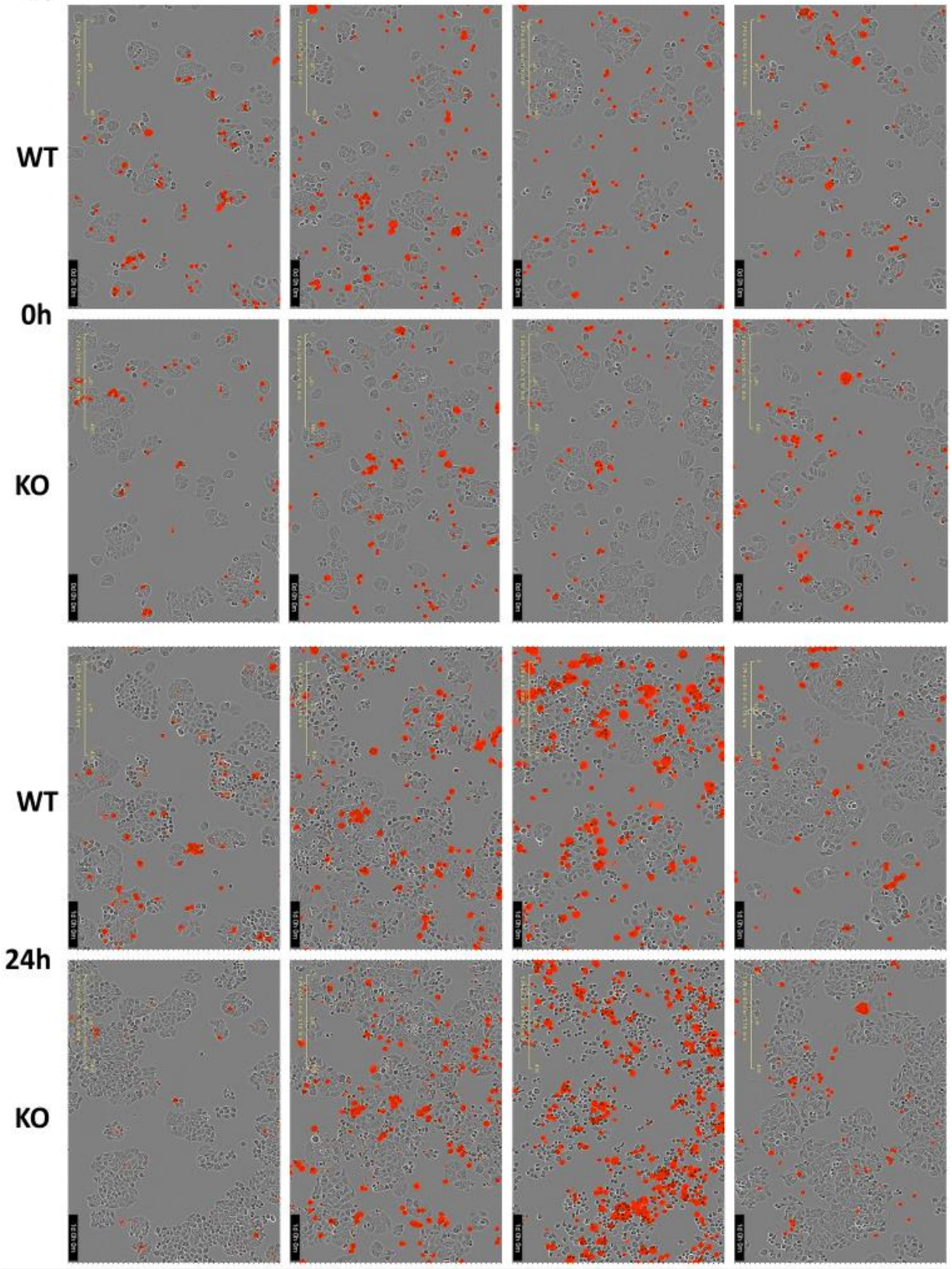
	Number of Peptides found (/4)				Ub Site	Amount of Protein	Significance
Protein Names	WT		ANKIB1 KO				
	UT	1h PIC	UT	1h PIC			
Caspase 8	0	3	0	0	130	Same	Non- significant
Caspase 8	0	3	0	0	132	Same	Non- significant
c-FLIP	0	4	0	0	192	NA	Non- significant
Gasdermin E	0	3	0	0	100	Upregulated	Significant
Gasdermin E	0	3	0	0	98	Upregulated	Significant
APAF-1	0	0	0	4	142	NA	Non- significant

Figure 3.7: Evaluation of the ubiquitinated proteins in whole proteome upon poly (I:C) induction. a) TLR3 signaling related proteins and their ubiquitination status with or without by poly (I:C) treatment. **b)** Cell death associated proteins and their ubiquitination status with or without by poly (I:C) treatment. Statistical analysis and significance were evaluated by ANOVA testing according to p value 0.05. n=4 NA: Not detected, PIC: poly (I:C), Ub: Ubiquitination, UT: Untreated. I would like to acknowledge Dr. Jan-Wilm Lackmann from proteomics facility in the CECAD, the University of Cologne, for the analysis of di-Gly enrichment.

3.4 Assessment of Role of ANKIB1 in Cell Death

Caspase 8, c-FLIP, and APAF-1 are important proteins in regulation of the cell death pathway. Despite their non-significant ubiquitinations upon poly(I:C) treatment, I evaluated that they may still have association with ANKIB1 in context of TLR3-dependent cell death in presence of proper cell death sensitizer agents. Also, I know that prolonged exposure time to pathogens or relevant molecules are needed to evaluate cell death perspective. Next, I decided to investigate cell death in WT and ANKIB1 KO HeLa, and HT29 cells. Then, I set up live imaging incucyte experiment in different cell death inducing and blocking conditions for 24 hours. HeLa cells allow us to explore only the apoptosis pathway due to the lack of RIPK3. On the other hand, HT-29 is mostly a necroptotic cell line, meaning that the inhibition of the caspases via Z-VAD pre-treatment both prevents apoptosis and strongly increases the induction of necroptosis after cell death stimulation. Inhibition of necroptosis was also accomplished by inhibitor of RIPK1, Necrostatin 1(Nec-1). Due to these two cell lines, I can assess both modalities of cell death. Prior the addition of poly(I:C) or TNF, cells were sensitized to cell death by SMAC mimetics BV6 that antagonizes cIAPs and XIAP. Without sensitization, HeLa and HT-29 do not die after TNF or poly(I:C) treatment (data not shown). 24 hours after treatment, viability of cells was evaluated by luminescent CTG assay. As a result, TNF induced cell death in HeLa and HT-29 was not significantly impacted by the perturbation of ANKIB1 expression in both cell line. Both WT and KO cells died to a similar extend via either apoptosis in HeLa or necroptosis in the HT-29 and they were completely rescued by Z-VAD and combination of Z-VAD and Nec-1 treatments respectively as shown in **Figure 3.8**. Nevertheless, ANKIB1 KO HT29 cells were more sensitive to poly(I:C)-induced cell death compared to WT HT29 cells, especially when apoptosis was inhibited by Z-VAD treatment. ANKIB1 KO HT29 cells were rescued by combination of Z-VAD and Nec-1 treatment. Accordingly, it seems that ANKIB1 plays a role in the control of the TLR3 dependent cell death in presence of BV6, possibly related to necroptosis.

a)



BV6		+	+	+
Poly(I:C)		+	+	+
Z-VAD			+	+
NEC-1				+

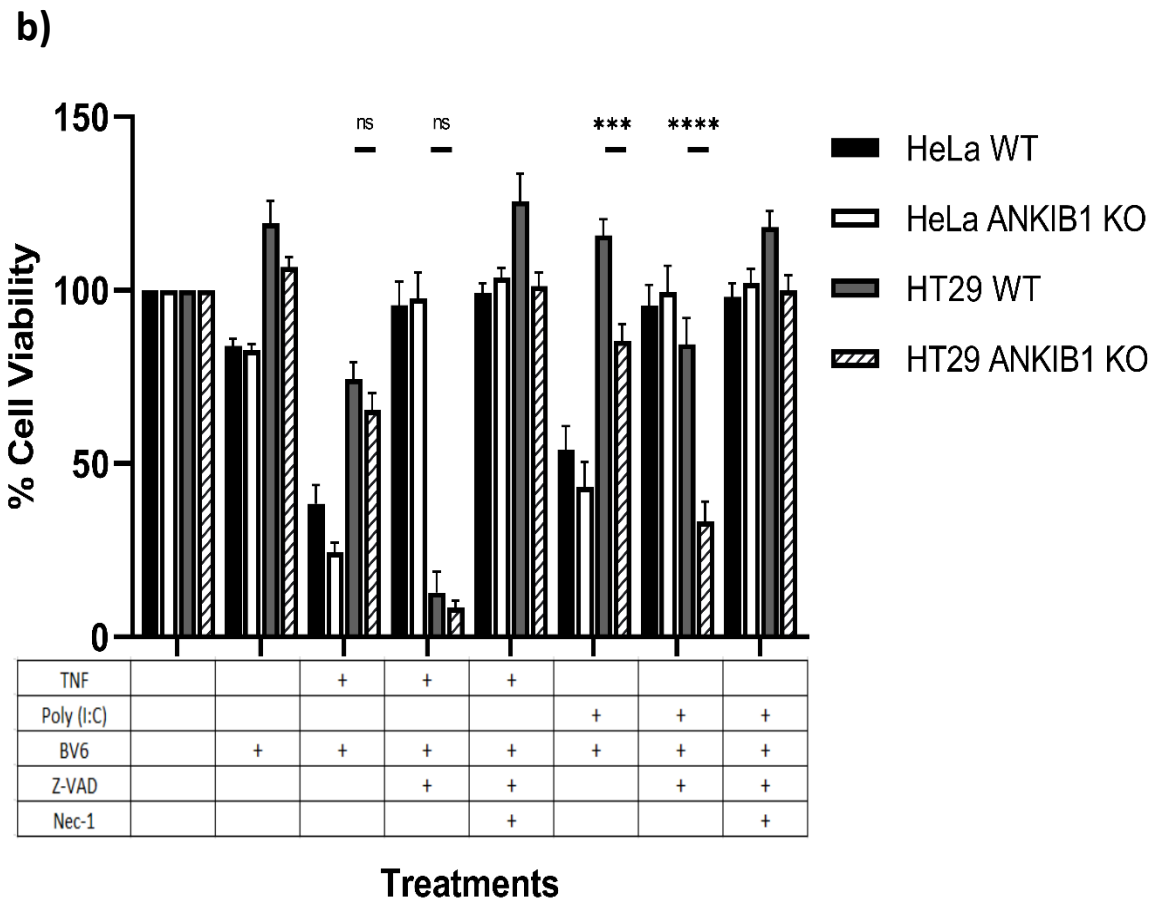


Figure 3.8: Effect of ANKIB1 on cell death context in HeLa and HT29. Cells were pre-treated with BV6, Z-VAD, and Necrostatin-1 (NEC-1) according to their conditions for 30 minutes, then treated with either 100 ng/ml TNFα or 10μg/ml poly(I:C) **a)** Phase-contrast images of the WT vs ANKIB1 KO HeLa and HT29 cells in different conditions after 24 hours from the treatments. **b)** Cell Titer Glow (CTG) cell viability assay was performed to determine the cell viability 24 hours after treatment. Unpaired two tailed Student t test was executed for statistical analysis. Scale bar at left top: 400 μm ns:non-significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, n=6.

Chapter 4: DISCUSSION AND CONCLUSION

Ubiquitination is one of the most vital PTM in the cells. Through the covalent attachment of ubiquitin to a targeted protein, the consequences of ubiquitination are various, such as protein quality control, degradation but also the regulation of the signaling pathways. The protein fate depends on the ubiquitination site of the targeted protein as well as the type of chain generated by combinational effect of specific E2-E3 enzyme pairs. To unravel biological significance of the ubiquitination, multiple E3 ubiquitin ligases have been identified over the last decades and their functions in different signaling pathways have been described. Nevertheless, there are still numerous E3 ubiquitin ligases that need to be characterized.

In this thesis, I focused on the role of a new RBR containing E3 ubiquitin ligase, ANKIB1, in TLR3 pathway. I have identified ANKIB1 as a primordial E3 ligase for the activation of TBK1, and consequently the activation of IRF3 in TLR3-SC. In numerous cell lines such as HeLa, HT29 and HaCaT, I showed that the deletion of ANKIB1 strongly impaired TBK1 and IRF3 phosphorylation in HeLa cells after treatment with dsRNA poly(I:C), synthetic ligand for TLR3 that mimic a viral infection. Interestingly, TBK1 phosphorylation and activation were not impacted following TNFR stimulation in absence of ANKIB1, leading to the conclusion that ANKIB1 does not any effect in TNF-induced TBK1 activation. Despite several similarities, TNFR and TLR3 signaling pathways contain differences, notably regarding distinct function of the non-canonical IKKs. As detailed in the introduction, TRAF3 generates K63 ubiquitin chain on itself and this serves as a platform for TBK1 recruitment to TLR3-SC via NEMO, thereby phosphorylation of TBK1 [78]. Also, OPTN can heterodimerize with TBK1 and facilitate trans or autophosphorylation and activation of TBK1. Both TRAF3 and OPTN enhance IFNα/β production via activation of TBK1 in TLR3 pathway, but not in the TNFR pathway [124]. Moreover, whether OPTN is recruited to TLR3-SC is still not clear. All together, I can hypothesize that those differences might be a safety mechanism during an immune response following infection. Innate immunity is stimulated through the direct recognition of the pathogens via the PRR. Once activated, type I interferon but also other pro-inflammatory cytokines are produced such as TNF in order to promote the recruitment and the activation of immune cells. I can therefore extrapolate that the requirement of two different E3 ligases for TBK1 activation (ANKIB1 in TLR3 pathway

and HOIP in TNF signaling pathway) allows the individual control of both pathways implicated in the immune response but with a different outcome. Then, I further investigated whether catalytic activity of ANKIB1 is essential to properly activate both IRF3 and TBK1 because ubiquitination of them by ANKIB1 may enhance their stability and activation. For this purpose, I created catalytically inactive RBR mutant of ANKIB1, and reconstituted expression of this RBR mutant ANKIB1 in HeLa ANKIB1 KO cells. Later, I evaluated gene activatory pathways after poly (I:C) treatment. It seems that RBR mut. ANKIB1 expressing cells are still able to activate slightly more TBK1 and IRF3 than the KO cell lines. Regarding our observation in these three proteins, I have to assess if ANKIB1 still has a residual catalytic activity or if this partial rescue of TBK1 phosphorylation is due to its scaffolding activity. In vitro ubiquitination assay may be performed for determination of its catalytic activity. To create efficient catalytically inactive mutant of ANKIB1, I should follow different mutagenesis strategy if it is necessary, such as completely removing catalytic RING2 domain of ANKIB1. In our results, I also saw that obvious reduction in phosphorylation of STAT1 (active form of STAT1) in the cells expressing RBR mut. ANKIB1 in comparison to rec. WT ANKIB1 one. STAT1 is downstream effector of IFN signaling. Indeed, once IFNα/β s are produced and secreted, they bind IFNAR and initiate a signaling cascade implicating the transcription factors STAT1-STAT2, associated as heterodimer and initiation of ISG expression. Consequently, ISGs enhance the anti-viral response [129]. Thus, this observation might translate the impairment of the IFNα/β production very likely when catalytic activity of ANKIB1 is absent. However, I need to confirm their production and secretion capacity via qPCR and ELISA. It is also possible that ANKIB1 is part of a complex with other E3 ligases and that its presence allows the stabilization and the activity of this latter such as the LUBAC complex [42]. Moreover, I pulled ANKIB1 down to detect its potential interaction partners and their functional relevance with our previous observations after poly (I:C) treatment. I have shown that ANKIB1 interacts with P-TBK1 and OPTN, which are required for TLR3- induced IFNα/β production. This result might be positively correlated with effect of ANKIB1 on TBK1 activation or maintenance of active TBK1. However, it needs to be determined if ANKIB1 contributes to this process and what consequences of these interactions are. Furthermore, ANKIB1 also seems to interact with NEMO, the key protein for the activation of the NF-kB and IFN production. Although I have strong evidence to validate our initial bioinformatic

analysis for substrate of ANKIB1, whether these interactions are direct or not should be elucidated through yeast two hybrid system, fluorescent based protein complementation assays or ELISA. It is also important to mention that TBK1 is a required kinase for the interferon production not only in TLR3 signaling but also in many others such as Toll like receptor 4 (TLR4), retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5) and cyclic GMP-AMP synthase-stimulator of IFN genes (cGAS-STING) [132]. In addition to this all these pathways have components including pLxIS IRF3 binding motif. For example, TLR4 recognizes bacterial lipopolysaccharide (LPS) and recruits IRF3 through TRIF. On the other side, Myeloid Differentiation Primary Response 88 (MYD88), gets involved as an adaptor protein in all TLRs except TLR3, and does not contain pLxIS IRF3 binding motif, therefore IRF3 cannot be recruited to their signaling complexes and get activated [132]. Also, MDA5 and RIG-I can sense cytosolic dsRNA and IRF3 is recruited via MAVS [81,133]. cGAS is the sensor for viral DNA and the activation of this pathway leads to the recruitment of IRF3 via STING [134]. Due to their similarity of RIG-I, MDA-5 and cGAS system in terms of nucleic acid sensing and stimulation of IFN production, there is a possibility that ANKIB1 may have a role in those pathways as well. This hypothesis needs to be addressed in the near future due to the potential massive relevance for antiviral immune response. For this purpose, I can directly deliver analogs of RNA or DNA virus to cytosol of TLR3-KO cell lines by transfection and evaluate TBK1 activation and IFN response. In addition, I showed that ANKIB1 did not interact with P-IRF3 suggesting that the interaction seen with TBK1 and other components is occurring in the signaling complex and not in the cytosol. Because all ubiquitinated proteins harbor di-Gly motif after tryptic digestion, I then followed an unbiased proteomics approach and analyzed peptides with di-Gly motif in order to dissect our previous observations and to potentially determine which proteins are impacted in terms of ubiquitination in absence of ANKIB1 after TLR3 stimulation. According to the results obtained, many proteins implicated in the IFNα/β production were listed. Among them, IRF3 and A20 were significantly ubiquitinated in poly (I:C) treated WT HeLa cells but not in ANKIB1 KO HeLa cells. K193 modification site for IRF3 has already been reported to be required for its proper activation and consequently the IFN production [135]. I should confirm functional importance of this site and modification type, either ubiquitin or ubiquitin like modifiers. Nevertheless, if ANKIB1 is directly responsible for this modification needs to be clarified via in vitro ubiquitination assay. Additionally, even

though A20 ubiquitination sites have not been described yet, A20 is known to negatively regulate TLR3 induced IFN signaling, notably by physically interfering interaction between TRAF3- TBK1 independently its DUB activity [136]. I therefore could hypothesize that ANKIB1 ubiquitinates A20 in order to control its stability or association with TBK1. Besides, considering the importance of E3 ubiquitin ligases in regulation of cell death pathways and our apoptosis related indication after GOBP analysis, I decided to evaluate cell death pathway relevant proteins and differences in their ubiquitination status upon perturbation of ANKIB1 expression and TLR3 stimulation. I could just show that Gasdermin E, a pore complex protein in the pyroptosis pathway was significantly ubiquitinated in WT but not in ANKIB1 KO cells, following the induction. Therefore, there is a possibility that ANKIB1 might regulate function of Gasdermin E in pyroptosis by inhibiting its activation to increase cell survival through ubiquitination, but further clarification is necessary. For that purpose, I can assess status of Gasdermin E and cellular behaviors upon poly(I:C) treatment after sensitization of ANKIB1 WT and KO cells to pyroptosis. In addition to this, Gasdermin E is upregulated in ANKIB1 KO cells without the stimulation as a result of the whole proteome analysis. There is a possibility that ANKIB1 may restrict epigenetic repressors of Gasdermin E and enhance its expression, because Gasdermin E expression is negatively correlated with cell survival due to its ability to induce pyroptotic cell death after activated by caspase 3 [131]. Therefore, cancer cells usually downregulate Gasdermin E [138]. Nevertheless, whether this consequence directly depends on ANKIB1 must be investigated. Subsequently, I aimed deciphering whether ANKIB1 might play a role in regulation of cell death in presence of appropriate cell death sensitizer. Because several differences on the ubiquitination of proteins implicated in cell death identified via di-Gly enrichment, and their accumulations may be necessary to induce cell death after prolonged exposure to stress. For that aim, I set up live imaging experiment in WT and ANKIB1 KO cancer cells (HeLa and HT29) sensitized by BV6 for apoptosis and monitored them for 24 hours after TNFR and TLR3 stimulation. I observed that neither TNFR nor TLR3 induced WT and ANKIB1 KO HeLa cells displayed a significant difference in terms of cell viability. Despite the di-Gly enrichment results, it can be speculated that ANKIB1 might play a minor role in the regulation of the apoptosis even at later time points. Also, TNFR induced WT and ANKIB1 KO HT29 cells died in similar ratio. Nonetheless, I could identify that ANKIB1 KO HT29 cells were more sensitive to cell death upon TLR3 stimulation compared to

WT HT29 cells as indicated in **Figure 3-7**. This sensitivity was emphasized when caspases are inhibited by Z-VAD pre-treatment. Then, cells were rescued by the total inhibition of cell death through combination of RIPK1 kinase inhibitor Necrostatin-1 and Z-VAD, suggesting that this cell death is necroptotic because RIPK1 can phosphorylate and activate RIPK3, leading to phosphorylation of MLKL and necroptosis. In conclusion, the deletion of ANKIB1 seems to sensitize the cells to a RIPK1 kinase activity dependent cell death (probably necroptosis), particularly in context of TLR3-induction. Nonetheless, another confirmation is necessary for determination of cell death type via western blot, flow cytometry. For example, I can evaluate status of cleaved caspase 3, caspase 8 for apoptosis, phospho RIPK3 and phospho MLKL for necroptosis pathway by western blot. Of note, I could not assess potential substrates for ANKIB1 in the necroptosis pathway because the di-Gly enrichment has been performed in HeLa cells that lack RIPK3. Thus, I need to repeat this di-Gly enrichment experiment in WT and ANKIB1 KO HT29 cells. Regarding ANKIB1-Gasdermin E association and potential role of ANKIB1 in pyroptosis, the question remains to be addressed. Indeed, it has been shown that caspase 8 and 3 can promote pyroptosis by the cleavage of gasdermins in pathological conditions, such as in case of prevention of innate immune signaling NF-kB after infection [139, 140]. In this regard, further experiments need to be performed in order to unravel potential association between pyroptosis and ANKIB1. For instance, I can induce pyroptosis in WT and ANKIB1 KO HT29 and HeLa cells in presence of IKKα/β inhibitor and assess cellular behavior in terms of cell death.

To summarize, I explored that ANKIB1 positively regulates TBK1 and IRF3 activation in TLR3-dependent manner and I suggest that its catalytic activity seems to be required. Its putative interaction/engagement with TBK1, OPTN and NEMO provides further functional correlation for this regulation. In addition, ubiquitination sites of A20, TBK1 and IRF3 appear to be impacted by the deletion of ANKIB1. However, I need to dissect in depth its putative role and the biological significance of these ubiquitination sites. As another perspective, I also described that ANKIB1 may play a role in the regulation of necroptosis. Our study provides, for the first time, several primordial activities for ANKIB1 in type I IFN production but also in cell death mediated by the TLR3 signaling pathway.

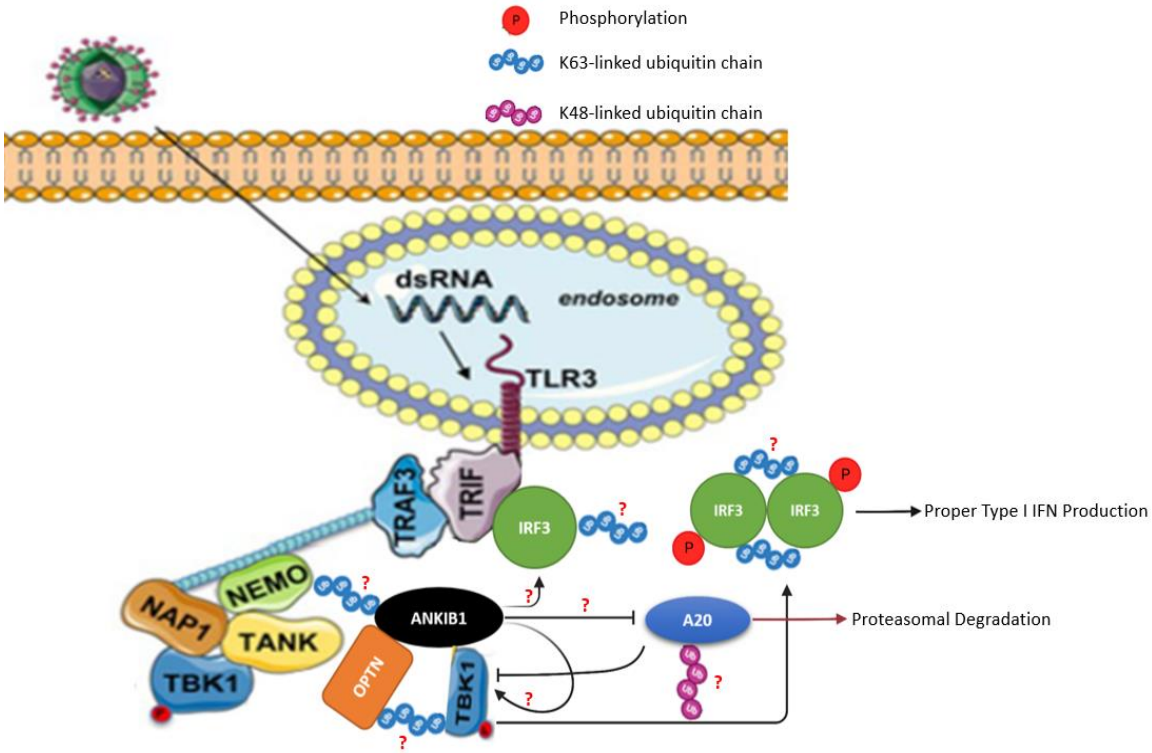


Figure 4.1: Graphical abstract of the study. After TLR3 stimulation, TRIF is recruited to TLR3 and it can further recruit TRAF3. TRAF3 generates K63 ubiquitin chain on itself and it serves as a platform for the recruitment of NEMO. NEMO binds adaptor proteins NAP1 and TANK; thus, they facilitate recruitment of TBK1 to TLR3-SC and trigger its autophosphorylation and activation. In addition to this, an alternative way exists for the activation of TBK1. In this context, ubiquitination of NEMO is firstly executed after its recruitment to the TLR3-SC. This ubiquitination enables further recruitment of ANKIB1. Then, ANKIB1 interacts with OPTN and TBK1; hence ANKIB1 can ubiquitinate TBK1, which triggers its interaction with OPTN and autoactivation. Also, ANKIB1 can ubiquitinate IRF3 and this enhances stability of IRF3 homodimers after its phosphorylation by active TBK1. In addition, ANKIB1 can create ubiquitin chain on A20 that is a negative regulator in TBK1 activation; consequently, it blocks interference of A20 on the activation of TBK1 by triggering its proteasomal degradation. Overall, ANKIB1 regulates activation of TBK1 and proper type I IFN production. However, there are some unclear points which illustrated with red question mark. One of them is whether ANKIB1 directly ubiquitinate TBK1, A20 and IRF3 or it just influences their ubiquitination status indirectly. Another question is which type chain is generated by ANKIB1 because this directly affects fate and function of the proteins. They must be clarified in future.

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Appendix A: CELL CULTURE

HeLa, HaCaT and HT29 Cells

Roswell Park Memorial Institute (RPMI) 1640 (PAN-Biotech p0416500) with phenol red was supplemented with 5% FBS and 1% Penicillin-Streptomycin. Cells were grown up to 4 days in the same medium prior to passaging. To passage cells, they were firstly washed with 37 ºC PBS once and detached by using 0.05% /0.02% Trypsin/ EDTA and incubating them at 37 ºC after diluting 0.5% /0.2% Trypsin/EDTA (PAN-Biotech P10-024100) with 1x PBS in 1:10 proportion.

Platinom Ampho Cells

DMEM phenol red supplemented with 10% FBS and 1% Penicillin/ Streptomycin. Cells were grown up to 4 days in the same medium. To passage cells, they were firstly washed with 37 ºC PBS once and detached by using 1x Trypsin/ EDTA and incubating them at 37 ºC.

Cryopreservation

90% FBS, and 10% DMSO.

Appendix B: OLIGONUCLEOTIDE SEQUENCES

Table B-1: Oligonucleotide Sequences used for Sequencing and Mutagenesis

Reference #	Name	Sequence
1	ANKIB1 Linker forward	5' GGGAGTGGGGGCGGGAGTCTCG AGAGATCTTGGTCTTC 3'
2	ANKIB1 Linker Reverse	5' GCCCCCACTGCCCCCGCCCACT AAATGTACTTGTTCAAG 3'
3	ANKIB1 RBR C to S forward	5'CACATGCAGAGTGCTAAGAGCAAGTATGACTT TAGCTGGATTAGCCTTGAAGAGTGGAAAAAACA TAG 3'

4	ANKIB1 RBR C to S Reverse	5' ATTGCTGCCTTCATTCTTCTGTATTGGAGAC TTACTGTTGGCACTAGGCTTGGAGTTAGTTAATA AC 3'
5	pBABE 5' Forward	5' CTTCCTCCATCCGCCCCG 3'
6	ANKIB1 internal forward 1	5' CTGAATATGCTGCATTAGACAAACG 3'
7	ANKIB1 internal forward 2	5' GTGAAGCACATGAGCCTTGTG 3'
8	pBABE 3' Reverse	5' GGACTTTCCACACCTGGTTGCT 3'