Characterisation of ANKIB1 as a new regulator of Toll-Like Receptor signalling

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I. Abstract

Interferon production triggered by the stimulation of the Pattern-Recognition-Receptors (PRR) following infection is primordial to induce an efficient immune response and prevent dramatic consequences for the host. Toll-Like Receptors (TLRs) play a crucial role in this process. Via TRIF, TLR3 and TLR4 are able to recruit IRF3 to the signalling complexes, thereby promoting its activation by TBK1. This latter is a crucial kinase involved in several immune signalling such as TNF, NOD, and TLRs where it contributes to the regulation of cell death or induces interferon production. However, its recruitment and activation to these receptors depend on different proteins, and post-translational modifications such as ubiquitination are primordial. In TNF pathway, TBK1 activation and recruitment to the TNF Receptor 1 Signalling Complex (TNFR1-SC) requires the presence of HOIP. This latter generates linear ubiquitin chains which are primordial to link NEMO to TBK1 via the adaptor molecules TANK and NAP1. On the other hand, in TLR3 pathway where TBK1 is required to induce type I and type III interferon, the presence of HOIP is not required suggesting that another E3 ligase is implicated in this process. However, this ubiquitin E3 is still not known. In this study, we have identified ANKIB1 as a new regulator of TBK1 activation in TLR3. The absence of this E3 ligase impairs TBK1 phosphorylation and, consequently, IRF3 activation, the transcription factor that mediates interferon production. Following TLR3 stimulation, ANKIB1 is recruited to TLR3-Signalling Complex (TLR3-SC) and interacts with TBK1, optineurin, and NEMO. Oppositely, ANKIB1 is not recruited to TNFR1-SC, therefore, is not involved in this signalling pathway. In order to promote TBK1 activation, ANKIB1 requires its catalytic activity which generates K11 and K63 ubiquitin chains. It also requires its UIM domain, which confers the ability to interact with ubiquitinated proteins. ANKIB1 is also involved in TBK1 and IRF3 phosphorylation in TRIF-mediated TLR4 signalling, but not by Myd88. Consequently, ANKIB1 is primordial to induce type I and type III interferon production following TLR3 and TLR4 stimulation. In summary, the results of this thesis identify ANKIB1 as a new member of TLR signalling pathways, which via its catalytic activity, contributes to the phosphorylation of TBK1 and consequently to the production of interferon-mediated by IRF3.

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1. Introduction:

1.1 Immune system

All phyla of life, ranging from simple prokaryotes to single and multicellular eukaryotes and plants ultimately rely on the availability of nutrients or energy to survive and proliferate. In heterotroph organisms, this is usually accomplished by hunting, scavenging, or a mixture of both. Some organisms, including fungi, bacteria ,or viruses, have devised strategies by which they invade other organism’s tissues or even their cells and reproduce by using their nutrients, resources and cellular machinery. This phenomenon of invasion of a host organism by a pathogen is called infection. Fortunately, eukaryotic organisms have the ability to defend themselves from these pathogens. As soon as these latter are infecting a host, they are recognised by the host cells as a non-self-organism and trigger an inflammatory response. This inflammation is crucial to alert the organism and activate an immune response that fights the pathogen in order to defend the host. An infection can lead to dramatic consequences and even death without an appropriate inflammatory response. Consequently, it is crucial to efficiently detect an infection and induce the appropriate activation of the immune system. This latter is tightly regulated and has a remarkable mechanism of action mediated by two systems that are linked together, which are the innate and adaptive immune systems.

1.1.1 The innate immune response

The innate immune system is considered as the first line of sensing and defence against pathogens. It is crucial to trigger the activation of the adaptive immune response with its target specificity and immunological memory. Pathogens are sensed by infected cells and surrounding innate immune cells through the binding of specific pathogen-associated molecular patterns (PAMPs) to specific pattern recognition receptors (PRR) (Akira, Uematsu, & Takeuchi, 2006). Pathogens that are able to disrupt this recognition or to delay the activation of the innate immune system thus have a survival advantage by delaying the potent adaptive immune response. The innate immune response is characterised as a non-specific defence.

It means that there are no immunological memories such as in adaptive immune response and therefore cannot be trained for a following similar infection. It comprises several cell types, including neutrophils, macrophages, dendritic cells, natural killer cells, mast cells, basophils, and eosinophils. Each of them has a specific role and contributes to an efficient anti-pathogen immune response (Janeway & Medzhitov, 2002). The innate immune system relies on different mechanisms of action. Following the sensing of a pathogen, they trigger a robust inflammatory response through the secretion of different chemokines and cytokines, essential to amplify the immune response. Some immune cell types are also phagocytic and endocytic, which enable the clearance of the pathogen and the antigen presentation to the immune cells of the adaptive immune response. In addition, they act on the physiology due to their ability to, for example, increase the temperature and lower the pH, such as in the stomach. Finally, they also form an anatomic barrier, for example in the skin or in mucous membranes such as in the intestine and therefore prevent the invasion of pathogens (Marshall, Warrington, Watson, & Kim, 2018).

1.1.1.1 The different cell types of the innate response

The most abundant type of innate immune cells are Neutrophils, which constitute between 40-75% of this cell population whilst maintaining a very high turnover. They are crucial to the first line defence, due to their ability to phagocyte pathogens or trap them by expelling their own DNA, named NETosis. Furthermore, they can release granules, a trait they share with granulocytes (eosinophils and basophils), another group of innate immune cells that are able to kill bacteria and fungi. Degranulation releases numerous proteases, enzymes, and reactive oxygen species that directly attack and degrade the pathogen. Another group of innate immune cells, mast cells, are also able to induce degranulation, they have, however, also been reported to be implicated in the development of allergies.

Another specialised innate immune cell class is the Natural Killer (NK) cells. Unlike most innate immune cell populations, which are derived from the myeloid lineage, NK cells are derived from lymphoid progenitors. They share features with adaptive immune cells but do not exert a pathogen-specific adaptation. They are present in different sites of potential infection but also in secondary lymphoid structures and blood.

After detecting infected cells through Major Histocompatibility Complex (MHC) class I, they release their granules containing different molecules inducing programmed cell death. These latter include death ligands such as FASL, TRAIL, and perforin and granzymes (Zamai et al., 1998).

Other cells that can phagocytose are the macrophages and dendritic cells. Both can be found in every body compartment and are considered antigen-presenting cells (APCs), even if dendritic cells are the main contributor to this process. Both contain almost all types of Toll-like Receptors (TLRs), allowing them to recognise all types of pathogens and trigger the adaptive immune response. The activation of the TLRs in macrophages, and dendritic cells to a lesser extent, induces a massive production of inflammatory cytokines and chemokines, including the crucial interferons. This inflammation promotes the recruitment of other innate-immune cells to amplify the inflammation, but most importantly, T-cells and B-cells, crucial cell types of the adaptive immune response.

Once activated, dendritic cells shift from a conventional state to a professional APC state. Then, they migrate to secondary lymphoid structures such as lymph nodes or the spleen in order to present MHC bound pathogen-derived epitopes to naïve B cells and T cells in order to activate them and trigger a specific antigen-immune response. Indeed, APCs trigger the differentiation of immune cells. In addition to antigen presentation, dendritic cells also secrete cytokines and chemokines that drive the differentiation and activation of B and T cells. Overall, dendritic cells play a critical role in coordinating the adaptive immune response to foreign pathogens.

1.1.1.2 The adaptive immune response

The innate immune response is able to contain the infection during the first days. However, their prominent role is to promote a strong inflammation that induces the recruitment and activation of B and T cells, members of the adaptive immune response. They are indispensable to trigger an efficient immune response due to their specificity of action regarding the pathogen.

T cells are generated from hematopoietic stem cells present in the bone marrow, mature in the thymus, and are activated and differentiated into the secondary lymphoid structure.

In order to be activated specifically to the pathogen present, they express a series of unique antigen-binding receptors on their membrane called T-cell receptors (TCR).

These latter will recognise the MHC of the APC that triggers their activation (Rossi et al., 2005). Following antigen recognition, T cells secrete cytokines, including Interleukine-2, 4, 12, which contribute to the immune response and will promote their differentiation into more specific subtypes. CD4+ T cells can differentiate into T follicular helper, Th1, Th2, Th17 and T reg. Regarding CD8+ T cells, they differentiate into different subtypes of CD8+ cytotoxic T cells (CTLs).

Cytotoxic T cells are then recruited to the infection site and efficiently kill infected cells through death signalling in a similar manner to NK cells. T-helper cells are in turn amplifying the immune response. They are not able to kill infected cells but efficiently trigger the maturation of other immune cells. Notably, alongside NK cells, they are the only cells able to secrete γ interferons that trigger the maturation of macrophages and promote their ability of pathogen clearance through phagocytosis (Schroder, Hertzog, Ravasi, & Hume, 2004). T helper cells also secrete cytokines that contribute to the activation of B cells. Once activated by APCs, B cells that contain specific antibodies for an antigen presented during the infection proliferate and become either antibody-secreting plasma cells or memory B cells. In contrast to innate immunity, subsets of T cells and B cells can become memory T and B cells that allow a faster adaptive immune response in case a similar pathogen infects the organism a second time.

Once the infection is over, effector cells have to be cleared and T-regs, a subtype of T helper cells mostly do this. They allow the clearance of immune cells by different mechanisms leading to the termination of the immune response. They can secrete anti-inflammatory cytokines such as IL-10 or 32 but also directly induce cell death by the release of perforin and granzymes (Vignali, Collison, & Workman, 2008). They also induce immune cells' exhaustion by the immune checkpoints including programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). Together, by a receptor-ligand mechanism, they target the activated T cells, B cells, macrophages and dendritic cells. A dysfunction or a lack of T-reg cells consequently leads to autoimmunity while an exacerbated activity would impair the immune response.

1.2 Pattern Recognition Receptors

The immune response is only possible through the detection of the pathogen by the resident immune cells and infected epithelial cells. This sensing in turn is accomplished by a set of so-called Pattern Recognition Receptors (PRRs). This family is composed of two types of receptors that are either cytosolic or transmembrane (Takeuchi et al., 2010). Cytosolic receptors are the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), the Nucleotide Oligomerization Domain (NOD)-like receptors (NLRs), Absent in Melanoma 2 (AIM2) and cGAS-STING cytosolic DNA sensor. The transmembrane receptors are the C-type lectin receptors (CLRs) and Toll-Like Receptors (TLRs).

1.2.1 CLR

CLRs are composed of more than 1000 proteins divided into 17 subgroups depending on their domain organisation and their phylogeny. These receptors are generally transmembrane proteins but some of them can be secreted and act as growth factors, antimicrobial proteins or even components of extracellular matrix (Iovanna & Dagorn, 2005). Their ligands can vary from proteins to lipids such as mannose or galactose that are present in numerous pathogens, including fungi, bacteria and viruses.

Once activated, these receptors mainly induce NF-kB and MAPK pathways to produce pro-inflammatory cytokines and chemokines such as TNF or IL-1β (Geijtenbeek & Gringhuis, 2009). However, they do not directly promote interferon production via the activation of IRFs. Other receptors such as PKD1 also activate mTOR and the Wnt-β-catenin pathway, implicated in the activation and differentiation of the cells sensing the pathogen. They are mostly expressed by the APCs such as dendritic cells and macrophages. Interestingly, once activated, these receptors are internalised and strongly contribute to the processing of the bound PAMP in order to be presented to the adaptive immune cells.

1.2.2 NLR

The family of NLRs has 22 known members that engage a variety of downstream signalling pathways. All members share common features including a Leucine-Rich repeat domain (LRR), a central nucleotide-binding domain (NBD) and they contain an ATPase activity. However, they can be divided into several subfamilies dependent on the domain present in the N-term of these receptors. The biggest subfamilies are NLRC which contains a Caspase recruitment domain (CARD) and the well-characterised NLRP subfamily that contains a pyrin domain (PYD) (Platnich & Muruve, 2019). The NLRPs have the ability to induce pyroptosis through the inflammasome formation mediated by a PYD-PYD interaction with Apoptosis-associated speck-like protein containing a CARD (ASC). Once ASC is recruited, Caspase-1 can also be part of the inflammasome by interacting with the CARD domain of ASC. Then, Caspase-1 is activated, leading to the induction of an immunogenic cell death called pyroptosis by processing and release of pro-IL-1β/18. Ultimately, Caspase-1 cleaves gasdermins that are effector proteins making membrane pores, promoting cell death.

The non-canonical NLRPs operate independently of the inflammasome due to the lack of the PYD or BIR domain. NOD1/2 are two well-characterised NLRs that induce the formation of a multi-protein complex following the binding of bacterial peptidoglycans. Under basal conditions, both receptors are in a close conformation. Once the ligand binds to the receptor, they change to an open conformation, leading to the recruitment of RIPK2. Consequently, several proteins are recruited, notably TAK1, leading to the formation of a multi-protein signalling complex (Caruso, Warner, Inohara, & Núñez, 2014). Thus, NOD1/2 promotes the activation of the gene-activatory NF-kB and MAPK pathways.

1.2.3 cGAS-STING

cGAS - STING is a new member of the PRR family. Cyclic GMP-AMP synthase (cGAS) is a nucleotidyl transferase that recognises cytosolic double-stranded DNA. The DNA can have different origins such as viral, bacterial but also endogenous. Interestingly, it has been shown very recently that large amounts of cGAS are also found in the nucleus.

Once DNA binds to cGAS, it switches to an open conformation and produces a second messenger, cyclic GMP-AMP (cGAMP). This new messenger is then recognised at the endoplasmic reticulum by the cyclic-dinucleotide sensor called STING and leads to its activation. Subsequently, TBK1 is recruited, activated and phosphorylates STING on the serine 366, creating a docking site to recruit IRF3 (Hopfner et al., 2020). Such as with RLR and several TLR members, IRF3 is phosphorylated by TBK1, leading to its dimerization and export to the nucleus to induce interferon production. It has been shown that STING can also promote NF-kB and MAPK pathway activation; however, the mechanism is still unclear. Possibly STING requires TBK1 which directly phosphorylates IKKs and activates the downstream effector proteins (Abe & Barber, 2014).

1.2.4 AIM2

AIM2 is a cytosolic receptor between the NLRP family and cGAS. Indeed, it senses cytosolic DNA, consequently leading to inflammasome formation and pyroptosis. It is mostly expressed in myeloid cells. Despite the tissue specific expression of this receptor, it has been shown that AIM2 is crucial to produce IL-1β, IL-18 and γ interferon following listeria monocytogenes (Rathinam et al., 2010).

1.2.5 RLR

RLR members sense RNA viruses through three different receptors that are the retinoic acid-inducible gene-I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). RIG-I recognises 5'-triphosphate (5'-PPP) or short double-stranded RNA (dsRNA), whilst MDA5 recognizes long dsRNA structures, which are often produced during viral replication. Conversely, methylated RNA, which can be found only in mammals, cannot activate these receptors. However, recently the role of mitochondrial RNA in the activation of these receptors has been actively studied (Dhir et al., 2018). The three members of the RLR family have a central DEAD helicase domain. An additional RNA-binding fold, called the carboxy-terminal domain (CTD), is also found in all RLRs. However, only RIG-I and MDA5 contain two Caspase activation and recruitment domains (CARDs) in tandem orientation in their N-

termini. It is important to note that LGP2 is not able to induce a signalling pathway on its own (Rehwinkel & Gack, 2020). However, several studies have shown that despite the absence of a CARD in LGP2, it has a major role in the regulation of the activation of the two other receptors. Indeed, it has been shown that LGP2 inhibits the recognition of RNA by RIG-I as a feedback inhibitor. On the other hand, it promotes the signalling through MDA5 by increasing its dimerization following the binding of its cognate ligand. LGP2-deficient mice failed to trigger a proper interferon response after infection with the EMCV virus. After binding of the viral RNA, both RIG-I or MDA5 oligomerize and bind to the adaptor protein MAVS through the CARD domain. This leads to a signalling cascade with the recruitment of the non-canonical IKKs TBK1 and IKKε. Their recruitment is still unclear but involves TRAF3 (Nakhaei et al., 2009). Moreover, other TRAFs have been reported to be implicated in their recruitment but this observation seems controversial and needs further evidence (Fang et al., 2017). NEMO, key protein involved in the recruitment of the canonical IKKs, has been shown to be required for TBK1 and IKKε activation (T. Zhao et al., 2007). Additionally, the requirement for other adaptor proteins such as TANK, NAP1 or SINTBAD have been reported but need further scrutiny. Once recruited, TBK1 and IKKε phosphorylate MAVS which consequently induces the recruitment of IRF3 through the *p*L*x*IS (where *p* is a hydrophilic amino acid and *x* is any amino acid) consensus motif (S. Liu et al., 2015). This latter is then phosphorylated by TBK1, detached from the complex and goes to the nucleus to induce interferon production. RIG-I or MDA5/MAVS are also able to induce the activation of the NF-κB pathway.

1.2.6 TLR

The family of Toll-Like-Receptors constitutes an essential part of the innate immune response. It was first discovered as a gene that controls the dorsal-ventral polarity of the drosophila embryo. A few years later, the first implication of these receptors for anti-fungal immunity was proved. Nowadays, we know that TLRs include ten members in humans and thirteen in mice, however, the tenth member is not functional in this latter. They are ubiquitously expressed, but their abundance varies. Due to their crucial role in pathogen sensing and the activation of the adaptive immune response, APC cells have the highest abundance of TLRs. Epithelial cells also express TLRs in a large amount, with some differences regarding their localisation.

For example, apical epithelial cells express fewer TLRs than basolateral epithelial cells due to their strong exposure to bacteria. To preserve the integrity of the physical barrier such as in the intestine, Basolateral epithelial cells express more TLRs in order to be able to trigger inflammation if pathogens are in an area where they should not be. As a positive feedback mechanism, most of the TLRs are upregulated following pathogen sensing, notably due to interferon production. After their synthesis in the Endoplasmic Reticulum, TLRs are transferred to the Cis-Golgy and in cooperation with chaperone proteins such as UNC93B1, gp96 or PRAT4A, they adopt their final configuration and are exported to their appropriate compartments (Lee et al., 2013; Takahashi et al., 2007; Yi Yang et al., 2007). UNC93B1 for example, is preferentially associated with trafficking of the nucleic acid-sensing TLRs that are found in the endosome. On the other hand, PRAT4A and gp96 are linked to trafficking and appropriate folding of the TLRs present on the cytoplasmic membrane. Each member of the TLR family recognises a specific pathogen pattern. TLRs implicated in the recognition of bacteria, fungi and protozoa such as TLR1, 2, 4, 5, 6, and 10 are localised at the cell surface. On the contrary, TLRs involved in viral sensing are rather localised intracellularly such as in endosomes, lysosomes and endoplasmic reticulum.

Table 1.1: The different TLRs in human and their ligands.

Receptor	Localisation	Ligand	Origin of Ligand
TLR1/TLR2	cell surface	Triacyl lipoproteins	Bacteria
TLR2	cell surface	Lipoproteins, Zymosan(beta-glucan)	Bacteria Fungi
TLR3	cell compartment	Double-stranded RNA, poly (I:C)	viruses
TLR4	cell surface	LPS , heat shock proteins	Bacteria Host cells
TLR5	cell surface	Flagellin	Bacteria
TLR6/TLR2	cell surface	Diacyl lipoproteins	Bacteria (primarily mycoplasma)
TLR7	cell compartment	Single-stranded RNA	viruses
TLR8	cell compartment	Double-stranded RNA, Single-stranded RNA	viruses
TLR9	cell compartment	CpG-DNA	Bacteria, viruses, protozoa, host cells
TLR10	cell surface	Pili	Bacteria

TLRs are type I transmembrane glycoproteins that contain 20-27 N-terminal extracellular leucine-rich repeats (LRR) which bind and recognise their specific ligands. A transmembrane helix is followed by an intracellular toll-interleukin 1 receptor (TIR) domain that is required to trigger the recruitment of the main adaptor proteins myeloid differentiation primary response gene 88 (Myd88) or TIR domain-containing adaptor inducing IFN-β (TRIF) and consequently trigger the formation of the signalling complexes. Apart from TLR3, every TLR recruits Myd88 with a particularity for TLR4 that is able to recruit both. Other proteins facilitate the binding of these adaptor molecules. Mal or TIR Domain-Containing Adapter Protein (TIRAP) accompanies Myd88 recruitment while TRIF-related adaptor molecule (TRAM) is recruited before TRIF to facilitate the interaction with the TIR domain for TLR4.

1.2.6.1 TLR3 signalling pathway

TLR3 is widely expressed among cells. It localises on endosomal membranes and is crucial for the anti-viral response. After its synthesis in the endoplasmic reticulum and N-glycosylation, it travels to the Golgi apparatus where it is fully glycosylated in order to be resistant to endoglycosidase H (Endo H). Then, through UNC93B1, it leaves the Golgi and goes to the endosomes where it is cleaved by different cathepsins at the N-terminus (Qi, Singh, & Kao, 2012). This cleavage, which does not fully dissociate the receptor, increases the binding affinity to double-stranded RNA (Fitzgerald & Kagan, 2020). As a consequence of a viral infection or dsRNA binding, TLR3 dimerizes to enhance the receptor-ligand complex. Successively, a TLR3 homodimer recruits TRIF to relay signals to different downstream effectors in order to form the TLR3-signalling complex **(Figure 1.1**). Conversely, it has been shown that Full-Length TLR3 is not able to bind TRIF (Toscano et al., 2013). This latter is essential to recruit RIP1, but also TNF receptor-associated factor 6 (TRAF6) and TRAF3, both RING-type E3 ubiquitin ligases. While TRAF6 and RIP1 induce the MAPK and NF-kB pathways, TRAF3 is responsible for the induction of interferon production (Oganesyan et al., 2006). RIP1 binds homotypically via a RHIM - RHIM interaction with the C-terminus of TRIF, while TRAF6 binds to the N-terminus of TRIF, collectively relaying the signal for NF-kB activation (Cusson-Hermance, Khurana, Lee, Fitzgerald, & Kelliher, 2005).

Both proteins require K63-linked polyubiquitination to bind to TAB2/3, facilitating the recruitment of TAK1. TAK1 is an essential protein that not only induces the MAPK pathway but also contributes to IKKα/IKKβ (Jiang, Mak, Sen, & Li, 2004). Although TRAF6 ubiquitinates both RIP1 and itself, RIP1 further needs the contribution of several E3 ubiquitin ligases in order to be fully ubiquitinated thus enhancing the stability of the signalling complex and recruiting other proteins involved in the complex such as Pellino-1 (M. Chang, Jin, & Sun, 2009). Similarly to other pathways such as TNF or TRAIL signalling, LUBAC is also recruited to TLR3-SC and generates linear ubiquitin chains on the components of the TLR3-SC, which are important for the proper recruitment and activation of the IKK complex that triggers phosphorylation and degradation of IkBα and nuclear translocation of the NF-kB (Zinngrebe et al., 2016). The precise substrate of LUBAC in this context has not been totally addressed. On the other hand, TRAF3 generates K63 polyubiquitin chain on itself, which forms a platform for the recruitment of NEMO and consequently of the adaptor proteins TANK and/or NAP1, shown to be important for the recruitment of the non-canonical IKKs, TBK1 and IKKε (Guo & Cheng, 2007; Sasai et al., 2006). Both interact with each other but their expression differs. While TBK1 is ubiquitously expressed, IKKε is mostly expressed in lymphoid cells and only induced by gene activation pathways in other cell lines. Consequently, while TBK1 has been shown to be crucial for IRF3 phosphorylation and interferon production, IKKε is not required for this process and is rather cell-type specific (Gleason, Ordureau, Gourlay, Arthur, & Cohen, 2011; Perry, Chow, Goodnough, Yeh, & Cheng, 2004; tenOever et al., 2007).

In contrast to Myd88 signalling, TRAF6 deletion does not impair TBK1 phosphorylation downstream of TLR3 activation (Jiang et al., 2004). However, the requirement of these adaptors for interferon production is still unclear, in part because these results were mostly done in an overexpression system. NAP1 knock-out mice did not show any defect in interferon production suggesting that its requirement for TBK1 activation is not validated in-vivo (Fukasaka et al., 2013). Moreover, it has been shown that BMDM isolated from TANK knock-out mice have impaired IKKε phosphorylation but not TBK1 and IRF3 activation. Therefore, they were able to induce, as efficiently as wild-type BMDM, the interferon production following TLR3 activation (Kawagoe et al., 2009). This goes in line with the dispensable role of IKKε in interferon production reported by others.

Oppositely, TBK1 knock-out MEFs were unable to activate IRF3 and consequently to produce interferon (McWhirter et al., 2004). Additionally, it has been shown in-vitro and in-vivo that Optineurin, another adaptor protein, is also very important for the activation of TBK1 and IRF3, but not IKKε (Munitic et al., 2013; Slowicka et al., 2016).

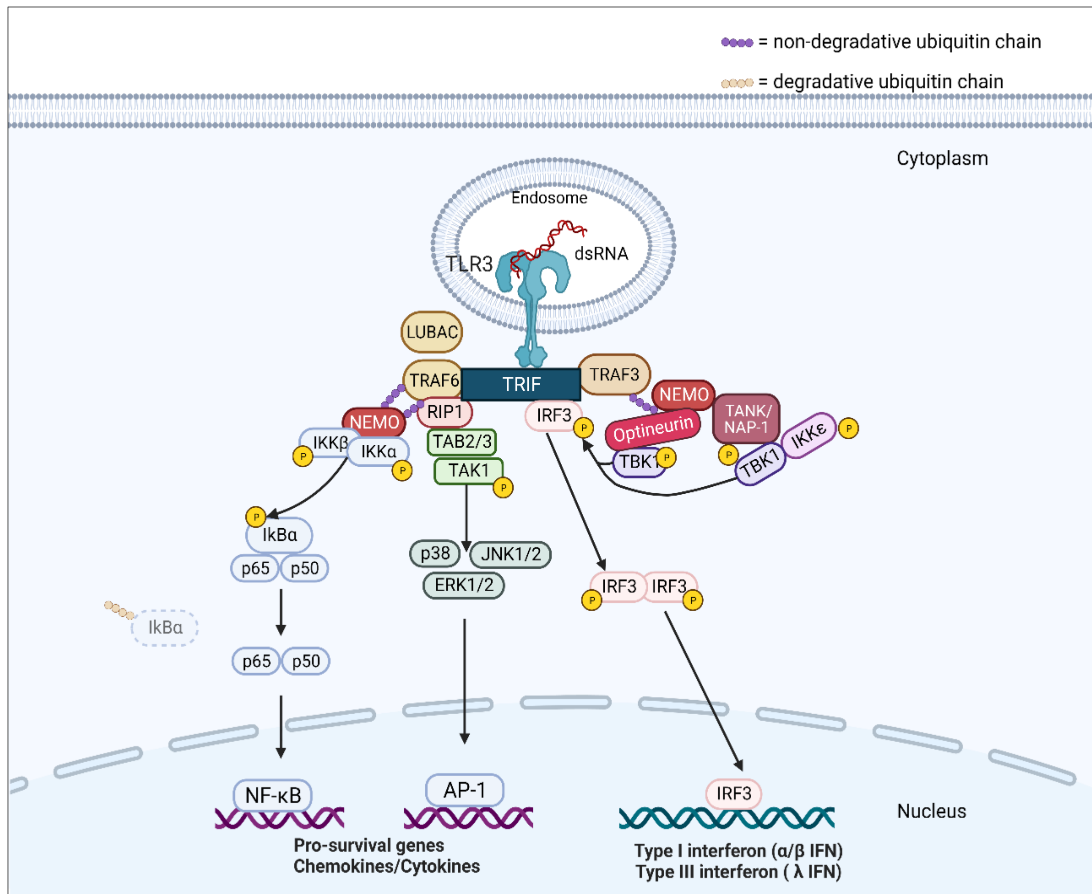


Figure 1.1. TLR3 signalling pathway:
After binding of the dsRNA to TLR3 triggering its dimerisation, TRIF is recruited via the TIR domain. TRAF6-RIP1 axis is responsible for the activation of the NF-κB and MAPK pathway while TRAF3-TBK1 axis is involved in interferon production via the activation of the transcription factor IRF3. Created with BioRender.

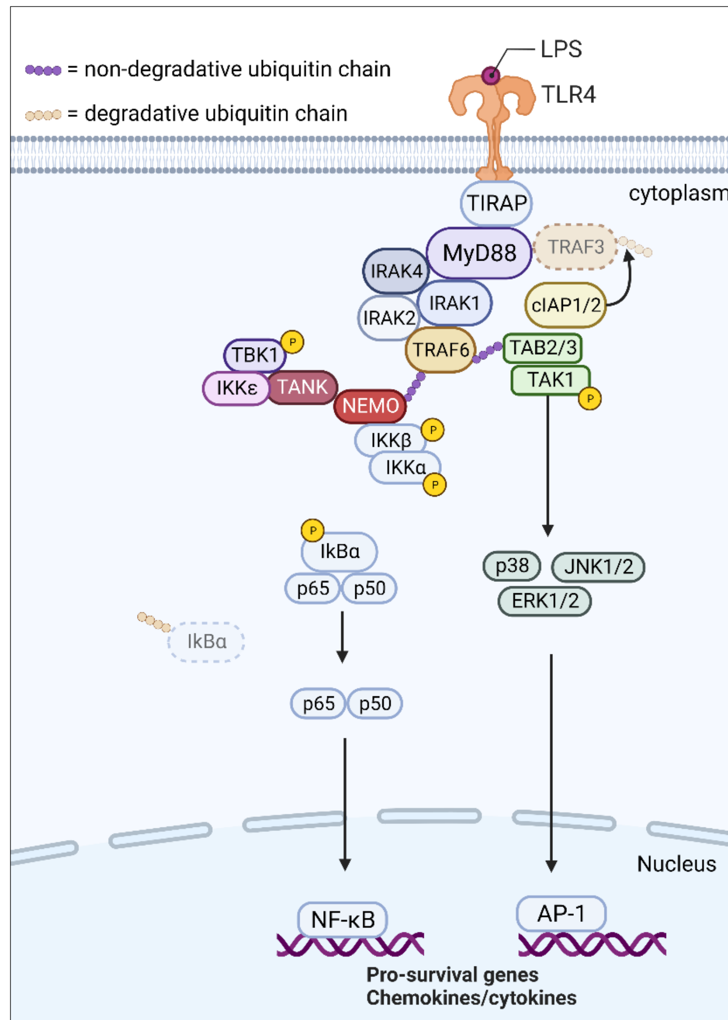
Interestingly, in order to efficiently interact with TBK1, Optineurin requires binding to polyubiquitin chain (Gleason et al., 2011). The role and requirement of these adaptor proteins have been addressed separately and therefore their requirement for this phenomenon is still unclear. Activated TBK1 firstly phosphorylates a serine of the TRIF's *p*L*x*IS IRF3-binding motif, crucial for the recruitment of IRF3 to the complex. Once recruited, IRF3 is phosphorylated by TBK1, which induces its homodimerization.

Thus, IRF3 detaches from the signalling complex and translocates to the nucleus to bind IFN-stimulated response element (ISRE) in order to promote type I (α/β) and type III (λ) interferon expression. TBK1 phosphorylation can also induce the heterodimer formation between IRF3 and IRF7, albeit IRF7 is expressed only in a few cells.

1.2.6.2 Myd88-mediated TLR4 signalling pathway

Except for TLR3, every TLR triggers the activation of downstream signalling through Myd88. TLR4, in turn, uses both Myd88 and TRIF. TLR4 localises to the plasma membrane and recognises lipopolysaccharide (LPS), a glycolipid found on the membrane of gram-negative bacteria. First, LPS binds to LPS-binding protein (LBP) that transfers the glycolipid to CD14 and then to the complex myeloid differentiation 2 (MD2)-TLR4, consequently triggering their dimerisation. While CD14 and LBP are not primordial, TLR4 alone is insufficient to trigger a signalling pathway following LPS binding and requires the binding of MD2. It has been reported that CD14 is, however, essential for the endocytosis of the receptor after LPS binding, which promotes the formation of a TRIF-mediated signalling complex (Zanoni et al., 2011). The dimerisation of the receptor allows the recruitment of TIRAP. Then, Myd88 is recruited and induces the formation of a signalling complex, which differs from the TRIF-mediated signalling complex seen in endocytosed TLR4 and TLR3. Myd88 signalling is executed through the recruitment of IL-1R-associated kinases (IRAK) 1, 2 and 4 and consecutively TRAF6 (**Figure 1.2**). Like TLR3-mediated signalling, TRAF6 auto-ubiquitinates itself, allowing the binding of TAB2/TAB3, TAK1 and NEMO binding and consequently leads to the activation of the NF-κB and MAPK pathways. However, the recruitment and activation of the non-canonical IKKs differ from TRIF-mediated signalling. Initially, TRAF3 is recruited to Myd88 but is rapidly degraded following the addition of K48 ubiquitin chains mediated by cIAPs (Tseng et al., 2010). Nonetheless, TBK1 and IKKƐ are subsequently activated and recruited to the Myd88-Signalling Complex. Interestingly, TANK deletion abolishes IKKε activation but only decreases slightly the phosphorylation of TBK1 following TLR4 activation (Clark, Takeuchi, Akira, & Cohen, 2011). However, IRF3 activation, dependent on TRIF-mediated signalling, is not impaired without TANK.

This confirms that TANK is crucial for IKKε activation and that the latter is not required for interferon production.



<u>Figure 1.2</u>. Myd88-dependent TLR4 signalling pathway:
After LPS binding to TLR4 triggering its dimerisation, TIRAP interacts with the receptor and promotes the formation of the Myd88-mediated signalling complex. IRAK 1/2/4 are then linking Myd88 to TRAF6, responsible for the activation of the NF-κB and MAPK pathway. TRAF3 is rapidly degraded by cIAP1/2, preventing its recruitment to the signalling complex. TBK1 and IKKε are recruited in close proximity to canonical IKKs via the involvement of TANK that can interact with NEMO. However, Myd88 cannot recruit IRF3, preventing its activation via the non-canonical IKKs and, consequently, interferon production. Created with BioRender.

Additionally, it shows that TBK1 activation depends on alternative adaptor proteins in TRIF-mediated signalling. It has been proposed that whilst TRIF activates TBK1 via TRAF3, Myd88-induced TBK1 phosphorylation depends on the canonical IKKs that form a complex with NEMO. TANK binds to NEMO and TBK1, allowing its recruitment into the signalling complex where it will be in close proximity to the canonical IKKs to undergo phosphorylation (Clark, Peggie, et al., 2011).

Because the depletion of TANK in mice leads to the development of a hyper-inflammatory phenotype caused by an exacerbated Myd88 signalling, it also suggests that TBK1 acts as a negative feedback to IKKs activation. Interestingly, the deletion of NAP1 does not have a similar effect on the gene-activation following LPS treatment, suggesting that NAP1 does not have a similar role than TANK in Myd88-mediated TLR4 signalling (Fukasaka et al., 2013).

It is important to note that Myd88, despite its ability to activate TBK1, is not able to activate IRF3 and therefore to induce interferon production. Recent work found that IRF3 cannot be recruited to the signalling complex, due to the absence of the IRF3-binding motif (*p*L*x*IS) on Myd88 (S. Liu et al., 2015).

1.3 NF-κB pathway

The importance of immune receptors is due to their ability to mediate the activation of several gene-activatory pathways. In order to adapt and react to changes in the environment, cells are often required to produce additional proteins. The sensing of stimuli (e.g., cytokines, pathogens, or chemical substances) relays the information through a signalling cascade involving numerous proteins. Consequently, their activation leads to the activation of several transcription factors. Depending on the stimulus and the set of transcription factors that are triggered, the cells trigger the production of protein selectively. One of the most important transcription factors is the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). After its discovery in B cells, NF-κB was identified as a member of a family of transcription factors that regulates gene activation in all cell types and is activated through various immune receptors including PRR and several members of the TNF superfamily such as TNF and TRAIL (Israël, 2010). The NF-κB family can be divided into two classes. Class I consists of the precursors p100 and p105 that are respectively processed to p52 and p50 following activation. Class II comprises Rel-family members RelA (also known as p65), RelB and cRel. Class I and class II members have an N-terminal Rel homology domain (RHD) that is composed of two immunoglobulin-like structures, which mediate homo- and hetero-dimerisation. Additionally, this conformation promotes the binding to DNA.

The RHD domains are subject to posttranslational modifications, including acetylation, phosphorylation and ubiquitination that consequently influence the protein's activity positively or negatively. All members except p50 and p52 also contain a transcriptional activator domain (TAD).

Therefore, p50 and p52 must dimerise with a member of the Rel family to form a functional transcription factor. In total, 12 combinations exist that can activate transcription and each of them can trigger the activation of different genes to a different extent (Napetschnig et al., 2013). In addition, dimers that lack a TAD, but still bind to DNA, can potentially function as inhibitors, thus adding increased complexity to the system. At a steady state, the NF-κB family members are sequestered in the cytosol via the binding of inhibitors of κB (IκBs) to the RHD through an ankyrin repeat domain. Consequently, the nuclear localisation sequence (NLS) is not accessible. There are six known proteins in the IκB-family: three typical members IκBα, IκBβ, IκBε, and three atypical members IκBδ, IκBζ and Bcl-3. Only Bcl-3 has been reported to exert an opposite role and over-activates p50 homo-dimers rather than inhibiting them. For class II NF-κB members to translocate to the nucleus, IκB must be removed. This form of NF-κB activation is termed canonical NF-κB signalling. In contrast, p100 is activated through proteolytic cleavage and is referred to as non-canonical NF-κB signalling. Different stimuli activate both signalling modalities and do not involve the same proteins. Nevertheless, both are crucial for an appropriate immune response.

1.3.1 Canonical NF-κB pathway

As mentioned previously, the canonical activation of NF-κB signalling requires the transfer of NF-κB dimers to the nucleus, which is typically blocked by IκB proteins. In order to activate NF-κB, the trigger of signalling through immune receptors such as PRR, TNF, IL-1β, and DAMPs leads to the phosphorylation of IκB kinases (IKKs) IKKα and IKKβ. These kinases interact with the regulatory subunit IKKγ/NEMO and are recruited to the different signalling complexes. IKK phosphorylation is the first step in the NF-κB activation cascade. The molecular basis for IKK activation is not fully understood, but it is suggested that TAK1 phosphorylates and activates IKKβ in a ubiquitin-dependent manner (C. Wang et al., 2001). Additionally, it was proposed that recruitment of NEMO

to ubiquitinated proteins like RIP1 might lead to oligomerisation and therefore, proximity-induced activation of IKK via trans-phosphorylation (Taniguchi & Karin, 2018). Once activated, IKKs phosphorylate IκBα on two serines in N-terminus (DiDonato, Hayakawa, Rothwarf, Zandi, & Karin, 1997).

These phosphorylations trigger its K48 polyubiquitination by the SCFβ-TrCP E3 ubiquitin ligase complex on lysines K21 and K22. Subsequently, IκBα is degraded by the 26S-proteasome, thereby releasing the NF-κB dimers (Spencer, Jiang, & Chen, 1999). Degradation of IκBα unveils the NLS in the NF-κB dimer, allowing it to enter the nucleus and bind to its cognate DNA binding sites (Ganchi, Sun, Greene, & Ballard, 1992). The pathway is tightly regulated, including negative feedback loops driven by proteins that are themselves NF-κB target genes. For example, DUBs, like CYLD and A20, can remove ubiquitins from signalling complex components, thus inhibiting continuous IKK activation and NF-κB signalling (Draber et al., 2015). The stabilisation of IκBα restores the inhibition of NF-κB by sequestering them in the cytosol. Regulation of canonical NF-κB activation and its termination is crucial to ensure correct gene transcription. This is particularly important given that the persistence of the NF-κB signalling can significantly alter the target gene landscape, with certain target genes showing an immediate response, while others require prolonged activation.

1.3.2 Non-canonical NF-κB pathway

The non-canonical NF-κB signalling pathway is activated by some PRRs such as c-GAS/STING but also by some TNF superfamily ligands, including CD40 ligand, receptor activator of NF-κB ligand (RANKL), lymphotoxin β, or B cell-activating factor (BAFF) (Ramakrishnan et al., 2004). This pathway plays a crucial role in cell differentiation, survival and organogenesis, and is regulated differently from the canonical pathway. The NF-κB-inducing kinase (NIK) is essential in this alternative pathway as it phosphorylates and thereby activates IKKα, which is the major IKK protein required for non-canonical NF-κB signalling. This, in turn, leads to p100 recognition by the E3 ligase complex SCFIκB and its K48-polyubiquitination, resulting in partial proteasomal degradation and the release of the p52 fragment. Together with RelB, it forms the primary NF-κB heterodimer in non-canonical NF-κB signalling.

Unlike canonical NF-κB signalling, the alternative pathway does not require the degradation of IκBα. This could be due to the low affinities that RelB-containing dimers show for canonical IκBα proteins, rendering them less dynamic. In non-canonical NF-κB signalling, most of the regulation occurs by modulating the activity of NIK, the central kinase in this pathway (S.-C. Sun, 2017).

NIK is negatively regulated by K48-linked poly-ubiquitination through proteins such as TRAF2, TRAF3 and cIAP1/2, which interact with NIK in a cytoplasmic complex and induce its proteasomal degradation (Zarnegar et al., 2008). Upon cellular stimulation, TRAFs and cIAPs are recruited to the respective receptor signalling complexes, followed by TRAF3 degradation, allowing NIK stabilisation and subsequent activation of RelB/p52(Liao et al., 2004). The non-canonical NF-κB pathway is a somewhat slower process but yields long-lasting NF-κB activity.

1.4 MAPK pathway

Mitogen activated protein kinases (MAPKs) play a vital role as signal transduction hubs downstream of various extracellular receptors. These kinases are evolutionarily well-conserved and are involved in a wide range of cellular processes, including metabolism, cell division, motility, cell death, gene expression, and survival (W. Zhang et al., 2002). While growth factors were initially found to activate MAPKs, it has since been discovered that a broad range of cell signalling receptors can activate them. MAPKs can be categorized into two groups: conventional and unconventional MAPKs. The conventional MAPKs include Extracellular Signal Regulated Kinase 1/2/5 (ERK1/2/5), c-Jun N-terminal kinase 1/2 (JNK1/2), and p38 (α, β, γ, δ), all of which are characterized by their N-terminal Ser/Thr-kinase domain. In contrast, unconventional MAPKs also have a Ser/Thr-kinase domain located at their N-terminus but possess additional distinct features that do not conform to the composition of conventional MAPKs (Braicu et al., 2019). The activation of conventional MAPKs is hierarchical and involves a cascade of kinases whereby a MAPK is activated by a MAPK kinase (MAP2K or MKK), which is previously activated by a MAPKK kinase (MAP3K) (Cargnello et al., 2011). MAP3Ks are activated in response to receptor stimulation and often interact with members of the Ras/Rho pathway.

Examples of MAP3Ks include MEK Kinase Kinase 2/3 (MEKK2/3), Rapidly Accelerated Fibrosarcoma 1 (Raf-1), Tumor Progression Locus 2 (TPL-2), and TAK1. The activation of MAPKs by receptors from the innate immune system often involves TAK1, which phosphorylates MKK3, -4, -6, and -7. Once activated, MKK3/6 activate p38, and MKK4/7 activate JNK.

Once activated, the MAPKs induce the activation of different transcription factors of the Activating protein-1 (AP-1) family such as c-Jun, Fos or ATF that lead to dimer formation in order to induce the transcription of several pro-survival genes (Plotnikov et al., 2011). The activation of TAK1 was found to require ubiquitination mediated by TRAF2/6 (Fan et al., 2010).

1.5 Interferon signalling pathway

Interferons were discovered in 1980 and are named after their ability to interfere with viral replication in host cells. This family is divided into three very distinct types that are type I (mostly α and β but also ε, κ, ω), type II (γ) and type III (λ) interferon.

Any cell type that is able to sense pathogens through the different PRRs can produce type I and type III interferon. α interferon is encoded by 13 genes in humans (14 in mice), while a single gene encodes β interferon. The same is true for γ interferon, and is mostly produced by immune cells, especially T-cells and NK-cells. 3 genes encode λ interferon in turn. In order to induce the production of interferon after the sensing of a pathogen, the cells need to activate a member of the transcription factor family named Interferon-Regulatory Factors (IRF). They were originally thought to be all implicated in interferon production. However, researchers have proven that this family is not only involved in this process and can even have an inhibitory role. Nine members compose this family and their expression strongly varies between cell types and members (Nguyen, Hiscott, & Pitha, 1997). As mentioned previously, IRF 1/5/3/7 promote the expression of type I and III interferons. However, even if IRF1 and 5 can be involved in this process, it has been shown that their deletion does not influence interferon production following virus infection (Reis, Ruffner, Stark, Aguet, & Weissmann, 1994). This is probably explained by their low expression or their mechanism of activation.

IRF3 is expressed ubiquitously therefore is the most described IRF. Unlike IRF3, IRF7 is barely expressed in cells at a steady state, except for pDC, monocytes and B-cells that constitutively express it (Marié, Durbin, & Levy, 1998). Indeed, IRF7 gene is an interferon-stimulated-gene (ISG) that is induced following interferon signalling produced by IRF3.

After activation of TLRs or other PRRs, TBK1 and IKKε phosphorylate IRF3 firstly on serine 396 which changes its conformation and allows the future binding to CREB binding protein (CBP) in the nucleus. Secondly, the non-canonical IKKs phosphorylate IRF3 on the serine 386 that leads to its dimerization and its release from the signalling complexes to go to the nucleus. The promoter region of β interferon is composed of four regulatory elements called positive regulatory domains (PRDs) I, II, III and IV. α interferon promoter region only contains 2 PRDs. PRD I and III are the binding sites for IRF1/5/3/7, whereas PRDII is for NF-κB and PRD IV is for AP-1 downstream transcription factor of the MAPK pathway. Once all are bound to their respective PRD, the enhanceosome is formed. This latter consequently recruits histone acetyltransferases and CREB binding protein (CBP) that promote the open conformation of DNA and therefore the transcription of the β interferon gene. Thus, β and λ interferon are produced. Interestingly, λ interferon production strongly depends on the activation of NF-kB, while type I is only slightly impacted by its absence. α interferon is mostly produced in the secondary response through IRF7 (Honda & Taniguchi, 2006).

Once type I or III interferons are produced by IRF3 following the sensing of a pathogen, they are released in order to activate interferon receptors in an autocrine and paracrine manner. Type I interferon binds to IFNAR1 and 2 whereas type III interferon binds to IFNLR1 with a strong affinity and IL-10Rβ with a low affinity. IFNARs are ubiquitously expressed while IFNLR and IL-10Rβ are mostly expressed at the plasma membrane of epithelial cells. Interferon binding leads to receptor dimerisation and subsequent recruitment of TYK2 to IFNAR1 or IL-10Rβ and JAK1 to INFAR2 or INFLR1. Then, JAK1 phosphorylates STAT2 and TYK2 phosphorylates STAT1, leading to their activation and the formation of a hetero-trimeric complex including both STATs and IRF9 (Mesev, LeDesma, & Ploss, 2019). Together, they form the IFN-stimulated gene factor 3 (ISGF3) that translocates to the nucleus in order to initiate the transcription of Interferon Stimulated Genes (ISGs) through the binding to IFN-stimulated response elements

(ISREs) (Platanias, 2005). ISGs are composed of approximately 90 different proteins that have diverse role. They can contribute to the anti-viral activity of interferon pathway by blocking the viral replication or contribute to the amplification of the immune response by coding notably for different IRFs or PRRs such as IRF1, IRF7 or RIG-I.

However, some ISGs are also important to control the interferon response by exerting a negative feedback role. For example, the ISGs SOCS1 and SOCS3 or USP18 are very important to control the interferon response by impairing the activation of STAT and JAK respectively (Ivashkiv & Donlin, 2014). In addition, several ISGs proteins are implicated in the regulation of cell death, representing a link between interferon response and cell death induction after sensing a pathogen. Type I and Type III interferon have very similar, albeit not identical transcription profiles. Consequently, they are not redundant and show class-specific signalling. While type I interferon triggers a strong and quick interferon response and ISG upregulation, type III interferon elicits a more indolent but long-lasting response. Due to the differential expression of the receptors, type I interferon response is systemic whereas type III interferon response remains only in epithelial cells and few immune cells (neutrophils and dendritic cells) (Lazear, Schoggins, & Diamond, 2019).

In contrast, type II interferon is not directly produced by PRRs such as the other types of interferons. It has been shown that type I interferon, Il-12, IL-15 and IL-18 are able to induce the production of type II interferon in NK cells through the activation of NF-kB, MAPK pathway and STAT3 activation. Once released, γ interferon binds to IFNγR1 and IFNγR2. Respectively, they recruit JAK1 and JAK2, which consequently induce the phosphorylation of STAT1. ISGF3 is not formed but rather a STAT1 homodimer that goes to the nucleus, binds the IFNγ activation site (GAS) DNA elements and activates the transcription of a totally different ISG repertoire (Platanias, 2005).

1.5.1 Role of type I and type III interferon in diseases

As discussed previously, interferons are essential to trigger an efficient and appropriate immune response to defend the host from any infection. However, an exacerbated or dysregulated interferon response can also be detrimental and lead to an over-sensitivity following infection or even the development of auto-immune diseases.

1.5.1.1 Beneficial effects during infection

The absence of efficient interferon production has been linked to increased mortality following influenza infection. IRF3 and IRF7 single knock-out mice have a decreased survival and double knock-out animals succumb very rapidly to the infection (Hatesuer et al., 2017). Interestingly, IFNAR knock-out mice were able to fight the infection, most likely due to redundancy between the type I and type III interferon response. Studies with SARS-Cov highlighted the importance of an immediate interferon response, as a delay led to a higher virus burden and increased mortality (Channappanavar et al., 2016). Consequently, it is not surprising that viruses frequently encode for proteins that can dampen and delay the interferon response (Cockram et al., 2021). Physiologically, interferons are crucial to enhance the activity of dendritic cells and macrophages, which then activate the adaptive immune response and promote their differentiation. Indeed, interferon promotes the cell surface expression of the different MHC molecules but also their co-stimulatory molecules such as CD80/86. They also directly upregulate the release of crucial chemokines implicated in their migration to secondary lymphoid structures. On T cells, interferons have also been shown to promote the differentiation into Th1 cells, implicated in the type II interferon production, crucial for the activation of NK cells (Huber & Farrar, 2011). Interestingly, type I interferons are also described to control the proliferation of CD8 T cells through an anti-proliferative effect via STAT1 activation. However, through other STATs, it promotes their survival and clonal expansion (Crouse, Kalinke, & Oxenius, 2015). Interferons are thus indispensable for a fully-fledged immune response, but equally ensure its appropriate regulation.

1.5.1.2 Detrimental effects mediated by interferons

While the importance of interferons during infections is undisputed, an exaggerated and uncontrolled activation can at times harm the host. For example, in the context of infection with Listeria monocytogenes, the absence of INFAR1 benefits the host, which survives longer than wild-type mice. This is in part explained by overwhelming cell death mediated by the upregulation of pro-apoptotic genes such as TRAIL (O'Connell et al., 2004). In parallel, it has been shown that an unleashed interferon response is detrimental for mice after influenza infection due to a strong upregulation of FASL (Davidson, Crotta,

McCabe, & Wack, 2014). Additionally, it has been shown that type I interferon, but not type II interferon, impairs the inflammasome activation following either poly(I:C) treatment in mice or infection with C. albicans.

Consequently, IL-1β was not processed by Caspase-1 and therefore cannot exert its inflammatory activity (Guarda et al., 2011). A similar observation has been reported for Mtb infection, where the secreted IL-1α and IL-1β were increased in IFNAR1 -/- mice (Mayer-Barber et al., 2011). Other models such as LPS shock, where IFNβ -/- mice were more resistant following high dose of LPS injection shows that interferon response is not always beneficial, especially in a context of high stimulation of PRRs.

Interferon can also lead to interferonopathies in different auto-immune diseases in absence of infection. Aicardi- Goutieres syndrome or systemic lupus erythematosus, have been shown to be driven by an exacerbated inflammatory phenotype caused by type I interferon (Bennett et al., 2003; Rice et al., 2013).

1.6 Programmed cell death

In addition to the induction of the gene-activatory pathways, TLR3 signalling has the ability also to induce cell death. Upon viral infection, viruses try to block interferon and proinflammatory cytokine production by either inhibiting functional proteins within the TLR3-SC or their downstream effectors in order to replicate themselves efficiently. As another host-cell defence mechanism, infected cells may trigger cell death pathways to counteract the infection, reduce viral replication and initiate inflammation. Studies showed that similar to complex II formation in TNF signalling, TLR3-induced Death-Inducing Signalling Complex (DISC) that leads to cell death originates from membrane-bound TLR3-SC and dissociates over time. Nevertheless, there is also the possibility that the TLR3-SC and the TLR3-induced DISC may be formed sequentially and independently from each other, but further work is necessary to understand the actual mechanism. Specifically, TLR3 recruits TRIF and RIP1 consecutively, facilitating RIP1-FADD interaction and further proCaspase 8 recruitment akin to TNF-induced complex II formation (Kaiser & Offermann, 2005). Subsequently, self-cleavage and activation of proCaspase 8 is triggered, which also causes activation of the executioner Caspases such as Caspase 3, leading to the induction of cell death through apoptosis.

As a regulatory mechanism, cIAPs may be found in TLR3-induced DISC complex and prevent cell death by K48-linked ubiquitination of RIP1 and its degradation (Estornes et al., 2012; Weber et al., 2010).

Moreover, LUBAC indirectly blocks TLR3-induced cell death by stabilizing TLR3-SC to promote the gene-activatory pathways that can increase pro-survival genes such as the cell death inhibitor c-FLIP (Zinngrebe et al., 2016). In case of the inhibition of any essential proteins like Caspases in the apoptosis pathway, cells may die via the alternative necroptotic pathway. In this pathway, the catalytic activity of RIP1 and RIP3 induces a conformational change of MLKL through phosphorylation, followed by pore formation in the plasma membrane and leakage of the cellular content.

1.6.1 Apoptosis

Apoptosis is a unique version of programmed cell death. It is implicated in important developmental processes but also contributes to tissue homeostasis. Once initiated, cells systematically display particular features, including chromosome condensation, DNA fragmentation and cleavage of structural proteins, followed by morphological changes like blebbing of the plasma membrane and eventually cellular disintegration without overt membrane rupture. Two main pathways lead to cell death by apoptosis, which are the extrinsic pathway triggered by immune receptors and 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). Even though some immune receptors like TLRs and TNF can initiate apoptosis signalling, the main receptors that trigger apoptosis are the Death Domain-containing TNFRSF. Whilst TLRs and TNF receptor can trigger apoptosis in a context of dysregulation of the signalling complex such as an impairment of the E3 ligases involved, TRAIL-R and FAS are able to initiate this modality of cell death directly. Indeed, after binding their respective ligands, both can recruit proCaspase 8 to a membrane-bound ligand-receptor complex via the adaptor protein FADD (Kischkel et al., 2000; Walczak et al., 1999). The colocalisation of several proCaspases-8 molecules leads to their self-cleavage and consequently their activation by forming a tetramer of the truncated p18-p10 peptides (Fuentes-Prior & Salvesen, 2004).

Once activated, Caspase 8 is released from the complex and then activates the effector proCaspases (Caspase 3/6/7) by cleavage. These in turn cleave a plethora of cellular substrates, such as cytoskeletal proteins and pro-survival proteins, which consequently leads to apoptosis and cell death.

Furthermore, Caspase 3 can activate Caspase 6/7 and accelerate cellular disintegration (Kumar, 2007). As a regulatory mechanism, a large amount of the negative regulator c-FLIP can block the initiation of apoptosis in cells by interfering with Caspase 8 activation. This inhibitory activity is partly facilitated by the close structural homology of these two antagonistic proteins, allowing cFLIP to bind FADD through its own death domain (D. W. Chang et al., 2002). This precludes Caspase 8 from being fully processed. There is, however, considerable interplay between the two proteins and cFLIP has been described to participate in the assembly of large filaments with Caspase 8, which ultimately enhance the activity of the latter, giving it a context-dependent pro-apoptotic role (Fu et al., 2016; Hughes et al., 2016). Also, Caspase 10, a Caspase 8 homolog, can negatively regulate Caspase 8 mediated cell death by interfering with its association to the DISC (Horn et al., 2017). However, only a few studies report this role, probably due to the absence of this homologue in mice. Furthermore, ubiquitination is also strongly implicated in the regulation of Caspases. Notably, TRAF2 may generate K48 ubiquitin chain on the active form of the Caspase 8, leading to its proteasomal degradation (Roberts, Crawford, & Longley, 2022).

Besides extrinsic apoptosis, active Caspase 8 can cross-signal to the intrinsic apoptotic pathway by inducing the cleavage of BH3 interacting domain death agonist (BID) (Li, Zhu, Xu, & Yuan, 1998). This facilitates its translocation to the outer mitochondrial membrane and results in an amplification of the apoptotic signal. In addition, the stress-activated intrinsic apoptosis is linked to mitochondria and the release of proteins from its intermembrane space along with mitochondrial outer membrane permeabilization (MOMP). This latter 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 (Kelekar & Thompson, 1998).

Anti-apoptotic proteins such as Bcl-2, Bcl-xL and Mcl-1 harbour 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.

Bak and Bax are indispensable components in the intrinsic apoptosis mechanism to create a pore on the outer mitochondria membrane (Kale, Osterlund, & Andrews, 2018). In a normal situation, these proteins are segregated by the anti-apoptotic Bcl-2 proteins and therefore prevent their activation. 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. 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 pro-Caspase 9 via a homotypic interaction mediated by the so-called CARD domain (Fulda & Debatin, 2006). After self-cleavage and activation of pro-Caspase 9, executioner Caspases are activated and an equivalent apoptotic cascade to the extrinsic one is initiated. In addition to cytochrome C, second mitochondria-derived activator of the Caspase/direct IAP-binding protein with low pI (SMAC/DIABLO) is released from mitochondria in order to bind and induce the proteasomal degradation of X-linked inhibitor of apoptosis (XIAP) and cIAP1/2 via their auto-ubiquitination (Du, Fang, Li, Li, & Wang, 2000; Verhagen et al., 2000). XIAP is able to inhibit active Caspase 3 by inducing its proteasomal degradation. 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 release perforin and granzymes via exocytosis to the targeted cell. Perforin enables pore formation on the plasma membrane of the cell whereas granzymes activate downstream Caspase 8 and Caspase 3 and trigger apoptosis (Trapani & Smyth, 2002). In the context of infection, apoptosis is essential due to its ability to promote activation of the immune system via the engulfment of the apoptotic cells, essential for an efficient antigen presentation by dendritic cells and macrophages. This is mediated by the promotion of intercellular adhesion molecule 3 (ICAM3) expression to the plasma membrane and the flip-flop of the phosphatidylserine. Those proteins and lipids act as an "eat-me" signal for the professional phagocytic cells (Ravichandran & Lorenz, 2007).

Additionally, despite early claims regarding the immuno-silent modality apoptosis, it is now clear that it is still able to release several chemokines and cytokines and therefore contribute to the inflammation during infection. Caspases are also very important physiologically to control the induction of other cell death modalities such as necroptosis and pyroptosis (Fritsch et al., 2019). Indeed, it is able to cleave RIP1 and RIP3. The deletion of Caspase 8 in mice leads to an embryonic lethality caused by an unleashed necroptosis induction (Kaiser et al., 2011; Oberst et al., 2011).

1.6.2 Necroptosis

Cell death with necrotic features such as swelling and loss of cellular integrity including the plasma membrane has traditionally been thought of as a passive process called necrosis. However, over the last two decades, it was discovered that cell death with necrotic features can indeed be the outcome of a highly regulated programme, which was subsequently termed necroptosis. Frequently observed during infections, it is an immunogenic alternative to apoptosis which causes the release of increased inflammatory cytokines and DAMPs (Pasparakis & Vandenabeele, 2015). Consequently, this release promotes immune cell infiltration to the inflammatory site. DRs and PRRs can induce necroptosis. It involves the activation of RIP1 that interacts with RIP3 via their RHIM domain, which promotes either directly or indirectly the phosphorylation and activation of RIP3. Active RIP3 can then phosphorylate the pseudo-kinase MLKL, followed by its oligomerization and translocation to the plasma membrane. MLKL oligomers create pores on the plasma membrane, resulting in Ca2+ and Na+ influx, disrupting the osmotic balance of the cells and rupture of the plasma membrane (L. Sun et al., 2012; J. Zhao et al., 2012). In spite of the fact that PRRs like TLR3 can induce the necroptotic pathway in a very similar manner, it still differs in certain ways and thus it is clear that a number of afferent pathways induce necroptosis. TRIF and ZBP1 also contain a RHIM domain like RIP3, mediating their interaction and promoting RIP3 activation independently of RIP1 (Kaiser et al., 2013). It should be noted that ZBP1 is an ISG protein, constituting a link between pathogen sensing, interferon induction and cell death (Jiao et al., 2020). Similarly to apoptosis, ubiquitination also regulates this modality of cell death.

For instance, TRIM25 can induce proteasomal degradation of RIP3 by adding K48 ubiquitin chain (Mei et al., 2021). In addition, several ubiquitination sites of MLKL have been reported and positively or negatively regulate its activity (Garcia et al., 2021; Z. Liu et al., 2021).

1.6.3 Pyroptosis

PRRs, including TLRs also contribute to another extremely inflammatory modality of cell death, which was consequently named pyroptosis. Gasdermin A, B, C, D and E are the five executioner proteins of this cell death. However, gasdermin D and E are the best-understood members of this group. In order to be activated, they need to be cleaved by Caspases. Classical inflammasome formation and pyroptosis require two phases, the first senses the infection, notably by PRRs. This induces the gene-activatory pathways that promote the expression of different proteins implicated in pyroptosis such as NLRP3 but also pro-inflammatory cytokines such as pro-IL-1β and IL-18 (Kelley, Jeltema, Duan, & He, 2019). Additionally, Caspase-11 (an analogue of Caspases 4 and 5 in humans) is upregulated following interferon signalling and allows the activation of the non-canonical inflammasome. The second phase is the activation of pyroptosis mediated by the sensing of the pathogen via NLR or osmotic changes such as K+ efflux and ATP influx. This leads to the formation of the classical inflammasome. As described previously, ASC is consequently recruited with Caspase-1 (Yu et al., 2021). Once the Caspase-1 is activated, it can cleave gasdermin A, C and D to trigger pyroptosis and process pro-IL-1β/-18 to allow their release. It is important to note that researchers have recently proven that other Caspases, so far only related to apoptosis, are also able to cleave several gasdermins. Caspase-8 is able to cleave the same gasdermins as Caspase-1, while Caspase-3 cleaves gasdermin E and induces pyroptosis (Sarhan et al., 2018; Yupeng Wang et al., 2017). Granzymes have also been linked to the cleavage of gasdermin B and E (Zhibin Zhang et al., 2020; Zhou et al., 2020). Altogether; we accumulate evidence suggesting that a cross-talk exists between the different cell death modalities detailed in this chapter. The non-canonical inflammasome is activated via cytosolic LPS and is sensed by Caspase-11. The binding leads to the oligomerisation and activation of Caspase-11 or 4/5 in humans that induces the downstream gasdermin cleavage.

However, these Caspases are not able to directly process pro IL-1β/-18. During LPS-induced sepsis or septic shock, it has been shown that either Caspase-1 or Caspase-11 knock-out prevent the lethality, proving a potential cross-talk between canonical and non-canonical inflammasome but most importantly, the crucial role of pyroptosis to counteract infection (Broz et al., 2012). Post-translational modification also tightly regulates this cell death modality, especially ubiquitination. Indeed, several ubiquitin E3s have been reported to control the activation or the expression of crucial components such as PARKIN that promotes the autophagic degradation of NLRP3 (Mouton-Liger et al., 2018). Oppositely, LUBAC and TRAF3 ubiquitinate ASC and induce its activation (Rodgers et al., 2014; Siu et al., 2019).

As discussed, immune receptor-mediated signalling pathways have the ability to trigger a robust inflammatory response via the activation of different gene-activatory pathways or cell death. This dual role of the receptors is strongly regulated by post-translational modification, notably ubiquitination. This latter is explained by the presence of several E3 ubiquitin ligases in the signalling that add ubiquitin molecules to different components of the signalling complexes. This post-translational modification is crucial for an appropriate anti-pathogen immune response.

1.7 Ubiquitin system

Ubiquitination is an essential post-translational modification that occurs only in eukaryotic organisms. A.Hershko, I.Rose and Aaron Ciechanover discovered it in 1975. It consists of the attachment of ubiquitin, either individually or in chains thereof, to specific target proteins (Ciechanover, Elias, Heller, Ferber, & Hershko, 1980; Ciechanover, Heller, Elias, Haas, & Hershko, 1980). Ubiquitin has 76 amino acids and a molecular weight of 8.6kDa. Four different genes, namely UBB, UBC, RPS27 and UBA52, encode this molecule. They all produce slight variations of pro-ubiquitin, which is then cleaved into mature ubiquitin by specific deubiquitinases (DUB), after which it is ready to be attached to its cellular targets. Ubiquitination requires the activity of three classes of proteins that are the E1 (ubiquitin-activating enzyme, two in humans), the E2 (ubiquitin-conjugating enzyme, around 30) and the E3 (ubiquitin ligase enzyme, around

600). E1 (UBE1 and UBA6, also called UBE1L2) are essential for the two-step activation of ubiquitin.

UBE1 is the predominant E1 for the ubiquitin molecule, while UBA6 more frequently acts as an E1 for ISGlytion (post-translational modification involving ISG15, a molecule similar to ubiquitin). Following binding to MgATP, the ubiquitin molecule is caught, adenylated on its C-terminus, and then transferred to a cysteinyl residue of the E1, leading to the formation of a thiol-ester linkage. Then, this activated ubiquitin molecule is transferred to another cysteinyl group on an E2. There are approximately 30 different E2 in humans. They are necessary to transfer the activated ubiquitin molecule from the E1 to the E3. Finally, the E2-ubiquitin molecule binds the E3 ligase (Scheffner, Nuber, & Huibregtse, 1995). This latter is able to interact with the substrate, allowing close proximity between all the components required for ubiquitination.

Depending on the family of the E3 ligase, the ubiquitin molecule is either transferred to the E3 which will then catalyse the transfer of ubiquitin onto the substrate, or the E2 directly transfers the ubiquitin molecule to the substrate **(Figure 1.3)**. The bond between the ubiquitin molecule and the substrate can differ but the most common one is the isopeptide bond (Kim et al., 2007). It is a bond between the C-terminal carboxylate glycine (Gly) of the ubiquitin molecule and the ε-amino groups of a lysine present on the substrate protein.

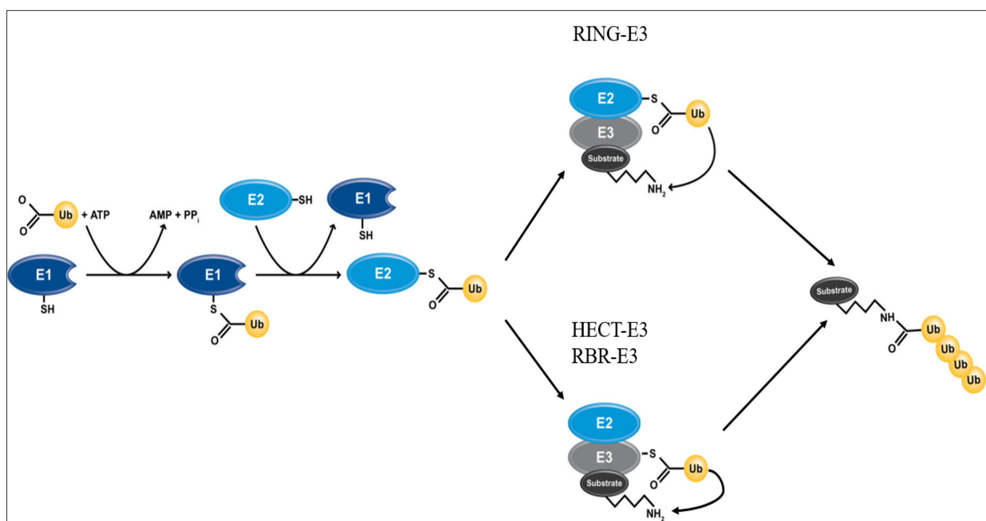


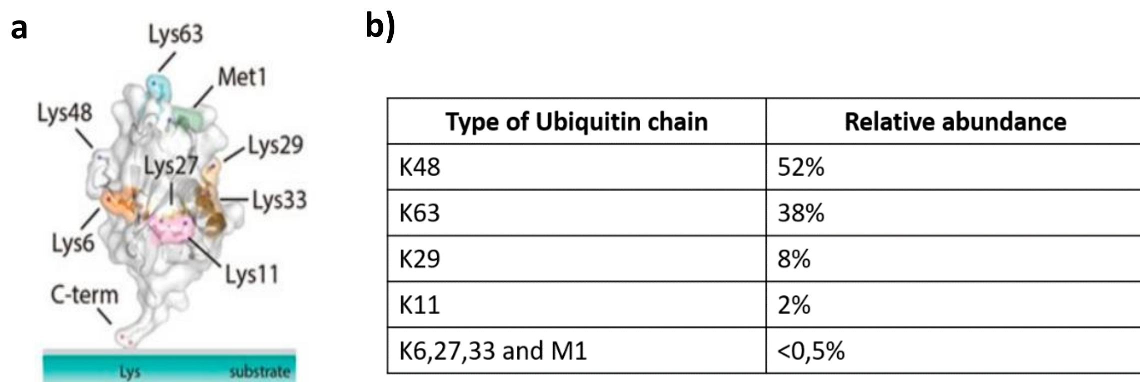
Figure 1.3. The ubiquitin system:
After activation of the ubiquitin molecule (Ub) by the ubiquitin-activating enzyme (E1), it is transferred to the ubiquitin-conjugating enzyme (E 2). Then, this latter interacts with a ubiquitin-ligase enzyme (E3). If it is a RING-E3, the E2 adds the ubiquitin directly to the substrate whilst if it is a HECT- or RBR-E3, ubiquitin is first transferred to the catalytic cysteine of the E3 and then added to the substrate protein.

1.7.1 Polyubiquitin chains

E3 ligases can either add only one ubiquitin molecule (monoubiquitination) or several ubiquitin molecules successively on the same site, leading to the formation of a polyubiquitin chain. In this case, the new ubiquitin molecule is added via a covalent bond between the C-term of the new ubiquitin molecule and a lysine from the already bound ubiquitin chain. A ubiquitin molecule contains the following seven lysines: K-6, K-11, K-27, K-29, K-33, K-48, K-63. The methionine M-1 can also be used to form a chain named linear ubiquitin chain (Kirisako et al., 2006).

Depending on the lysine that the new ubiquitin molecule is attached to, different polyubiquitin chains with different topology can be formed, leading to different consequences for the targeted protein (Swatek & Komander, 2016).

A globular and compact structure characterises K6, K11, K33 and K48 chains, while K29, K63 and M1 have an open and linear conformation. These different structures influence the interacting protein but also their recognition by the DUBs. The relative abundance of these different types of linkages differs widely and is summarised in the table below (Dammer et al., 2011) **(Figure 1.4)**.



Type of Ubiquitin chain	Relative abundance
K48	52%
K63	38%
K29	8%
K11	2%
K6,27,33 and M1	<0,5%

Figure 1.4. Ubiquitin molecule and relative abundance of the different polyubiquitin chains:
a) Ubiquitin molecule attached to the substrate protein. The first ubiquitin is attached through the glycine in c-Term. The position of the different lysines and the methionine that can be used to form polyubiquitin chains are marked. b) Abundance of the different types of chains found in human cells in normal condition. Adapted from (Swatek et al., 2016; Dammer et al., 2011)

K48 ubiquitin chains represent more than 50% of all ubiquitin chains that can be found in human cells. This modification labels proteins for destruction. Once a target protein has been marked with a K48 ubiquitin chain, it binds to adaptor proteins that through their UBA domains carry ubiquitinated proteins to the proteasome for their degradation (Chau et al., 1989). Inhibition of this proteasomal degradation has a drastic effect on cell viability. Misfolded protein, regulator proteins such as IκBα cannot be degraded, leading to the absence of pro-survival signalling and lead to cell death. It is involved in several cellular processes such as receptor signalling, cell cycle regulation and cellular stress (Glickman & Ciechanover, 2002).

Due to the overactivation of the proteasome in cancer cells, drugs such as bortezomib, a proteasome inhibitor, has been developed for cancer treatment to slow down cancer growth (Chen, Frezza, Schmitt, Kanwar, & Dou, 2011).

K63 ubiquitin chains represent 30% of the entire chains. These chains are non-degradative but rather allow for the formation of large scaffolds for proteins containing UBA domains and their downstream binding partners. They thus promote the formation of higher-order complexes and play a crucial role in regulating immune signalling. For example, TNF-receptor associated factor (TRAF) and cellular inhibitor of apoptosis protein 1/2 (cIAP1/2) are crucial E3 ligases that promote the activation of the NF-kB pathway in extensively ubiquitinated receptor complexes (Mahoney et al., 2008).

K6 polyubiquitin chains are also non-degradative but their role remains largely unclear. However, it has been shown that PARKIN, an RBR-containing E3 promotes this type of linkage in order to induce mitophagy. Following DNA damage caused by UV genotoxic stress, researchers could observe an upregulation of K6 linkage, translating a role in DNA repair (Michel, Swatek, Hospenthal, & Komander, 2017).

K11 linkage has been characterised as a degradative ubiquitin chain, generated by APC/C during cell cycle. However, it has been shown that homotypic K11 chains barely promote proteasomal degradation. More recently, K11 has also been linked to immune signalling. Notably, it has been reported that cIAP1 is able to add this type of linkage to RIP1 and promote the recruitment of NEMO, an essential protein for the activation of canonical and non-canonical IKKs (Dynek et al., 2010). Additionally, it is implicated in T cell activation and endocytosis of EGF receptors in heterotypic K11/ K63 chains.

K27 in turn has been shown to confer scaffolding properties to its substrates. For example, during interferon-mediated signalling, STING ubiquitination allows proper recruitment and activation of TBK1. In other immune signalling pathways such as RLR, TAK1 and NEMO have also been reported to be K27 poly-ubiquitinated (Arimoto et al., 2010). Additionally, several TRIM protein ligases (TRIM-23, 26) have been shown to auto-ubiquitinate themselves with K27 chains, promoting interferon production. This linkage has also been shown to be implicated in autophagy and DNA repair (Tracz & Bialek, 2021).

K29 has been linked to autophagy, mitophagy, cellular stress, and cell cycle regulation. K29 linkages, generated by ITCH, have also been shown to negatively regulate the activation of gene-activatory pathways like NF-kB (Chastagner, Israël, & Brou, 2008). In the WNT pathway, Smurf adds K29 chains to Axin, disrupting its binding with LRP5/6, thereby repressing the activation of this pathway.

K33 chains also have a regulatory activity. For example, STAT1 binding either to its DNA binding site or to TCR receptors is destabilised by this type of chain. Further, K33 is implicated in protein trafficking and AMPK signalling (Akutsu, Dikic, & Bremm, 2016).

Another type of chain, called linear chain or M1, can also be generated. The binding of the ubiquitin molecule occurs between the C-terminal glycine of the substrate and α-amino group of the methionine on the ubiquitin molecule via a peptide bond. Linear chains do not induce protein degradation but rather a conformational change, promoting a scaffold activity of the substrate similar to K63 chains (Haas et al., 2009). Ubiquitin molecules of linear chains are in an open conformation and do not interact between them. This specific type of chain is generated by HOIP, a member of the Linear Ubiquitin Assembly Complex (LUBAC). This E3 ligase is rather unique as it is, so far, the only E3 ligase known to be able to form this type of chain. Linear chains are involved in the control of cell death induction by immune receptors, where it biases the system towards a pro-survival response by the gene-activatory pathways (Gerlach et al., 2011). The deletion of HOIP leads to embryonic lethality due to an exacerbated TNF-induced cell death (Peltzer et al., 2018, 2014). More recently, it has been shown to contribute to bacterial degradation via autophagy (Noad et al., 2017).

Interestingly, there is evidence that heterogeneous chains are formed, with a mix of linkages between ubiquitin molecules as mixed or even branched ubiquitin chains. A mixed chain is a ubiquitin chain that has a different type of linkage but only to one specific lysine of the ubiquitin molecule. A branched chain contains multiple types of linkages and some ubiquitin molecules can link several other ubiquitin molecules on multiple lysines acceptor sites. The presence of these heterotypic chains greatly adds to the complexity of the ubiquitin code (Nakasone, Livnat-Levanon, Glickman, Cohen, & Fushman, 2013). The abundance of branched linkages is still unclear but seems to be between 5 and 20% depending on the cell type and the conditions.

Branched K48/K11 linkages have for example been shown to be significantly more efficient at triggering proteasomal degradation than K11- or K48-only chains (French, Koehler, & Hunter, 2021). A fascinating example is TRAF6 mediated NF-κB activation. When TRAF6 is only K63 auto-ubiquitinated, it activates NF-κB but this can be disrupted by deubiquitinases such as CYLD or A20 that cleave the chains (Ying Wang et al., 2017). However, when TRAF6 contains K48/K63 branched chains, neither DUB is able to cleave these chains and NF- κB activation is sustained.

1.7.2 E3 ligases

All E3 ubiquitin ligases comprise an E2 ubiquitin-binding domain and they are divided into 3 groups, regarding their structure and their mechanism of action to mediate ubiquitination. The three groups are the Really Interesting New Gene (RING) E3s, the Homologous to the E6-AP Carboxyl Terminus (HECT) E3s and the RING-in-between RING (RBR) E3s.

RING-type E3s is the largest family, containing 270 members in humans. They are defined by their catalytic domain, which can be either a RING or U-box domain. Both enable the interaction with the E2 and therefore allow the direct transfer of the ubiquitin molecule from the E2 to the target protein (Deshaies & Joazeiro, 2009). To do so, the RING domain contains an extremely conserved motif composed of seven cysteines and one histidine, which allows the binding of two Zn2+ ions and stabilizes their conformation. Conserved hydrophobic residues are also important to interact with the E2 appropriately.

Conversely, the U-box domain does not bind any Zn2+ ions but the mechanism of action is similar. Within this family, some RING-containing E3s can directly bind to E2 and the target protein as monomers, whereas others require the recruitment of different proteins, leading to a multimeric structure (Budhidarmo, Nakatani, & Day, 2012).

The second most abundant family of E3 ligases, with 28 members, is HECT-containing E3s. This domain has a binary structure involving an E2-binding domain in the N-terminal region and a catalytic cysteine in C-term (Huang et al., 1999; Verdecia et al., 2003). The particular property of this group is the formation of a ubiquitin-HECT intermediate during the catalysis of the ubiquitination.

Indeed, after binding of the E2 to the N-terminal region of the HECT domain, the ubiquitin molecule is transferred to the catalytic cysteine of the E3. Then, the ubiquitin is added to the substrate by an isopeptide bond (Komander & Rape, 2012).

Finally, the most recent and smallest family, the RBR-containing E3 ligase, is characterised as a hybrid family between the RING and the HECT E3s. It contains only 14 members, including the well-known PARKIN, HOIL-1 and HOIP. They have been reported to be implicated in the regulation of the immune signalling and inflammation and their dysregulation has been correlated with the progression of several neurodegenerative diseases such as Parkinson, Alzheimer’s or ALS but also in cancer (Giasson & Lee, 2001; Ishigaki et al., 2004; P. Wang, Dai, Jiang, Li, & Wei, 2020). This family contains two RING domains separated by an In-Between-RING domain (IBR). The first RING, in collaboration with the IBR, allows the recruitment of the E2. RING1 is not similar to the equivalent domain found in RING-E3 ligases, and so far all studied RBR-E3 ligases did not have the conserved cysteines and important hydrophobic amino acids that are involved in the interaction with the E2. Once they interact with each other, the ubiquitin molecules are transferred to the catalytic cysteine of the second RING domain. There are some prerequisites to have a functional RING2 domain, including five cysteine residues that are important to capture two Zn2+ ions needed for the RBR domain to adopt an active conformation **(Figure 1.5)**. Finally, a triad of residues is required for the catalytic activity of the RBR domain. It is constituted of a cysteine, a histidine at the +2 positioned and a negatively charged amino acid between the fifth and the sixth cysteines of the RING2 (Spratt, Walden, & Shaw, 2014). The third cysteine is the one responsible for the capture of the ubiquitin molecule.

Interestingly, the E3 ligases are not constitutively active but rather require specific post-translational modifications or protein-protein interactions to be fully activated (Dove & Klevit, 2017).

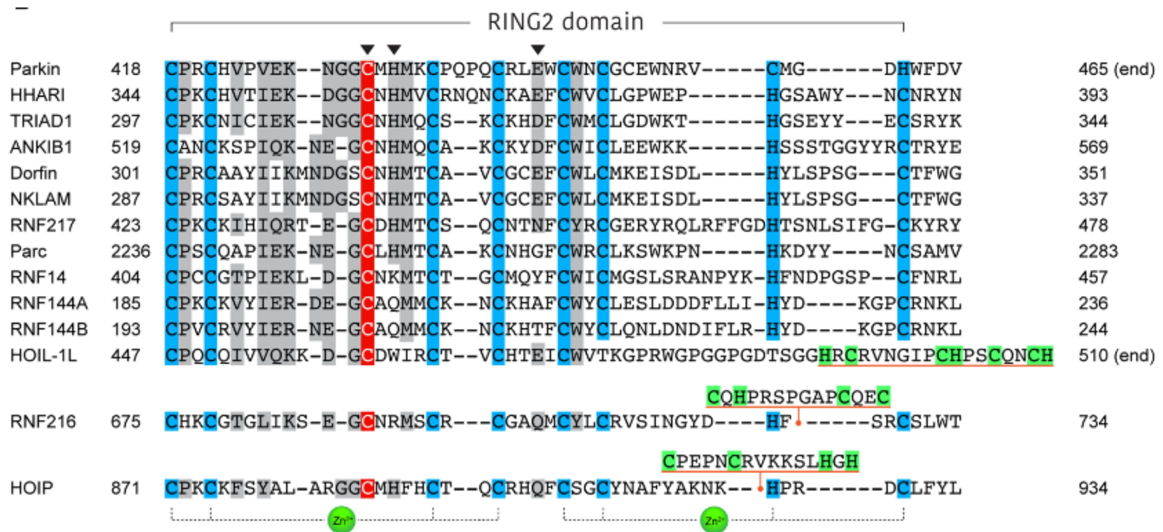


Figure 1.5. Sequence alignment of the RING2 domains present in the different RBR-E3s: *The cysteines and histidines involved in Zn^{2+} binding are underlined in blue. The triad of amino acid required for the ligase activity are marked with the black triangle and the catalytic cysteine is underlined in red.*

1.7.2.1 ANKIB1

Ankyrin repeat and IBR domain-containing protein 1 (ANKIB1) is, so far, described as a putative RBR-containing E3 ligase, meaning that it contains a RING-in between- RING domain. Not much is known about this protein of 122kDa, including whether it harbours E3 ligase activity. Structurally, it contains two large Ankyrin repeat domains on the N-terminus that confer a strong ability to bind other proteins (Mosavi, Cammett, Desrosiers, & Peng, 2004). This motif is followed by the RBR domain, which, judged by their structures, should confer the ability to generate ubiquitin chains. However, the IBR which is generally very similar to the RING2, lacks one cysteine compared to other members of the RBR E3 family, the consequence of this omission on the catalytic activity remains unclear. Previous findings as well as in silico structural prediction by AlphaFold suggest that an Ariadne domain follows the RBR.

This is an auto-inhibitory domain composed of four successive α-Helices, which in their native conformation mask the binding site of the RING2 for ubiquitin (Duda et al., 2013). Post-translational modifications or heterotypic protein interactions are known to lift this inhibition via a conformation chance.

This is suggestive of ANKIB1 requiring a stimulus that leads to the interaction with a protein or a post-translational modification such as phosphorylation or ubiquitination to be fully activated. Finally, close to the C-terminus of the protein, there is a Ubiquitin-Interacting-Motif (UIM) that indicates that ANKIB1 has the ability to interact specifically with ubiquitinated proteins.

Figure 1.6. The different domains of ANKIB1:

In fact, the work of *Miller SL et al.* even suggests that ANKIB1 preferentially binds polyubiquitin chains, omnipresent in immune signalling, including TNF and TLR3 (Miller, Malotky, & O'Bryan, 2004). In order to obtain an indication of the relevance of these different motifs and also to potentially find out other important parts of the protein, we used the bioinformatic tool ConSurf which is an online platform that assesses the sequence conservation of a protein between more than 150 species. Interestingly, we can see that every described domain is very well conserved **(Figure 1.7)**. The C-terminal part of ANKIB1 is the less conserved except for the UIM domain and another sequence of as yet unknown function. Additionally, it has been reported that ANKIB1 is N-myristoylated on the glycine just after the first methionine. This post-translational modification can be very informative to understand the localisation of the protein. Indeed, a myristoylated protein is in general localised to cellular membranes, including the plasma membrane, the endoplasmic reticulum or even mitochondria. This could indicate that ANKIB1 has an activity close to membranes, we could therefore hypothesise that it can play a role in immune signalling since most of the signalling happens at membranous compartments.

Regarding its physiological role, very little is known about this protein. Interestingly, *Wang F et al.* report an upregulation of the ANKIB1 gene after bacterial infection suggesting that ANKIB1 might be implicated in immune pathways recognising an infection such as the Pattern-Recognition-Receptor pathways like the TLR family (F et al., 2017). Another recent paper proposed ANKIB1 as a new biomarker for Alzheimer's disease, as the protein is upregulated in patients compared to healthy controls (Sharma & Dey, 2021). Other articles report a role for non-coding RNA from ANKIB1 in cancer.

Figure 1.7. Conservation of ANKIB1 between species:
Analysis done with the bioinformatics website Consurf.

1.7.3 Deubiquitinase

Ubiquitination plays an essential role in the control of the signalling pathways triggered by immune receptors. However, disassembling the ubiquitin chain generated to confer stability to the signalling complex is crucial to prevent an uncontrolled and exacerbated inflammatory response. This is mediated by a family of enzymes called deubiquitinase that are able to hydrolyse isopeptide and peptide bonds generated between ubiquitin molecules.

More than 100 different DUBs exist and are classified into six different families based on their structure and function. These are the cysteine proteases that include ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), ovarian tumour proteases (OTUs), Machado-Joseph disease proteases (MJDs) and monocyte chemotactic protein-induced (MCPIPs). Additionally, some metalloproteinases act as DUBs including JAB1/MPN/MOV34 (JAMMs) (Komander, Clague, & Urbé, 2009). Some DUBs are able to cleave all types of ubiquitin chains but most of them, similarly to E3s, have a chain-specific activity. In TLR3, but also other signalling pathways such as the TNF pathway, several DUBs are indispensable to control the inflammatory response. CYLD removes K63 polyubiquitin chains found on TRAF6 or TRAF3 and destabilises TLR complex, consequently turning off signal transduction (Kinsella, Fichtner, Watters, König, & Prehn, 2018; Yoshida, Jono, Kai, & Li, 2005). The metalloproteinase MYSM1 also acts similarly and degrades both TRAFs (Panda, Nilsson, & Gekara, 2015). DUBA (or OTUD5), another K63 DUB, prevents TBK1 and therefore IRF3 over-activation by removing the chains on TRAF3 (Kayagaki et al., 2007). Conversely, USP25 removes K48 chains on TRAF3, promoting its stability to induce interferon production following viral sensing (Zhong et al., 2013).

Overall, DUBs are essential to control immune signalling and their presence in signalling complexes adds another mechanism of regulation to promote an appropriate immune response.

1.8 Ubiquitination in TLR3 and other immune signalling pathways

Ubiquitin E3s are essential components of the TLR3-signalling pathway. Most of them are recruited to the complex to promote its stability. However, their overall contribution and mechanism of action remain unclear compared to better-studied signalling pathways such as the TNF pathway. TRAF3/6 are RING-containing E3s that generate K63 polyubiquitin chains on themselves. This auto-ubiquitination promotes the activation of the gene-activatory pathways and interferon production. TRAF3 deletion disrupts interferon production, whilst the absence of TRAF6 downregulates gene-activatory pathways but does not disrupt it. It has been proposed that Peli1 is responsible for the ubiquitination of RIPK1, leading to the activation of the downstream pathways (M. Chang et al., 2009). However, the relevance of the E3 ligase activity of Peli-1 and its implication in interferon production mediated by TLR3/4 remains controversial (Enesa et al., 2012). In contrast to the TNF signalling complex, the role and recruitment of TRAF2, and the downstream cIAP1/2 to TLR3-SC remain unclear.

Several papers report different contributions of these E3s, which are required for gene-activatory pathways in Myd88-induced signalling while they are not implicated in TRIF-mediated signalling. A result that underpins this hypothesis is that TRAF3 is only transiently recruited to the Myd88 signalling complex due to its degradation by cIAPs (Tseng et al., 2010). This is not observed in the TLR3 pathway where TRIF is responsible for the formation of the signalling complex. However, their contribution to prevent cell death after activation of TLR3/4 or TNFR1 seems universal (Estornes et al., 2012). In line with cIAPs, LUBAC is also involved in TLR3-SC, where it acts similarly than in TNF-signalling pathway. M1 chains generated by HOIP prevent TLR3-induced cell death while it promotes the activation of the gene-activatory pathway (Zinngrebe et al., 2016). However, the mechanism of recruitment of LUBAC and the targeted proteins remain unknown. Overall, even if several E3 ligases have been reported to be involved in TLR3 signalling pathway, a lot still needs to be addressed to understand the regulation of this pathway.

1.9 Aim of the project

As discussed in the previous section, many E3s are implicated in both TNF and TLR3 pathways. However, they do not have necessarily the same roles and their ubiquitin ligase activity is not equally required. One of the main differences is the role of linear chains in the activation of the non-canonical IKK, TBK1. In the TNF pathway, our lab has reported that the presence of M1 ubiquitin chains on RIP1 is crucial for the recruitment and activation of TBK1 and IKKε (Lafont et al., 2018). Indeed, linear chains promote the recruitment of NEMO to the signalling complex, consequently leading to the recruitment of different adaptor proteins such as TANK and NAP1 that are essential for TBK1 recruitment.In contrast to other signalling complexes such as TLR3, the recruitment of these non-canonical IKKs does not induce type I interferon production and is not mediated by TRAF3. However, they phosphorylate RIP1, which prevents its autophosphorylation and activation, thus contributing to the inhibition of TNF-induced cell death.

Intriguingly, despite the same general role for LUBAC in TLR3 in terms of cell death inhibition and MAPK/NF-kB pathway, our lab has demonstrated that in absence of HOIP, the activation of TBK1 and its recruitment to the signalling complex was barely impacted (Zinngrebe et al., 2016).

Because of its essential role in interferon production, this thesis aimed to decipher the requirement for TBK1 recruitment and activation in the TLR3 pathway. More precisely, we aimed to find out if another E3 ligase could be implicated in this process, in collaboration with TRAF3, which would explain the discrepancy between TNF and TLR3 signalling regarding the requirement of HOIP.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals & reagents

Every chemical and standard regents used in this thesis were purchased from the following companies: Anatrace, Invitrogen, Merck, Pierce, Roche, Roth, Sigma Aldrich.

2.1.2 Buffers & solutions

Blocking Buffer	5% milk powder
	0.05% Tween-20 in PBS.
Freezing medium	90% FBS (v/v)
	10% DMSO (v/v)
IP-lysis buffer	30 mM Tris-Base (pH 7.4)
	120 mM NaCl
	2 mM EDTA
	2 mM KCl
	10% Glycerol (v/v)
	1 % Triton X-100 (v/v) (IP-LB1)
	or 1,7mM DDM (v/v) (IP-LB2)
	COMPLETE Protease Inhibitor Cocktail
	Phosphatase Inhibitor Cocktail
Macrophage medium	500 mL IMDM
	5 mL Pen/Strep
	50 mL FBS decomplemented
	50ng/mL m-CSF
PBS (pH = 7.4)	137 mM NaCl

	8.1 mM Na2HPO4
	2.7 mM KCl
	1.5 mM KH2PO4
PBS/Tween	137 mM NaCl
	8.1 mM Na2HPO4
	2.7 mM KCl
	1.5 mM KH2PO4
	0.05 % Tween-20 in PBS (v/v)
Stripping Buffer (pH = 2.3)	50 mM Glycin in H2O
Ubiquitin Buffer	30 mM Tris-HCl (pH 7.5)
	5 mM $MgCl_2$
	2 mM DTT
Wash Buffer	0.05% Tween-20 in PBS (1x)

2.1.3 Biological agents

Table2.1: Biological agents purchased

Agent	Source
Murine M-CSF	Peprotech
E1	R&D
UBE2D3	R&D
UBE2L3	R&D
UBE2L6	R&D

ERS	R&D
Ubiquitin	R&D

2.1.4 Antibodies

Table 2.2: List of the primary antibodies used for Immunoblotting.

Antibody	Isotype	Source	Dilution
A20	Rabbit	Cell Signalling 5630	1:1000
ARIH1	Rabbit	Novus Biology NBP1-55039	1:1000
ARIH2	Rabbit	Abcam ab133744	1:1000
Actin	mIgG1	Sigma A5441	1:10000
ANKIB1	Rabbit	Atlas Antibody HPA021780	1:1000
Cullin 9	Rabbit	Thermofisher A300-098A-T	1:500
GAPDH	Rabbit	Sigma G9545	1:10000
IRF3	Rabbit	Cell Signalling 4302	1:1000
IkBα	Rabbit	Cell Signaling 9242S	1:1000
HOIP	Sheep	Ubiquigent 68-0013-100	1:1000
NEMO	Rabbit	Cell Signaling 2685	1:500
RIP1	Rabbit	Cell Signaling 9493	1:1000

Optineurin	Rabbit	Cell Signaling 58981	1:500
Phospho IkBα	mIgG1	Cell Signaling 9246	1:1000
Phospho ERK 1/2	Rabbit	Cell Signaling 4695	1:1000
Phospho IRF3	Rabbit	Abcam EPR2346	1:1000
Phospho-JNK	mIgG1	Cell Signaling 9255S	1:1000
Phospho-P38	Rabbit	Cell Signaling 9215S	1:1000
Phospho-P65	Rabbit	Cell Signaling 3033	1:1000
Phospho-TBK1	Rabbit	Cell Signaling 5483P	1:1000
RNF14	Rabbit	Abcam ab134927	1:500
RNF216	mIgG1	Abcam ab226215	1:500
TBK1	Rabbit	Cell Signaling 3013	1:1000
TLR3	Rabbit	Cell Signaling 6961	1:1000
TNFR1	mIgG2b	Santa Cruz 8436	1:1000
TRIF	Rabbit	Cell Signaling 4596	1:1000
Ubiquitin	Rabbit	Sigma-Aldrich	1:1000

Table 2.3: Conjugated antibodies

Antibody	Source	Dilution
Donkey-anti-Sheep HRP	EMD Millipore AP184P	1:10000
Rat-anti-mIgG2a HRP	Southern Biotech 1186-05	1:10000
Goat-anti-mIgG1 HRP	Southern Biotech 1070-05	1:10000
Goat-anti-Rabbit HRP	Southern Biotech 4050-05	1:10000

2.1.5 Oligonucleotides

Primers were used for different purposes. Guide RNA (gRNA) labelled oligonucleotides were used for CRISPR-Cas9 knock-out. hGAPDH, hIFNB1 and hIFN2/3 were used for RT-qPCR and the others were used for cloning and mutagenesis.

Table 2.4: Oligonucleotides

Name	Sequence (5’- 3’)
hANKIB1 gRNA Fw	CACCGGCGTGCCAAGCGAGGATGA
hANKIB1 gRNA Rv	AAACTCATCCTCGCTTGGCACGCC
hTLR3 gRNA Fw	CACCCAGCTAACTAGCTTGGATGT
hTLR3 gRNA Rv	AAACACATCCAAGCTAGTTAGCTG
Linker pBABE ANKIB1 Fw	gggagtgggggcgggagtCTCGAGAGATCTTGGTCTTC
Linker pBABE ANKIB1 Rv	gcccccactgcccccgccCACTAAATGTACTTGTTCAAG
ΔRING2 Fw:	CTTGAAGAGTGGAAAAAACATAG
ΔRING2 Rv:	AGGCTTGGAGTTAGTTAATAAC
ΔUIM Fw	CTCGATGAAGAAACTAGAG

ΔUIM Rv	ATCCAAGGAACTCAGAGC
ΔRBR Fw	CTTGAAGAGTGGAAAAAAC
ΔRBR Rv	CAAACTGGTGTCTAAATCC
hGAPDH fw	ACCCAGAAGACTGTGGATGG
hGAPDH rv	TTCTAGACGGCAGGTCAGGT
hIFNB1 Fw	ACATCCCTGAGGAGATTAAGCA
hIFNB1 Rv	GCCAGGAGGTTCTCAACAATAG
hIFNL2/3 fw	AGTTCCGGGCCTGTATCCAG

2.1.6 Cell Culture media and additives

Dulbecco’s Modified Eagle Medium	PAN biotech, Aidenbach; Germany
RPMI 1640	PAN biotech, Aidenbach; Germany
Iscove’s Modified Dulbecco’s Medium	ATCC, Manassas; Virginia-USA
Trypsin/EDTA	PAN biotech, Aidenbach; Germany
Penicillin/Streptomycin	PAN biotech, Aidenbach; Germany
Opti-MEM® Reduced Serum Medium	Invitrogen, Karlsruhe; Germany
Ampicillin	Sigma-Aldrich, Munich; Germany
Puromycin	Sigma-Aldrich, Munich; Germany

2.1.7 Kits and ready-to-use solutions

DNA isolation kits

QIAprep Maxi Kit	Qiagen, Hilden; Germany
QIAprep Spin Mini Kit	Qiagen, Hilden; Germany
QIAquick Gel Extraction Kit	Qiagen, Hilden; Germany

RNA isolation kit

RNEasy mini kit	Qiagen, Hilden; Germany

Other

Pierce BCA	ThermoFisher, Munich; Germany
ECL Western blotting detection kit	Perkin, Hamburg; Germany
Q5 site-directed mutagenesis kit	New England Biolabs, Frankfurt; Germany
Iscript Reverse Transcription Supermix	Bio-Rad, Düsseldorf; Germany
Itaq Universal SYBR	Bio-Rad, Düsseldorf; Germany

2.1.8 Instruments

Blotting equipment	Bio-Rad, Düsseldorf; Germany
Countess Automated Cell Counter	ThermoFisher, Munich; Germany
Developper	Agfa, Mortsel; Belgium
Freezer -20°C	Liebherr, Hauberhaussen, Germany
Freezer -80°C	Panasonic, Neuss; Germany
Incubator	ThermoFisher, Munich; Germany
Light Microscope	Zeiss, Oberkochen; Germany
Multichannel pipettes	Sartorius, Göttinger; Germany
Nanodrop One/One	ThermoFisher, Munich; Germany
PCR	Eppendorf, Hamburg; Germany
RT-PCR	Bio-Rad, Düsseldorf; Germany

pH meter	Knick, Berlin, Germany
Pipettes (2 µl, 10 µl, 200 µl, 1 ml)	Gilson, Bad Camber; Germany
Pipetboy	Brand, Wertheim; Germany
Trans-Blot Turbo transfer system	Bio-Rad, Düsseldorf; Germany
Scanner	Epson, Meerbusch; Germany
Table Centrifuge	Eppendorf, Hamburg; Germany
Thermomixer	Eppendorf, Hamburg; Germany
Vortex	

2.1.9 Software

Benchling	Benchling, California, USA
BioRender	BioRender, Ontario; Canada
Microsoft® Excel 2016	Microsoft, Redmont; USA
Microsoft® PowerPoint 2016	Microsoft, Redmont; USA
Microsoft® Word 2016	Microsoft, Redmont; USA
Zotero	Zotero, Virginia, USA

2.2 Methods of Cell Biology

2.2.1 Cell lines

HeLa

HeLa was established in 1951 from a patient named Henrietta Lacks. They were originally isolated from an adenocarcinoma of the Cervix. It is the first cancer cell line for cell culture. These cells have a hypertriploid chromosome number (75 to 80 chromosomes in total) and contain several integrations of human papillomavirus 18.

HaCaT

HaCaT are human keratinocyte cells derived from a 62-year-old female that had psoriasis. They have been immortalized spontaneously, which provides a non-modified and non-cancerous cell line. Hacat cells are hypotetrapoloid (<4N).

HT-29

HT-29 cells are human colon adenocarcinoma cell line derived from a 44-year-old male. These cells have a hypertriploid chromosome number.

2.2.2 Cell culturing conditions

All cell lines were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. HeLa and MEFs were maintained in Rosewell Park Memorial Institute medium (RPMI) with 5% fetal calf serum and 1% Pen/Strep. To prevent confluency or seed for an experiment, cells were washed with PBS, and detached by incubation in 1X Trypsin (PAN Biotech) in PBS solution.

2.2.3 Freezing and thawing cell lines

80% confluent cells were collected, washed once with RPMI, and resuspended in freezing medium (90% FCS, 10% DMSO). 2 x 10^6 cells/mL were transferred into cryogenic vials and frozen down in -80 °C with a freezing box containing isopropanol.

For long-term storage, vials were transferred to liquid nitrogen at -196°C. To thaw the cells, cryogenic vials were heated at 37 °C, and cells were transferred in 10 mL of medium, centrifuged, and resuspended in fresh medium.

2.2.5 Generation of primary bone marrow-derived macrophages (BMDMs)

Femur was isolated from 6 to 8-week-old mice. Femur and tibia were isolated and opened on each side using scissors. Bone marrow was flushed out using a 25-gauge needle and syringe with PBS. Red blood cells were lysed using a red blood cell lysis buffer. Cells were washed with 50 mL of PBS, cells were counted and resuspended in BMDM medium (IMEM with 10% decomplemented FCS, 1 % penicillin/streptomycin and 50ng/mL of M-CSF). Cells were plated in non-coated 10cm dishes with a concentration of 2 x 10^6 cells/mL for seven days. Cells were then detached with 10 mM EDTA for 10 minutes and plated in a 12-well plate for experiment.

2.2.6 siRNA-mediated knockdown

Cells were plated the day before transfection at a confluency between 50-60% for the day of transfection. The following day, the medium was changed to a medium without FCS. For a well of a 6-well plate, 20 nM of siRNA was incubated 5 min at room temperature with 100 µl Opti-MEM. In parallel, 2.5 µl of Lipofectamine was incubated with 100 µl Opti-MEM for a ratio of 1:3 siRNA/Lipofectamine. Then, both DNA and lipofectamine 2000 (Invitrogen) were pulled for 20 minutes at room temperature. Thus, the mixture was added dropwise to the cell. The next day, the medium was changed to 2 mL of medium with 5% FBS and antibiotic. Experiments were performed 48h or 72h after transfection.

2.2.7 Retroviral infection of eukaryotic cells

In order to generate viral particles and stably reconstitute ANKIB1 expression in cells, 5 million Platinum Ampho cells were seeded per 10 cm dishes in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). Platinum Ampho cells constitutively express gag-pol and the envelope protein for amphotropic viruses. The next day, 5 µg retroviral pBABE vector including the construct of interest were mixed with 20 µl at 1mg/ml polyethyleneimine (PEI) (Sigma-Aldrich 764965) in Opti-MEM and they were incubated at room temperature for 20 minutes. Then, cells in each dish were transfected dropwise. The next day, the medium was removed and replaced with 5 mL of fresh medium. 48 hours later, supernatants were collected and filtered with a 0.45 µm filter. Then, the supernatant comprising viral particles was mixed with fresh RPMI supplemented by 5%FBS and 1% penicillin-streptomycin. Additionally, 8 µg/ml polybrene (Sigma-Aldrich TR-1003) was added to increase the efficiency of the transduction. Plates were subjected to spin-infection at 2500rpm for 45 minutes at 30°C. After two days, cells were either selected via GFP-positive cell sorting by Fluorescence-activated cell sorting (FACS) or via puromycin selection.

2.2.8 Generation of knock-out cell lines

Specific guide RNAs (gRNA Table 2.4) were inserted into PX458-Cas9-2A-mCherry. Direct transfections were performed on cells with 10 µg/mL of plasmid premixed as detailed previously with 30 µl of Lipofectamine 2000.

2 days after transfection, mCherry positive cells were single-cell sorted by Fluorescence-activated cell sorting (FACS) in a 96-well plate containing conditioned media previously filtered with 0.45µm filter mixed 1:1 with fresh media containing 10% FBS. Single cells were then analysed by immunoblotting, confirming the depletion of the gene of interest.

2.3 Method of Molecular Biology

2.3.1 DNA restriction digest

DNA restriction for analytic or preparative purposes was performed with FastDigest restriction enzymes) and buffers (Thermo Fisher Scientific). Per reaction, DNA was diluted in water, mixed with buffer 10X solution and 1µl of the appropriate enzyme(s) was added. The total volume was kept at 30µl. Samples were incubated for 30 minutes at 37 ˚C.

2.3.2 DNA purification

To purify the produced DNA, QIAquick PCR Purification Kit (Qiagen) was used according to the manufacturer’s protocol. The DNA was eluted in 30 µl water. The purity and the concentration were measured by Nanodrop.

2.3.3 Agarose gel electrophoresis of DNA

DNA was separated and isolated by agarose gel electrophoresis. 2% agarose gels were in Borax buffer using a microwave oven. Samples were then loaded in the wells of the gel, electrophoresis was carried out in Tris Borate EDTA buffer (TBE).

2.3.4 Gel extraction of the DNA fragments

After electrophoresis, DNA fragments were visualized with a GelStick imager (Intas) in order to excise them with a scalpel. DNA isolation was achieved by using QIAquick Gel Extraction Kit (Qiagen) and the manufacturer’s protocol was followed. The DNA was purified by using the spin columns and eluted in 20 µl of water.

2.3.5 Ligation of DNA fragments

DNA inserts were ligated to the plasmid of interest by using T4 DNA ligase (ThermoFisher Scientific) with its buffer. The molar ratio between insert and plasmid were 4:1. Master mix contained 1µl of T4 ligase (5U) and 1 µl of Buffer 10X for a total volume of 10µl. Sample stayed overnight at room temperature for efficient ligation.

2.3.6 Competent E. coli transformation

NEB 10-beta competent E.Coli (High efficiency; NEB) were transformed with 10 ng of plasmid following the manufacturer's recommendation. First, bacteria and plasmid were incubated on ice for 15 minutes. Subsequently, they were heated in a water bath for 1 minute at 42°C followed by a cooling step of 5 minutes on ice. 500 µl of SOC medium was added and bacteria were shaken for 1 hour at 37°C. Finally, they were plated on LB agar plates with the appropriate antibiotic and incubated overnight at 37°C. The next day, several colonies were picked for liquid culture in 5 ml of LB medium containing the antibiotic. 12 hours after incubation at 37°C on a bacterial shaker, plasmids were isolated with QIAprep Miniprep Kit (Qiagen).

For protein production, a similar protocol was used, but BL21 (DE3) E.Coli bacteria were used instead. The heating step was at 42°C for 45 seconds.

2.3.7 Polymerase Chain Reaction (PCR)

DNA fragments of interest were amplified with a Mastercycler pro PCR machine (Eppendorf). Every amplification for cloning was prepared with the following mix:

Table 2.5: PCR master mix

Component	Amount (µl)
2X Kapa-HiFi master mix	10 µl
Cresol Red PCR dye	2 µl
Forward Primer (10µM)	1 µl
Reverse Primer (10µM)	1 µl
Nuclease-free dH_2O	6 µl
Total Volume	20 µl

Then, the master mix was placed into PCR-reaction tubes, and the following program was used:

Table 2.6: Reaction Conditions for classical PCR

<table>
<tr><th>Steps</th><th>Temperature</th><th colspan="2">Time</th></tr>
<tr><td>Initial Denaturation</td><td>98°C</td><td>2 minutes</td><td></td></tr>
<tr><td>Denaturation</td><td>98 °C</td><td>20 seconds</td><td rowspan="3">33 cycles</td></tr>
<tr><td>Annealing</td><td>50-65°C</td><td>15 seconds</td></tr>
<tr><td>Extension</td><td>72 °C</td><td>1 min/kb</td></tr>
<tr><td>Final Extension</td><td>72 °C</td><td>30 seconds</td><td></td></tr>
</table>

2.3.8 cDNA preparation

Cells were lysed and complementary cDNA was generated from the mRNA using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's manual. RNA-binding spin columns were used and water was added to the column to elute the purified RNA.

Then, cDNA was generated by using Iscript Reverse Transcription Supermix (Biorad). 1 µg RNA was added to the master mix. The priming occurred for 5 minutes at 25°C, the reverse transcription for 20 minutes at 46°C, and the reverse transcriptase inactivation was performed by heating at 95°C for 1 minute.

2.3.9 qRT-PCR

From the cDNA generated, quantitative real-time PCR was performed using Itaq Universal SYBR (Bio-RAD) following the manufacturer's recommendations. The master mix was prepared as detailed in Table 2.6. Each sample was loaded three times in a 96-well plate and run on a Bio-Rad CFX Opus 96. The initial polymerase activation step lasted 30 sec at 95°C, followed by 35 amplification cycles of 5 seconds at 95°C and 30 seconds at 60°C. GAPDH was used as reference gene and the relative expression of the gene transcripts was analysed using $2^{-\Delta Ct}$.

Table 2.7: qRT-PCR preparation:

Component	Amount (µl)
Universal SYBR Green Supermix 2x	5 µl
Forward Primer (10µM)	0,5 µl
Reverse Primer (10µM)	0,5 µl
Template DNA (50ng/µl)	2 µl
Nuclease-free dH_2O	2 µl
Total Volume	10 µl

2.3.10 Site-directed Mutagenesis

In order to modify a plasmid already generated, we used NEB Q5® Site-Directed Mutagenesis Kit E0554S. This kit allows the insertion, deletion, or substitution of DNA fragments on a plasmid of interest. A mixture was prepared as described in **Table 2.8** and the reaction conditions are detailed in **Table 2.9**.

Table 2.8: Mutagenesis master mix:

Component	Amount (µl)
Q5 Hot Start High Fidelity 2x Master Mix	12.5 µl
Forward Primer (10µM)	1.25 µl
Reverse Primer (10µM)	1.25 µl
Template DNA	1 µl
Nuclease-free dH_2O	9 µl
Total Volume	25 µl

Table 2.9: Reaction Conditions for mutagenesis (for pBABE plasmid)

<table>
<tr><th>Steps</th><th>Temperature</th><th colspan="2">Time</th></tr>
<tr><td>Initial Denaturation</td><td>98ºC</td><td>30 seconds</td><td></td></tr>
<tr><td>Denaturation</td><td>98 ºC</td><td>10 seconds</td><td rowspan="3">25 cycles</td></tr>
<tr><td>Annealing</td><td>60 ºC</td><td>30 seconds</td></tr>
<tr><td>Extension</td><td>72 ºC</td><td>3 min 15 seconds</td></tr>
<tr><td>Final Extension</td><td>72 ºC</td><td>2 minutes</td><td></td></tr>
</table>

After the PCR, the plasmid template was degraded by KLD reaction, and transformation was subsequently performed as manufacturer's instructions. Bacteria were spread to an 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 overnight. Finally, plasmid isolation from bacteria was executed with QIAprep Spin Miniprep Kit (Qiagen). After validation of the mutagenesis, plasmids were amplified and isolated using QIAprep Maxi Kit (Qiagen).

2.4 Methods of Biochemistry

2.4.1 DNA concentration

The concentration of DNA isolated and eluted in water was determined by using a NanoDrop spectrophotometer.

2.4.2 Sample Preparation for Immunoblotting

Following treatment with specific ligands, the medium was removed from the wells, and cells were washed with ice-cold PBS. Subsequently, they were lysed in lysis buffer including (30 mM Tris-Cl pH 7.5, 120 mM NaCl, 1% Triton-X-100, 2 mM KCl, 2 mM EDTA, 10% Glycerol) phosSTOP (Roche), EDTA-free protease inhibitor cocktail (Roche) and tumbled at 4 ºC. for 20 minutes before centrifugation at 13,600 RPM at 4 ºC for 30 minutes. Supernatants were collected for protein quantification.

2.4.3 Quantification of the protein concentration

Total cell lysates were calibrated by using Pierce Bicinchoninic Acid (BCA) Protein Assay (Thermo Fischer Scientific). Component A and component B were mixed to a ratio of 50:1, and 100 µl was added per well in a 96-well plate. 2 µl of every sample was added in a unique well in triplicate and the plate was incubated for 20 minutes at 37°C to reach a violet colour. Thus, the absorbance was measured using a Tecan at A562nm.

2.4.4 Western Blot

Before sample loading, NuPAGE lithium dodecyl sulfate (LDS) Sample Buffer 4X (Thermo Fisher Scientific) was added to reach 1X. 25 mM of DTT were also added and samples were incubated for 5 minutes at 95°C on a Thermomixer. Samples were then vortexed, spin down, and loaded on 4-15% Precast gels with TGX running buffer to separate the proteins according to their size. Proteins were separated at 210 V for 35 minutes by SDS-PAGE. Later, they were transferred to a nitrocellulose membrane in 1.3A-25V for 10 minutes by Turbo trans-blot (Bio-Rad) and the membranes were blocked with 2.5% milk solution in PBS-Tween 20 (PBS-T) for 1h. After that, proteins were blotted with the respective antibody (**Table 2-3**) in 2.5% bovine serum albumin (BSA) and 0.025% NaN_3 in PBS-T overnight. The next day, membranes were washed 3 times with PBS-T for 10 minutes and incubated with the respective HRP conjugated- secondary antibody in **Table 2-4**. Finally, membranes were washed 3 times with PBS-T for 10 minutes and developed by ECL solution (Western Lightning Plus ECL 0RT265).

2.4.5 ANKIB1 and TLR3 pull-down

5 million HeLa or 7 million HT-29 cells were seeded in 15 cm dishes in RPMI. The following day, cells were treated with 10 µg/ml poly(I:C) (Invivogen) for 0, 30 minutes, and 1 hour. The medium was removed and cells were washed with 5 mL ice-cold PBS. Subsequently, they were lysed in 1 mL lysis buffer including (30 mM Tris-Cl pH 7.5, 120 mM NaCl, 1,7 mM DDM, 2 mM KCl, 2 mM EDTA, 10% Glycerol) phosSTOP (Roche), EDTA free protease inhibitor cocktail (Roche) 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 the 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 and 25 mM DTT at 95 ºC for 5 minutes. The rest of the cell lysates were incubated with 15 µl of M2 anti-Flag magnetic beads (Sigma Aldrich) per conditions overnight after pre-washed the beads three times with the lysis buffer. The following day, beads were washed with lysis buffer 5 times with 1mL of lysis buffer and boiled with 2x Laemmli buffer involving 50 mM DTT at 95 ºC for 5 minutes. Samples were analysed by previously described western blotting.

2.4.6 Production of recombinant moTAP-TNF

moTAP- consisting of a his-tag followed by 3X Flag-tag and 2X Strep-tag fused to the N-term of the extracellular part of TNF (from the amino acid 78 to amino acid 233). This construct was inserted into a pQE30 vector. This plasmid was transfected into BL21 (DE3) and the protein production was induced by addition of 1 mM Isopropyl-β-D-thio-galactoside (IPTG) and incubated overnight at 30 °C. Bacteria were then lysed and the moTAP-TNF was purified from the His-tag with nickel beads. The elution was performed by addition of 250 mM imidazole and dialysed against PBS. The final protein concentration was measured by Nanodrop (Thermofisher), and 30% glycerol was added and stored at -80°C.

2.4.7 TNFR1-SC pull-down

5 million HeLa were seeded in 15 cm dishes in RPMI. The next day, cells were treated with 1µg/ml moTAP-TNF for the indicated times. The medium was removed and cells were washed with 5 mL ice-cold PBS. Subsequently, they were lysed in 1 mL lysis buffer including (30 mM Tris-Cl pH 7.5, 120 mM NaCl, 1% Triton-X-100, 2 mM KCl, 2 mM EDTA, 10% Glycerol) phosSTOP (Roche), EDTA free protease inhibitor cocktail (Roche) and tumbled at 4 ºC for 20 minutes before centrifugation at 13,600 RPM at 4 ºC for 30 minutes. Then, all steps are similar to the ones detailed in 2.4.5.

2.4.8 Di-Gly enrichment analysis by Mass Spectrometry

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). Then, they were washed with ice-cold PBS before lysis and the manufacturer’s instructions in PTMScan® HS Ubiquitin/SUMO Remnant Motif (K-ε-GG) Kit #59322 were followed. As a note, the manufacturer’s protocol was modified by using 40 mM chloroacetamide to alkylate Sulphur groups.

Additionally, 1% formic acid was employed instead of 0.1% trifluoroacetic acid. The experiment was repeated four times and samples were prepared for MS analysis.

2.4.9 In-vitro ubiquitin assay

moTAP ANKIB1 WT or moTAP ANKIB1 ΔRING2 were purified by modified tandem affinity purification. After protein purification, the efficiency of the purification and the amount of ANKIB1 enriched were assessed by Coomassie brilliant blue G-250. Then, the different versions of ANKIB1 were incubated for 1 hour at 37 °C in a 30 µL reaction mixture containing 200 nM UBA1, 1 µM UBE2L3 or 1 µM UBE2D3 or 1 µM UBE2L6, 100 µg/mL ubiquitin, 2 mM DTT, 30 mM Tris-HCl, pH 7.5 and 5 mM $MgCl_2$ and 1x ERS containing ATP/ADP, $MgCl_2$ and ATP regenerating enzymes. For negative controls, ubiquitin buffer was added instead of the E2. The reaction was terminated by adding reducing sample buffer and boiling at 95°C for 5 min. Afterwards, the samples were subjected to analysis by western blot.

2.4.10 Statistical analysis

Data from Di-Gly Proteomics Analysis were analysed by ANOVA, Student t-test, and Fisher exact test. Data from qPCR were analysed 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.

2.4.11 Data accessibility

The raw data generated by Mass Spectrometry regarding the di-gly enrichment and the characterisation of the ubiquitin chains generated in vitro by ANKIB1 with UBE2D3 and UBE2L3 have been uploaded on OSF and are available upon request.

3. Result:

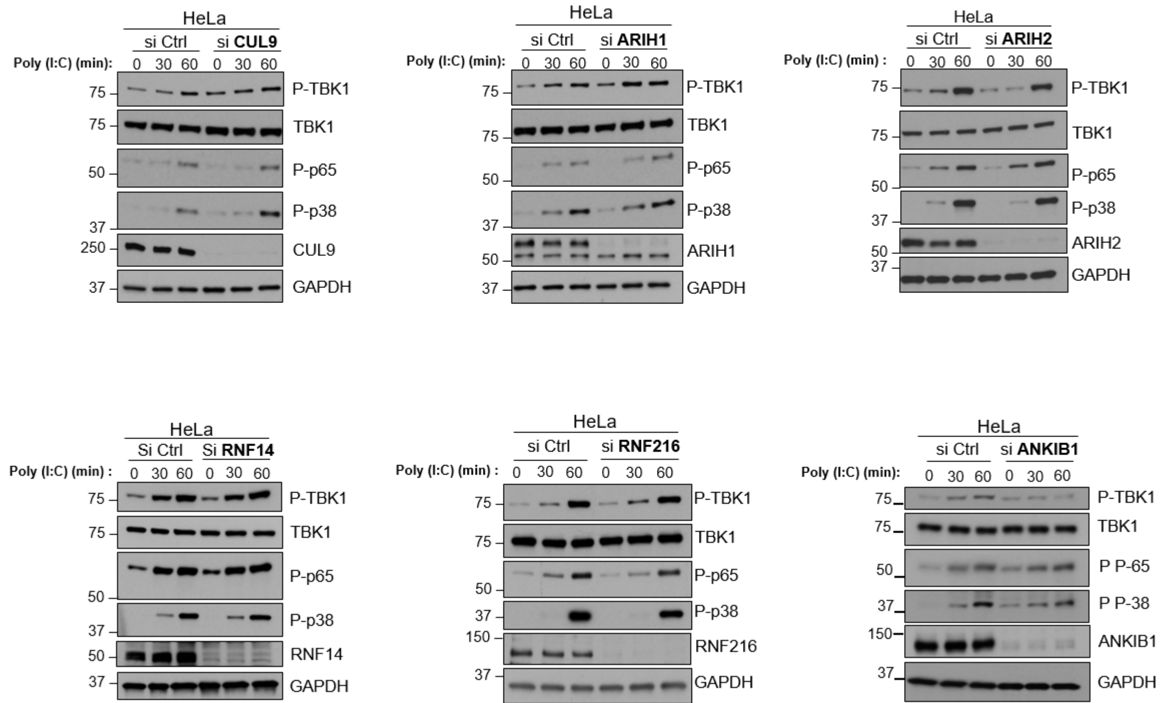
3.1 Identification of a new E3 ligase so far undescribed

Regarding the extensive implication of the RBR-containing E3 ligase in the regulation of immune signalling, we decided to address the potential implication of this particular subfamily for the activation of TBK1 in TLR3.

3.1.1 Role of the RBR-containing E3 in TLR3-induced TBK1 activation

Because very little is known about the majority of this subfamily, we aimed to do a knock-down screen of every member susceptible to contribute to TBK1 activation. Regarding our previous finding that HOIP is not required for TBK1 activation in TLR3, we also excluded HOIL-1 (Zinngrebe et al., 2016). Without HOIP, HOIL-1 cannot be recruited to the signalling complex. PARKIN was also discarded from our potential candidate due to the already published role in the negative regulation of TBK1 activation in the TLR pathway mediated by the degradation of TRAF3, an essential E3 required for TBK1 activation in TLR3 (Xin, Gu, Liu, & Sun, 2018). RNF144b has also been reported to interact with TBK1 in order to disrupt its activation and therefore IRF3 phosphorylation (Zhen Zhang et al., 2019). More recently, RNF19a was reported to trigger proteasomal degradation of TBK1 in RIG-I signalling pathway (Yingyun Yang, Cao, Huang, & Yang, 2022). Then, after analysis of the literature, we decided to knock-down the remaining RBR containing-E3s and assess the consequence on the TLR3 signalling pathway, especially TBK1 phosphorylation after poly(I:C) treatment **(Figure 3.1)**.

While Cullin-9, RNF14 and ARIH2 knock-down do not seem to have any impact on TLR3 signalling, ARIH1 and RNF216 down-regulation interestingly seem to rather slightly increase TBK1 activation. However, in HeLa cells transfected with siRNA targeting ANKIB1 TBK1 activation upon poly(I:C) treatment is almost completely abolished. However, ANKIB1 knock-down did not impact the phosphorylation of p65 or p38, respectively associated to the activation of the other gene-activatory NF-kB and MAPK pathways.



<u>Figure 3.1</u>. ANKIB1 knock-down impairs TBK1 phosphorylation upon TLR3 activation:
a) HeLa cells were transfected for 48 hours or 72h (depending on the protein) with 20 nM of non-targeting siRNA Control or siRNA targeting Cullin-9, ARIH1, RNF14, ARIH2, RNF216 or ANKIB1. Cells were then treated for 30 and 60 minutes with 10µg/ml of poly(I:C). Lysates were analysed by western blotting.

In order to find out if ANKIB1 seems to be equally involved in TBK1 phosphorylation in TNF signalling, we performed a similar knock-down experiment and treated the cells with TNFα. Strikingly, the down-regulation of ANKIB1 did not affect the gene-activation, including TBK1, upon TNFR1 activation (**Figure 3.2**). This suggests that, as hypothesised, TBK1 phosphorylation does not occur through a similar process and does not involve the same proteins between both pathways. ANKIB1, so far an uncharacterised E3 ligase, seems to be involved in TLR3-induced TBK1 activation, while this is not the case in TNF signalling pathway.

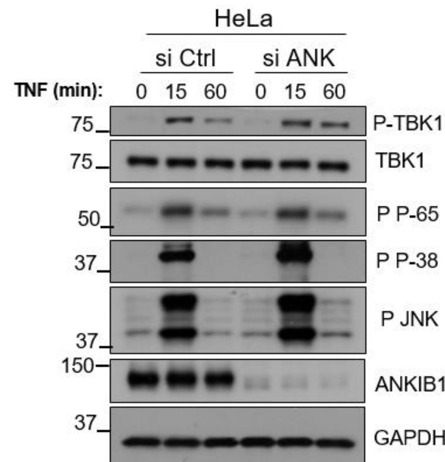


Figure 3.2. ANKIB1 knock-down does not impact TNF signalling pathway:
a) HeLa cells were transfected for 48 hours with 20 nM of non-targeting siRNA Control or si ANKIB1. Cells were then treated for 15 and 60 minutes with 100ng/ml of TNF. Lysates were analysed by western blotting.

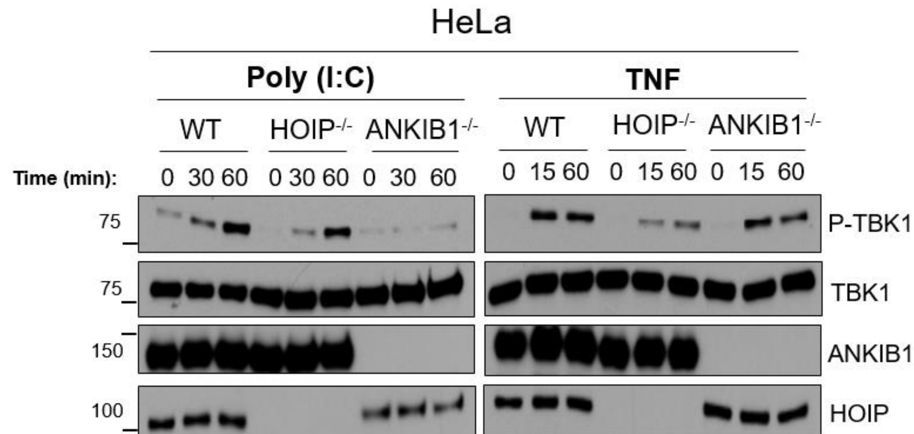
In order to obtain some potential supplementary information concerning ANKIB1, we used a bioinformatic platform that is able to predict putative substrates for an E3 ligase. Interestingly, the predicted substrates for ANKIB1 by Ubibrowser were NEMO and Optineurin, both reported to be implicated in TBK1 recruitment and activation in TLR3 signalling.

Regarding our results and information obtained, we decided to focus on ANKIB1 which seemed an interesting protein to understand the discrepancy of TBK1 activation between TLR3 and TNF pathway.

3.1.2 Differential implication of ANKIB1 and HOIP in signalling

To validate our previous observation, we have generated several knock-out cell lines for ANKIB1 by applying the CRISPR-Cas9 technique. Once obtained, we decided to compare TBK1 activation in HeLa wild-type, HeLa HOIP knock-out and HeLa ANKIB1 knock-out following TNF or poly(I:C) treatment **(Figure 3.3)**. As expected, the depletion of HOIP strongly decreases TBK1 activation after TNF treatment but not after poly(I:C) treatment. Oppositely and as observed previously with the siRNA-mediated knockdown approach, ANKIB1 knock-out cells were not able to activate TBK1 after TLR3 activation. Immunoblotting for total TBK1 allows us to confirm that only the activation of TBK1 is impacted in absence of ANKIB1 and not the amount of total protein present in cells.

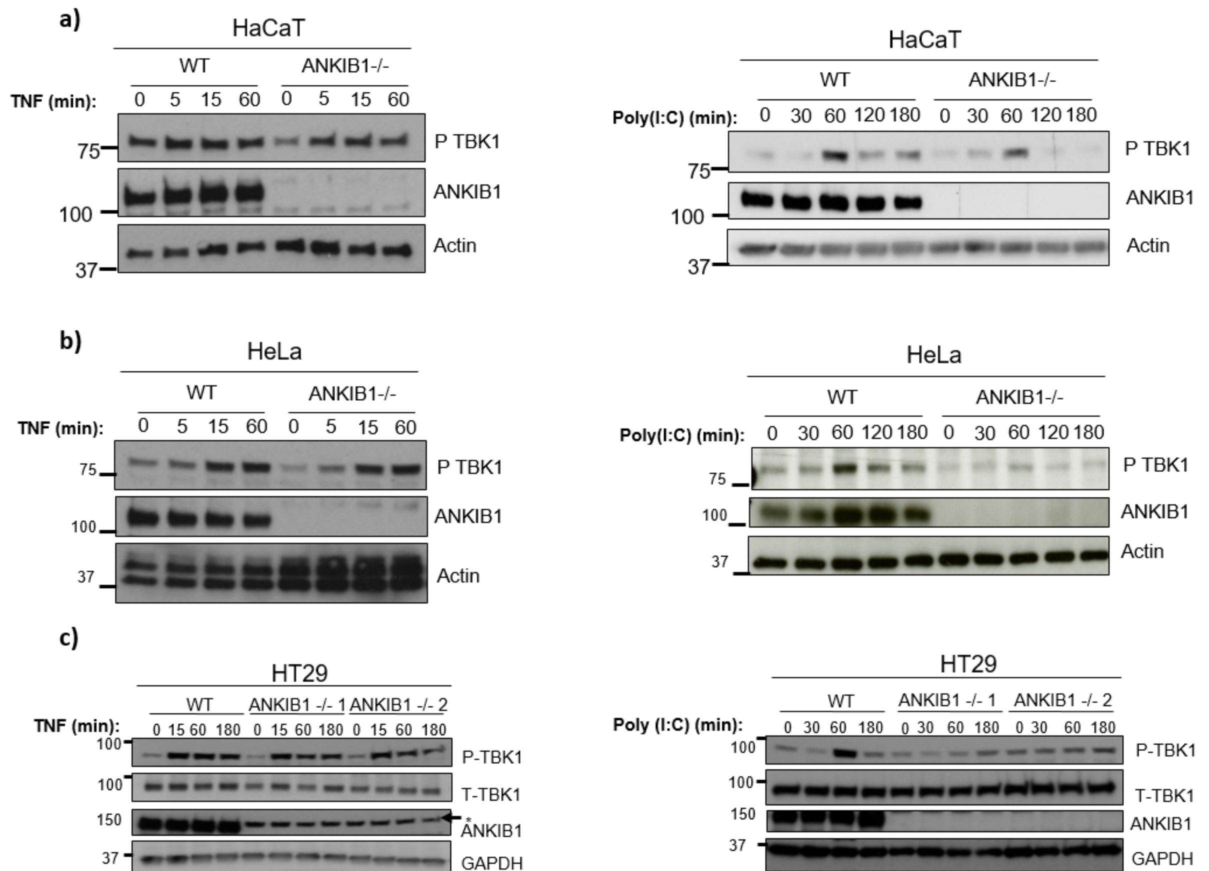
In conclusion, it seems that TBK1 requires HOIP in TNF pathway, and ANKIB1 in TLR3 pathway, in order to be properly activated.



<u>Figure 3.3</u>. In contrary to HOIP, ANKIB1 is required for TBK1 activation in TLR3 but not TNF pathway:
HeLa wild-type, HOIP knock-out or ANKIB1 knock-out cells were stimulated with 100ng/mL of TNF for 15 and 60 minutes or 10µg/mL poly(I:C) for 30 and 60 minutes, respectively. Cells were lysed and proteins were analysed by western blotting.

3.1.3 ANKIB1 is involved in TBK1 activation in TLR3 pathway

We then investigated the kinetic of TBK1 activation by western blotting, comparing WT and ANKIB1 KO in different cell lines such as HeLa, HaCaT, and HT29. Indeed, we wanted to confirm that TBK1 activation is not only delayed in knock-out cell lines but rather impaired. We compared the ability of the cells to induce TBK1 phosphorylation (the active form of TBK1) upon TNF-α and Poly(I:C) treatment. In all cell lines, we did not observe any changes regarding TBK1 phosphorylation after TNF-α treatment in ANKIB1 knock-out cells compared to wild-type cells (**Figure 3.4**). However, activation of TBK1 was strongly impaired after poly(I:C) treatment in ANKIB1 knock-out cells. Hence, we suggest that ANKIB1 is strongly implicated in TBK1 activation following TLR3 stimulation.



<u>Figure 3.4</u>. ANKIB1 is required for TBK1 activation in TLR3 but not TNF pathway:
HaCat in a), HeLa in b) and HT-29 in c) wild-type or ANKIB1 knock-out cells were stimulated with respectively 100ng/mL of TNF for 5, 15 and 60 minutes in a), b) and for 15, 60, 180 minutes in c) or 10 µg/mL poly(I:C) for 30, 60, 120 and 180 minutes in a), b) and 30, 60, 180 minutes in c). Cells were lysed and proteins were analysed by western blotting.

3.1.4 ANKIB1 is similarly required for TBK1 activation in mouse

Following the reproducibility of the data in different cancer cell lines and human keratinocytes, we decided to generate a mouse line that is flanked with loxP site on the exon 4 of ANKIB1 sequence. Therefore, by crossing this strain with a CMV- Cre (Total Cre) positive mouse strain, we obtain mice depleted for ANKIB1 in the full body. Mice were born in the Mendelian ratio suggesting that the deletion of this protein does not cause embryonic lethality **(Table 3.1)**. Thus, we aimed to assess whether in vivo we see a similar effect for ANKIB1 in TLR3 signalling as we did in vitro.

Table 3.1: Number of mice obtained regarding their genotype

	ANKIB1$^{+/+}$	ANKIB1$^{+/-}$	ANKIB1$^{-/-}$
Observed number	31(30%)	51(49%)	22(21%)
Expected number (Ratio 1:2:1)	26(25%)	52(50%)	26(25%)

To do so, we first studied the expression levels of ANKIB1 in various tissues. After isolating and preparing the different organs, we immunoblotted for ANKIB1 **(Figure 3.5)**. Interestingly, we could see that ANKIB1 is strongly expressed in spleen and lungs. Colon, intestine and Peyer's patches also expressed ANKIB1. The comparison to knock-out animals allowed us to validate the specificity of the antibody used **(Figure 3.5, b)**. Interestingly, stomach, skin, heart and liver seem to express ANKIB1 barely. This could be correlated with the presence of immune cells in the different organs. Indeed, where ANKIB1 is mostly expressed, immune cells are also present in a large amount. Thus, we think that ANKIB1 might play an important role in immune cells.

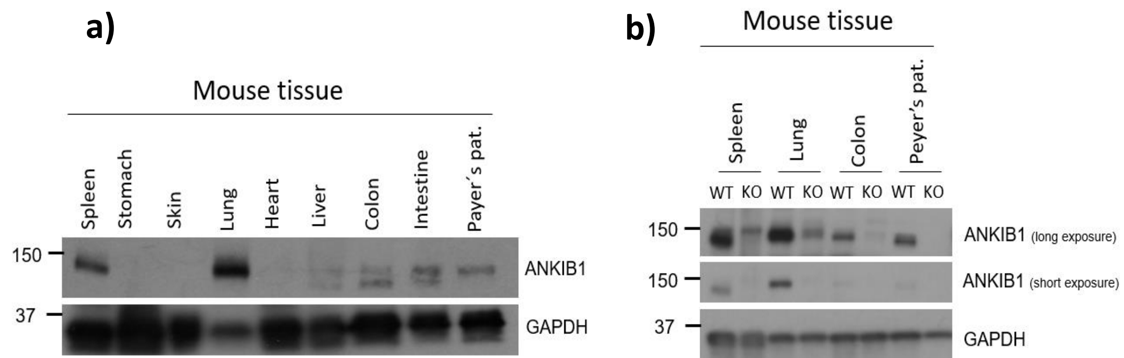


Figure 3.5. ANKIB1 expression in mouse:
Organs were isolated from 8 weeks old in a) or 7 months old in b) mice and were lysed in order to analyse them by western blotting. a) ANKIB1 expression in wild-type mouse in different organs. b) ANKIB1 expression in wild-type or ANKIB1 knock-out mouse in organs where ANKIB1 was mostly expressed.

Together with the high expression of Toll-Like Receptors and the ability to trigger a potent interferon responses that characterise Antigen-Presenting Cells, we expected that BMDMs would be an appropriate primary cell type to assess the relevance of ANKIB1 in mice. Therefore, we generated Bone Marrow-Derived-Macrophages (BMDMs) from

heterozygote mice and ANKIB1 knock-out mice in order to treat them either with poly(I:C) or TNF (**Figure 3.6**). Similarly than in human cells, BMDMs treated with TNF can activate TBK1 and other gene-activatory pathways equally. However, following TLR3 activation, ANKIB1 knock-out BMDMs barely activate TBK1 in comparison with the wild-type. It is important to notice that phosphorylation of p65, read-out for NF-kB activation and phosphorylation of JNK, a member of the MAPK pathway, are similar in presence or absence of ANKIB1. This suggests that ANKIB1 is not involved in the regulation of these gene-activatory pathways as seen previously. Overall, ANKIB1 plays a similar role in primary mouse cells, confirming its essential role for TBK1 activation in TLR3 signalling pathway in mammals.

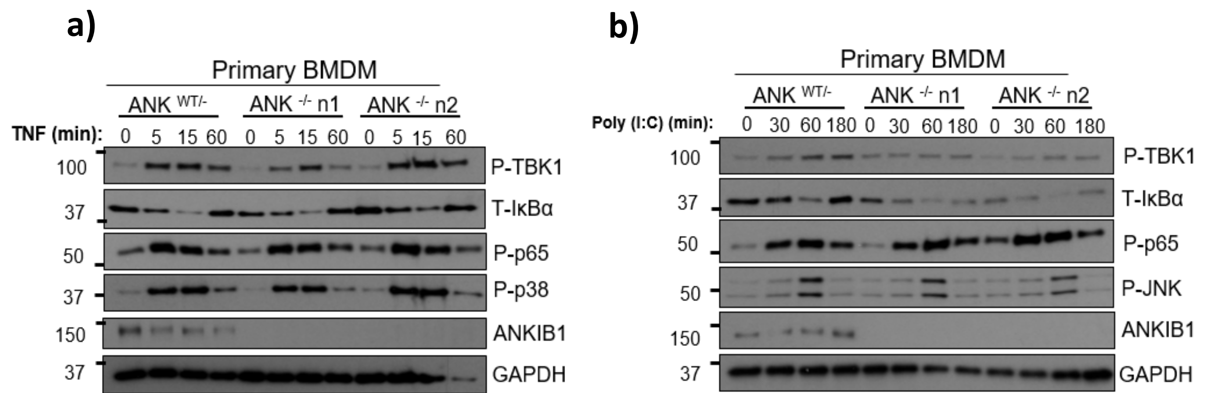


Figure 3.6. **ANKIB1 is equally important for TBK1 activation in mice:**
Primary Bone Marrow Derived Macrophages isolated from 8 weeks old mice either heterozygous for ANKIB1 or knock-out. Cells were stimulated with respectively a) 100ng/mL TNF for 5, 15, 60 minutes or b) 1µg/mL poly(I:C) for 30, 60 and 180 minutes. Cells were lysed and proteins were analysed by western blotting.

3.2 Identification of the modality of action of ANKIB1

After confirming the role of ANKIB1 for TBK1 activation following TLR3 activation, we wanted to know if this contribution is taking place at the signalling complex or not. To do so, we generated HeLa TLR3 knock-out cells that we stably reexpressed with Flag-tagged TLR3, allowing us to immunoprecipitate the receptor and study the formation of the signalling complex following poly(I:C) treatment.

3.2.1 ANKIB1 is recruited to TLR3 signalling complex

After treatment with poly(I:C), we blotted for known components of the TLR3-SC such as RIPK1 or HOIP, confirming that our system allows us to study this signalling complex **(Figure 3.7)**. Similarly, ANKIB1 immunoblotting shows that upon TLR3 activation, it is recruited to the complex. This suggests that ANKIB1 is a new member of the TLR3-SC and that it probably acts at the complex in order to promote TBK1 phosphorylation.

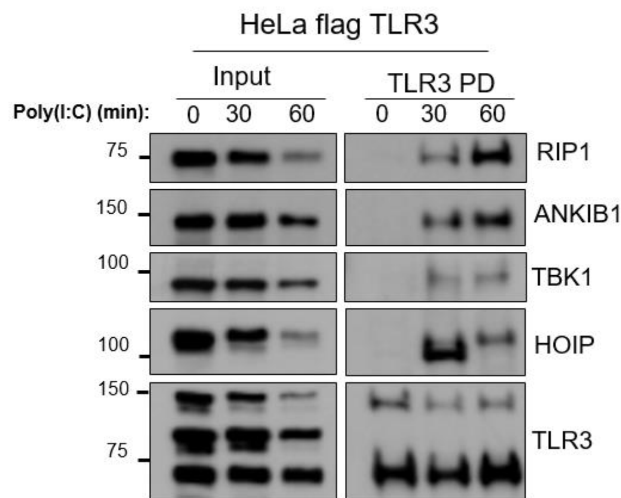
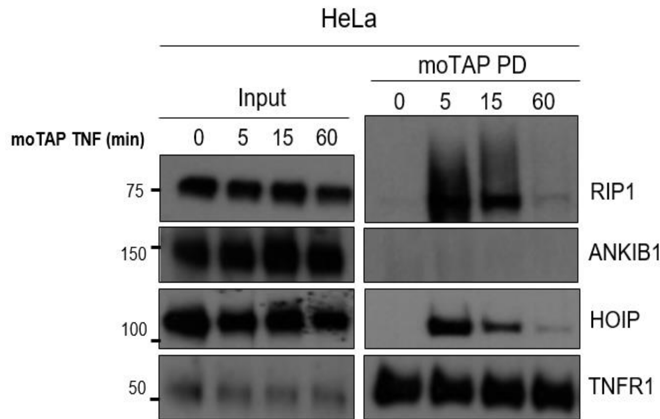


Figure 3.7. ANKIB1 is recruited to TLR3-SC following poly(I:C) treatment:
HeLa TLR3 knock-out stably reexpressed with Flag-tagged TLR3.Cells were stimulated with 15µg/mL poly(I:C) for 30 and 60 minutes. Cells were lysed, TLR3 was immunoprecipitated via Flag-tag and coimmunoprecipitated proteins were analysed by western blotting.

In Parallel, we have performed a similar analysis by treating HeLa wild-type cells with TNFα containing a moTAP-tag (composed of 1 Flag-tag and 2 Strep-tag). The use of this tagged TNF allows us to visualise the formation of TNFR1-SC at different time points. Western blotting shows some of the known proteins implicated in this signalling complex, proving that the complex has been properly formed **(Figure 3.8)**.



<u>Figure 3.8</u>. ANKIB1 is not recruited to TNFR1-SC:
HeLa wild-type cells were stimulated with 1µg/mL moTAP-TNF for 5, 15 and 60 minutes. Cells were lysed, TNF was immunoprecipitated via Flag-tag and coimmunoprecipitated proteins were analysed by western blotting.

However, ANKIB1 has not been detected, suggesting that ANKIB1 is not recruited to TNFR1-SC, providing some explanation concerning the absence of consequences on the gene-activatory pathways in absence of ANKIB1. Therefore, it suggests that, in order to be recruited to a signalling complex, ANKIB1 requires a protein or a post-translational modification that occurs in TLR3-SC but not in TNFR1-SC.

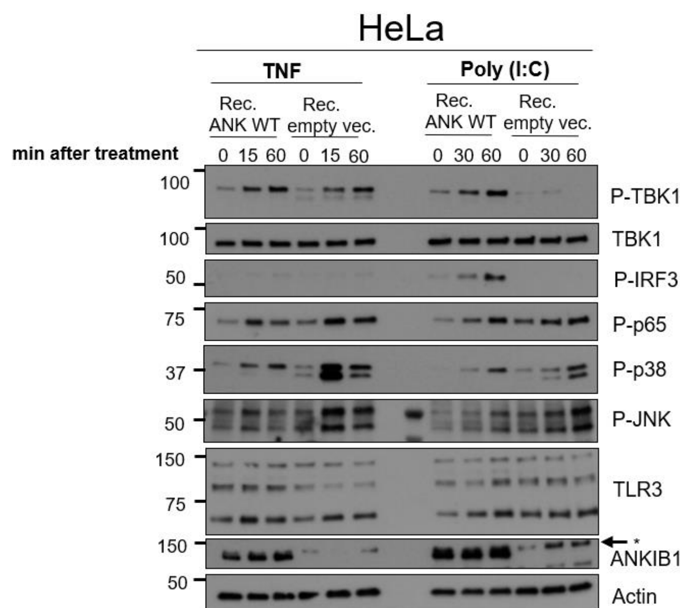
3.3 Decipher the interacting partners of ANKIB1 in TLR3 signalling

Next, we restored ANKIB1 expression in the different knock-out cell lines that we generated. This reexpression allows us to not only assess the ability to rescue TBK1 activation but also to pull-down our protein of interest in order to identify interaction partners following treatment. To do so, we added a moTAP-tag on the C-terminus of ANKIB1 (1x Flag- and 2x Strep-tag) with a flexible linker motif in between. The addition of a flexible linker is important for both, the prevention of any conformational changes of the protein but also to prevent ANKIB1, a relatively large protein (122kDa) to hide the moTAP-tag and therefore be able to efficiently immunoprecipitate it. The use of the pBABE plasmid allows us to reconstitute the expression of our protein of interest in a similar amount to the endogenous levels.

Overexpression of the protein could modify its natural distribution in cells and therefore modify its activity. We then reconstituted ANKIB1-moTAP by retroviral transduction in ANKIB1 knock-out HT-29 and HeLa cells.

3.3.1 The reexpression of ANKIB1 rescues TBK1 and IRF3 phosphorylation

We then compared ANKIB1 wild-type reconstituted HeLa cells to the empty vector transduced HeLa cells (Knock-Out for ANKIB1) in terms of activation of the different gene-activatory pathways following TNF and poly(I:C) treatment (**Figure 3.9**). Concerning the TNF pathway, proteins involved in NF-κB (p65) and non-canonical IKKs (TBK1) were similarly activated in both wild-type and ANKIB1 knock-out cells. However, proteins involved in the MAPK pathway, such as JNK and p38, were upregulated in cells depleted for the E3 ligase. Unfortunately, it is an inconsistent observation that was not always as clear as in Figure 3.9, and we therefore think that it is not relevant. Overall, this confirms that ANKIB1 does not play a role in TNF signalling pathway.



<u>Figure 3.9</u>. TBK1 phosphorylation is rescued after ANKIB1 reexpression in TLR3 signalling:

HeLa ANKIB1 knock-out cells stably reexpressed with moTAP-tagged ANKIB1 wild-type or with moTAP empty vector. Cells were stimulated with respectively 100ng/mL TNF for 15 and 60 minutes or 10 µg/mL poly(I:C) for 30 and 60 minutes. Cells were lysed and proteins were analysed by westernblot. blotting.

However, the reexpression of ANKIB1 rescues the activation of not only TBK1 but also of the downstream transcription factor IRF3 following TLR3 activation. Consequently, we can confirm that, in TLR3 signalling, ANKIB1 is crucial, not only for TBK1 activation but more importantly for IRF3 activation, an essential transcription factor to induce type I and III interferon production following the sensing of pathogens by TLR3.

3.3.2 Optimisation of ANKIB1 pull-down and interacting partners.

Next, we aimed to understand whether ANKIB1 potentially interacts in close proximity with TBK1 and other components of the TLR3-SC or if it is involved rather indirectly in TBK1 activation. To address this question, we used the Flag-tag on ANKIB1 in the reexpressed cell lines which allowed us to pull down ANKIB1 and all its interaction partners following poly(I:C) treatment.

However, before applying this new tool, we first had to optimise the sample preparation of the ANKIB1 pull-down. Indeed, different lysis buffers can be used for immunoprecipitation. The concentration of salt can differ but also, and more importantly, the type of detergent used. Additionally, sonication steps and the addition of SDS have also been reported to improve the disruption of membrane compartments such as endosomes, where TLR3 is localised.

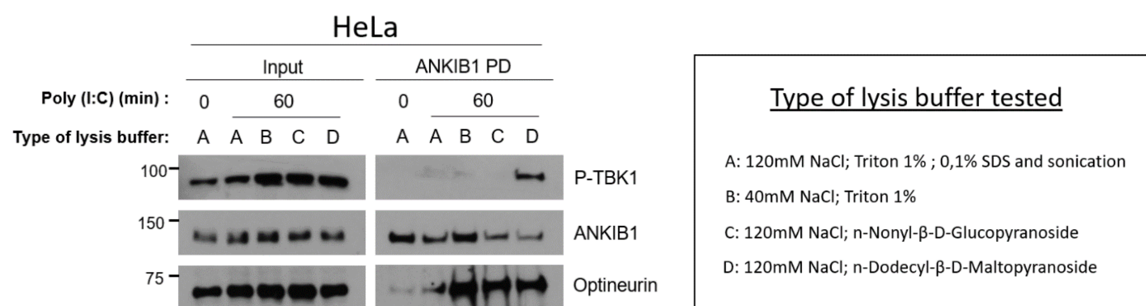


Figure 3.10. n-Dodecyl-β-D-Maltopyranoside allows an efficient ANKIB1 isolation without disrupting protein-protein interactions:
HeLa ANKIB1 knock-out cells stably expressing moTAP ANKIB1 were stimulated with 10µg/mL poly(I:C) and ANKIB1 was immunoprecipitated via Flag-tag following lysis. Different sample preparations were tested and are detailed in the table. Coimmunoprecipitated proteins were analysed by western blotting. ANKIB1 PD: ANKIB1 Pull-down

All these different sample preparations were tested in HeLa moTAP ANKIB1 cells **(Figure3.10)**. As expected, Triton 1% seemed the most efficient to isolate ANKIB1 as compared to the other detergents n-Nonyl-β-D-Glucopyranoside (NG) and **n-Dodecyl-β-D-Maltopyranoside** (DDM), which are also non-ionic detergents but less harsh. However, despite less pull-down of ANKIB1, DDM shows a remarkable ability to preserve the interaction between ANKIB1 and activated TBK1 in comparison to other lysis buffers containing Triton or NG which seem to disrupt this interaction. Optineurin also interacts with ANKIB1 following poly(I:C) treatment. This interaction is equally preserved between NG, DDM or Triton (40mM NaCl) however it seems that the addition of SDS and sonication seems too harsh to preserve the interacting partners of ANKIB1. Overall, DDM seems the best detergent to study the interaction partners of ANKIB1 and will be used for the different ANKIB1 pull-downs. Additionally, ANKIB1 seems to interact in close proximity with TBK1 and Optineurin, an important adaptor in TLR3 pathway.

3.3.3 ANKIB1 interacts with several components of the TLR3-SC, notably TBK1, NEMO and Optineurin

Once the sample preparation has been optimised, we addressed the interacting partners following poly(I:C) treatment. Empty vector reexpressed cell lines were used in parallel as a negative control for the specificity of the pull-down. As displayed in Figure 3.10, in both HeLa and HT-29 reexpressed cell lines, ANKIB1 interacts with phosphorylated TBK1 following poly(I:C) treatment.

In line with our previous observation that ANKIB1 is recruited to TLR3-SC, it interacts also with the main components of the signalling complex such as TRIF and A20 (Figure 3.11, a). This interaction is treatment dependent and does not occur basally. Total TBK1 seems also to interact with ANKIB1. In addition, NEMO, an essential protein for TBK1 activation in different signalling pathways such as TNF and TLR3 also interacts with ANKIB1 upon TLR3 activation (Figure 3.11, b).

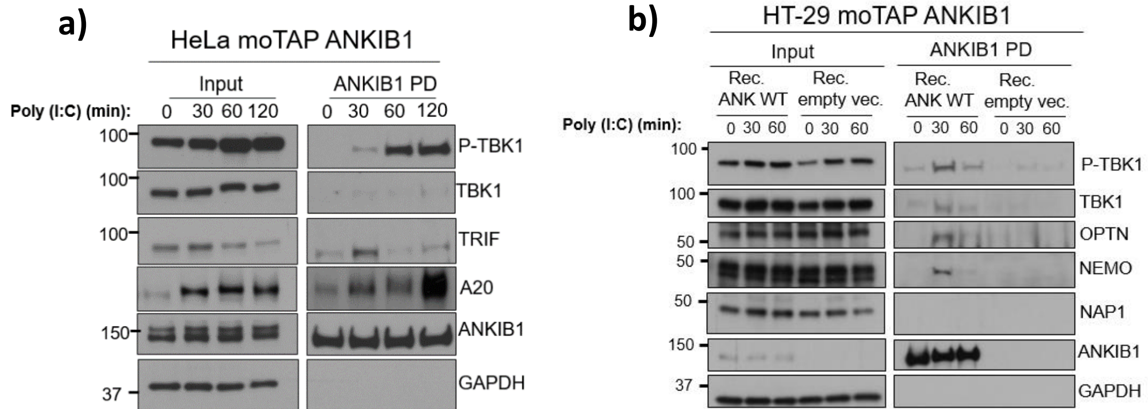


Figure 3.11. ANKIB1 is a new member of the TLR3-SC and interacts with components of the interferon-inducing pathway:
a) HeLa ANKIB1 -/- cells stably expressing moTAP ANKIB1 or b) HT-29 ANKIB1-/- stably expressing moTAP ANKIB1 or moTAP empty vector were stimulated with 10μg/mL poly(I:C) for 30, 60 and 120 minutes or 30 and 60 minutes respectively. ANKIB1 was immunoprecipitated via Flag-tag and coimmunoprecipitated proteins were analysed by western blotting.

Interestingly, as seen previously, it interacts with Optineurin, an adaptor protein for TBK1 in TLR3-SC, and seems to be an interacting partner following treatment. Oppositely, ANKIB1 does not seem to interact with NAP1, another described adaptor protein for TBK1, which could suggest that the pull of TBK1 interacting with Optineurin and NAP1 are different. Consequently, we elicited that ANKIB1 interacts with TBK1, OPTN and NEMO at the signalling complex. We could extrapolate that ANKIB1 is required for these proteins to promote TBK1 phosphorylation in TLR3-SC.

3.4 Molecular and functional characterisation of the role of ANKIB1 in TLR3 signalling.

Next, we wanted to understand the mechanism by which ANKIB1 is recruited to the TLR3-SC and how it interacts with TBK1, TRIF, NEMO and Optineurin. ANKIB1 is a protein that contains several domains that are potentially involved in its role as a regulator of TLR3 signalling.

Ubiquitin E3s are known to have a catalytic activity but they frequently also harbour scaffold functions. Structural analysis has revealed that ANKIB1 contains domains which would suggest both activities. We, therefore, aimed to address which domains and consequently which activity of ANKIB1 is required for TBK1 phosphorylation.

3.4.1 RBR domain of ANKIB1 enables the production of ubiquitin chains

ANKIB1 is part of the RBR-containing E3 ligase family. However, so far it has not been shown that the RBR domain is able to generate polyubiquitin chains. Thus, we performed an in-vitro ubiquitin assay. We used UBA1, the classical E1, different type of E2 and ANKIB1 as potential E3. ANKIB1 was purified from the reexpressed cells by using the Flag-tag. Bacterial production of purified ANKIB1 has been tried yet it turned out to be challenging to obtain higher yields, probably due to the large size of ANKIB1. Moreover, the main advantage of purifying ANKIB1 from eukaryotic cells and not in bacterial cells is the abundance of post-translational modifications such as ubiquitination or disulphide bond formation. These modifications are essential for proper protein conformation and for conserving its activity. Post-translational modifications can also occur in bacteria but to a less extent. ATP and MgCl2 are added in an in vitro ubiquitin assay for their role in the process of activation of the ubiquitin molecules. Because we did not know which E2 preferentially cooperates with ANKIB1, we tested three different E2s. UBE2L3 (the most reported E2 for RBR-containing E3 ligases), UBE2D3 (acts with cIAP1/2, TRAF6, the classical E2 for the RING-containing E3 ligases but is also able to cooperate with RBR-E3s) and UBE2L6 (responsible for the ISGylation, after type I interferon production and also reported to cooperate with RBR-containing E3 ligases). After 1h incubation at 37°C, we analysed the samples by western blotting. By blotting for total ubiquitin, we observed a smear blot, translating the ability of ANKIB1 to generate polyubiquitin chains when an E2 is added to the reaction (Figure 3.12, a).

The signal is stronger with UBE2D3 than with UBE2L3, translating a potential preference for UBE2D3 in vitro. However, in combination with UBE2L6, ANKIB1 poorly induces ubiquitin chain formation compared to ANKIB1 alone.

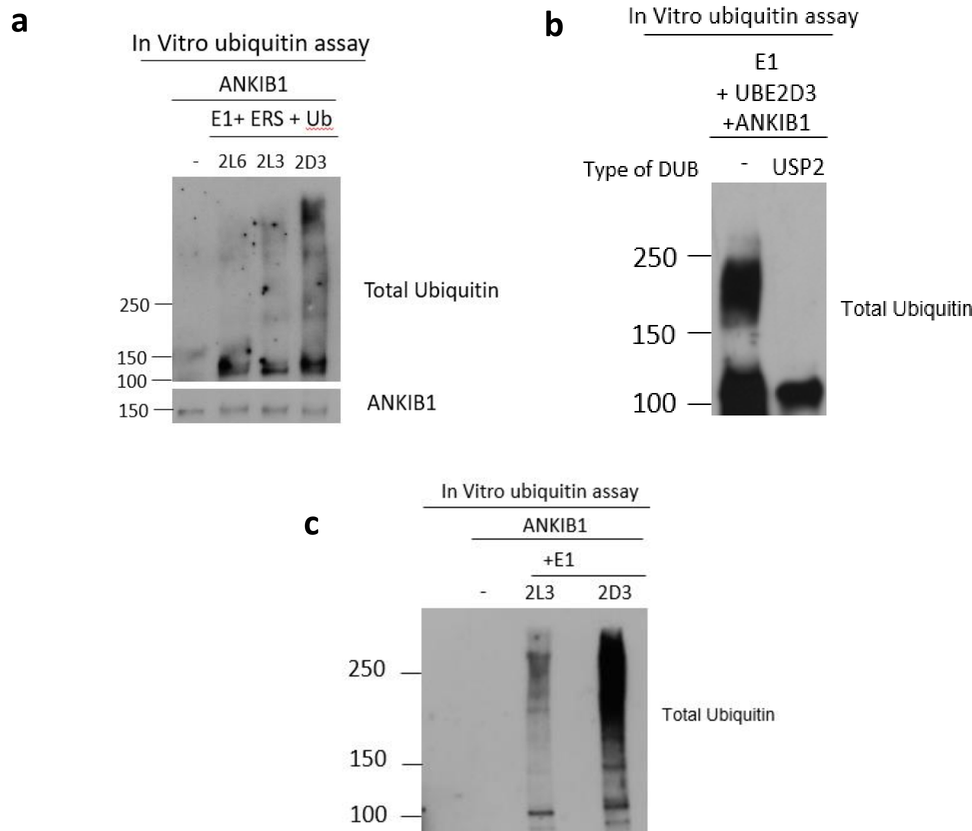
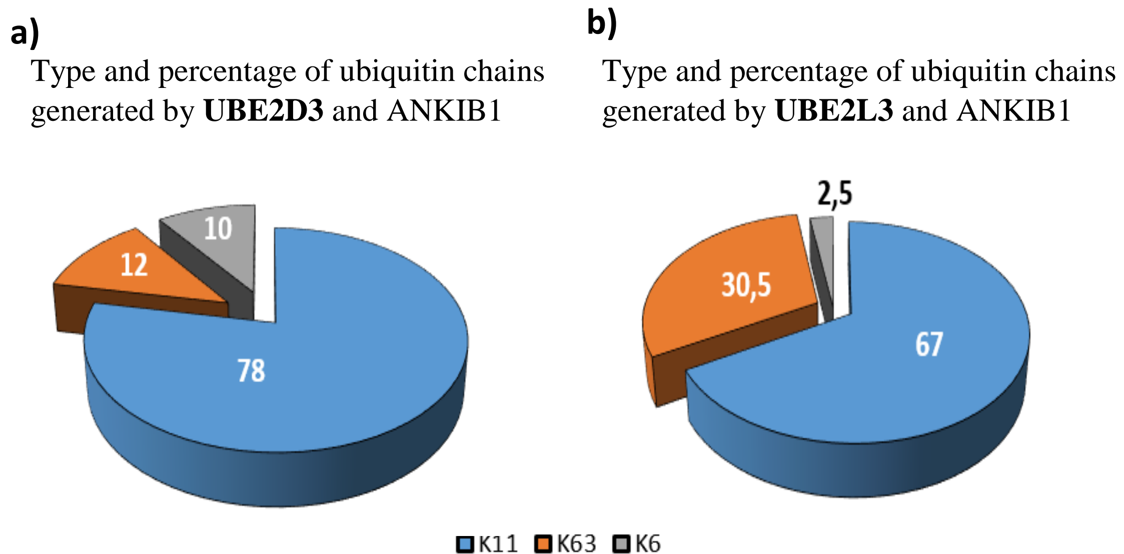


Figure 3.12. ANKIB1 has a functional RBR domain and generates polyubiquitin chains:
In vitro ubiquitin assay performed with purified moTAP ANKIB1 in combination with different E2s, incubated for 1h at 37 °C. Samples were analysed by western blotting. b) USP2 was added in the reaction. c) after 1h at 37 °C, moTAP ANKIB1 was isolated with M2 beads and samples were prepared for western blotting for analysis.

The addition of USP2, a deubiquitinase that cleaves every type of polyubiquitin chain, leads to a strong degradation of the ubiquitin smear generated by ANKIB1 in combination with UBE2D3, confirming that ANKIB1 generates polyubiquitin chains (Figure 3.12, b). Next, we wanted to understand if ANKIB1 was generating these ubiquitin chains by adding ubiquitin linkages on itself or if it is generating free polyubiquitin chains. To do so, we used the Flag-tag on ANKIB1 to purify it after the in vitro ubiquitin assay (Figure 3.12, c). With both UBE2L3 and UBE2D3, ubiquitin chains were visible suggesting that in this reaction, ANKIB1 auto-ubiquitinates itself. To summarise, we showed that, despite a missing cysteine in the IBR domain, ANKIB1 has an efficient RBR domain that is able to generate polyubiquitin chains in cooperation with UBE2D3 and to a less extent with UBE2L3.

3.4.2 ANKIB1 preferentially generates K11 polyubiquitin chains

After we obtained evidence that the RBR domain of ANKIB1 is functional, we aimed to identify the type of ubiquitin chain generated either with UBE2L3 or with UBE2D3. To do so, we performed an in vitro ubiquitin assay experiment similar to Figure 3.11. However, the samples were not analysed by western blotting but by mass spectrometry.



<u>Figure 3.13</u>. ANKIB1 preferentially generates K11 polyubiquitin chains: *Samples were generated by in vitro ubiquitin assay (as described in figure 3.11, a) with purified ANKIB1 and either a) with UBE2D3 or b) with UBE2L3. In-gel digestion was performed on the samples and successively analysed by Mass spectrometry.*

Indeed, depending on the lysine used to generate the polyubiquitin chains, the peptides obtained after trypsin digestion will have a specific amino acid sequence that is identified precisely by mass spectrometry. After analysis, we could identify K11 ubiquitin chains as the most abundant chains generated by ANKIB1 with both E2s (Figure 3.13). K63 and K6 were also found but to a less extent.

Interestingly, in combination with UBE2L3, the amount of K63 chains generated were higher than with UBE2D3. It could suggest that ANKIB1 might have a different role depending on the E2 that provides the activated ubiquitin molecules.

3.4.3 ANKIB1 requires the UIM domain, but not the RING2 domain, to promote TBK1 activation

Once we have proven that ANKIB1 is able to generate polyubiquitin chains, we wanted to address the relevance of its catalytic activity but also its scaffold property for the activation of TBK1 in TLR3 signalling pathway. Thus, we generated different mutants of ANKIB1 in order to reexpress the mutated versions of ANKIB1 in the previously knock-out cell lines, similarly done for the WT version (Figure 3.15). The first mutant, ANKIB1ΔRING2, lacks the entire RING2 domain, the catalytic subunit of the RBR domain. According to what has been published about RBR E3s, we expect that this construct disrupts its E3 ligase activity, allowing us to assess the requirement of the ubiquitin chains generated by ANKIB1. The second mutant generated was ANKIB1ΔUIM. Ubiquitin-interacting motifs have been shown to be necessary for the recruitment of different proteins to signalling complexes such as the UBAN domain of NEMO in TNFR1-SC (Laplantine et al., 2009; Rahighi et al., 2009). Therefore, we expect that this mutant version of ANKIB1 would not be able to interact with ubiquitinated proteins.

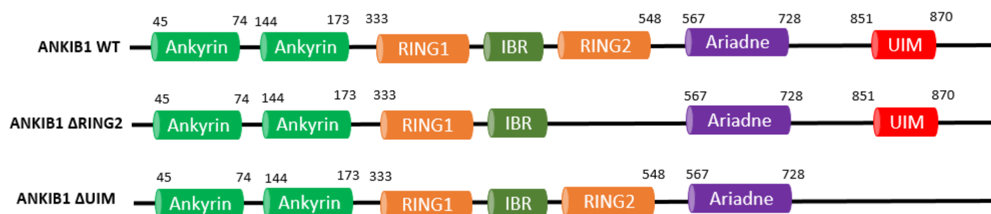


Figure 3.15. The different versions of ANKIB1 generated:
ANKIB1 was reexpressed in HeLa and HT-29 knock-out cells with the following mutations. These constructs were cloned into a pBABE linker-moTAP.

After reexpression of the different versions of ANKIB1, we compared them to HeLa ANKIB1 knock-out (empty vector) in terms of activation of the different gene-activatory pathways after treatment with poly(I:C) **(Figure 3.16)**. IκBα degradation, read-out for the activation of NF-κB pathway, is similar between the different cells confirming that ANKIB1 does not seem to be involved in this pathway. Phosphorylation of p38, members of the MAPK pathway, is however inconsistent between experiments and despite the

differences, cannot be interpreted in a constructive manner. This variability between cells and experiments can be explained by their role in sensing cell homeostasis and cell cycle regulation, an important process that can differ between experiments. More importantly, as expected and shown before, empty vector reexpressed cells behave like HeLa ANKIB1 knock-out cells in terms of TBK1 and IRF3 activation. Interestingly, HeLa ANKIB1ΔUIM cells were not able to phosphorylate TBK1 and consequently IRF3 after TLR3 activation. This result suggests that the UIM domain of ANKIB1 seems essential for its activity in TLR3 pathway. However, and very surprisingly for us, HeLa ANKIB1ΔRING2 were able to activate TBK1 to the same extent as HeLa cells reexpressed with the wild-type version of ANKIB1. To conclude, this result suggests that ANKIB1 needs to interact with a ubiquitinated protein in order to promote TBK1 activation in TLR3 signalling pathway; however, it does not seem to be dependent on its RBR-E3 ligase activity.

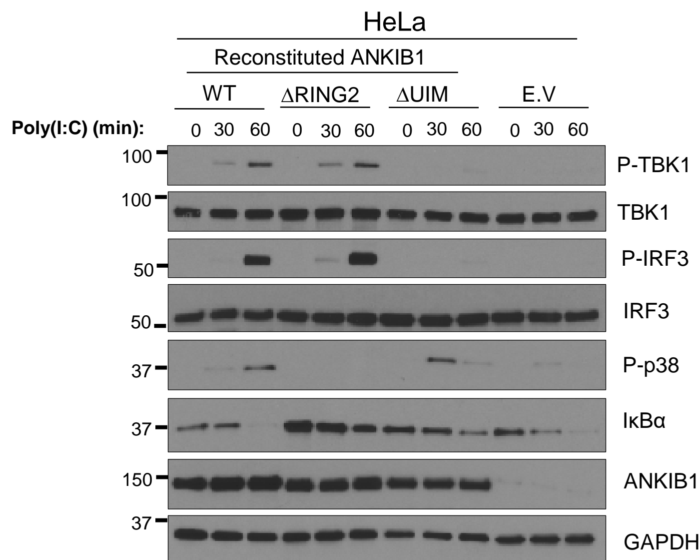
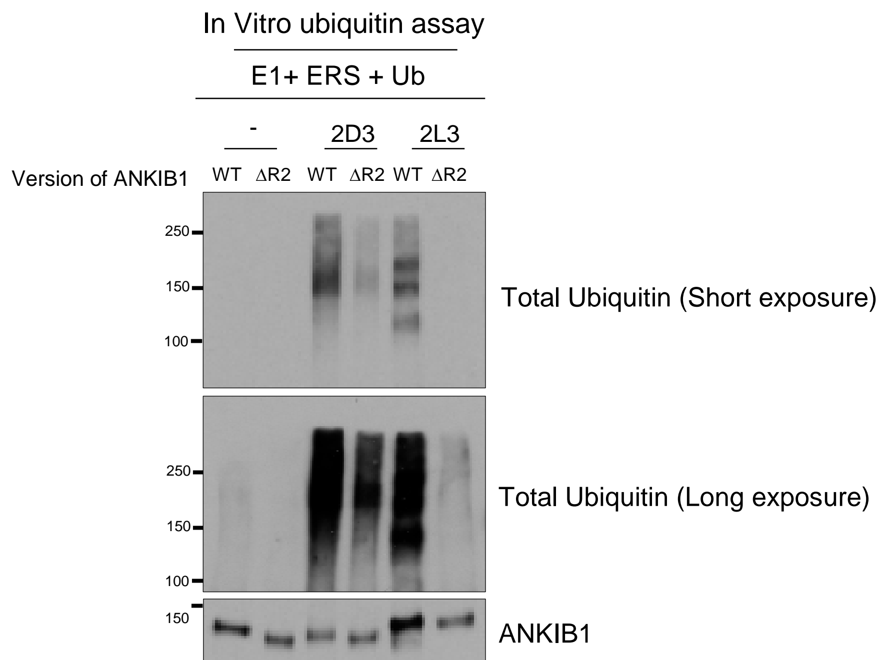


Figure 3.16. UIM domain, but not RING2 domain, is essential for ANKIB1 activity:
HeLa ANKIB1 knock-out stably reexpressed with moTAP ANKIB1 wild-type, ΔRING2, ΔUIM or with moTAP empty vector. Cells were stimulated with 10μg/mL poly(I:C) for 30 and 60 minutes. Cells were lysed and proteins were analysed by western blotting.

3.4.4 Deletion of RING2 domain does not totally abolish ANKIB1 catalytic activity

In order to validate that ANKIB1ΔRING2 promotes TBK1 phosphorylation despite the disruption of its catalytic activity, we wanted to confirm that this version of ANKIB1 is not able to generate polyubiquitin chains. Thus, we compared purified ANKIB1 WT and ANKIB1ΔRING2 in an in-vitro ubiquitin assay in cooperation with both UBE2D3 and UBE2L3, previously shown to act with ANKIB1. As expected, the analysis by western blotting showed that ANKIB1ΔRING2 could not generate ubiquitin chains in association with UBE2L3, the classical E2 for the RBR-containing E3 ligases **(Figure 3.17)**. However, and very surprisingly, when ANKIB1ΔRING2 is associated with UBE2D3, ANKIB1 is still able to generate polyubiquitin chains. This result could suggest that despite being characterised as an RBR containing E3 ligase, ANKIB1 is able to make ubiquitin chains via its RING1, similar to the RING E3 ligases such as cIAP1/2.

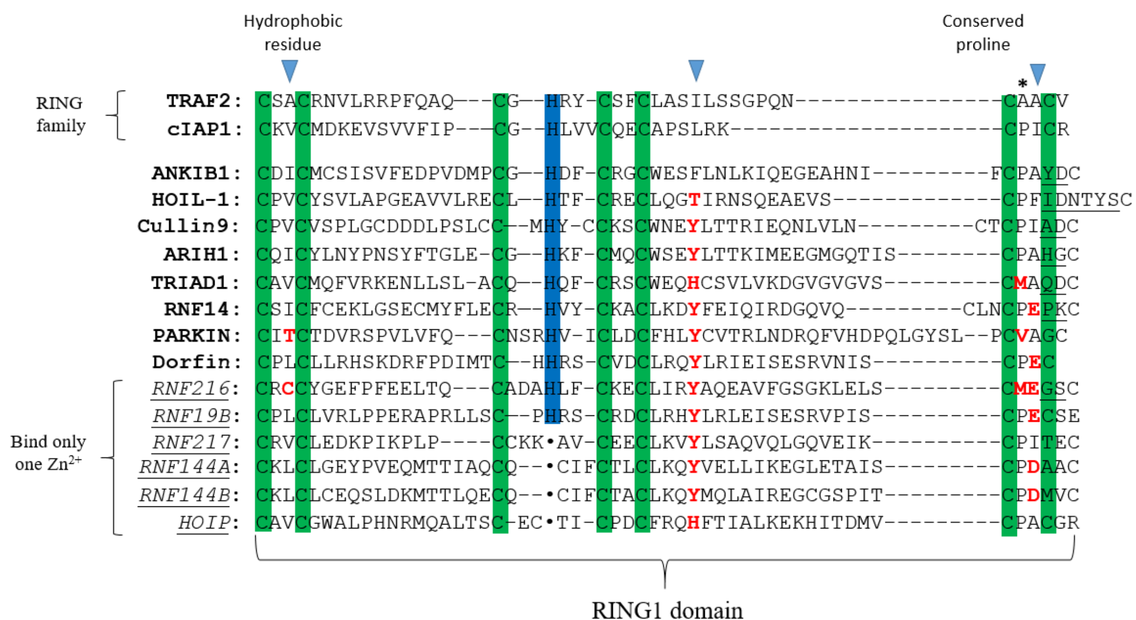


<u>Figure 3.17.</u> RING2 is required to generate ubiquitin chain with UBE2L3 but not with UBE2D3:
In vitro ubiquitin assay with isolated moTAP ANKIB1 wild-type (WT) or ΔRING2 (ΔR2) in combination with UBE2L3 (2L3) or UBE2D3 (2D3). 60 minutes after incubation at 37°C, samples were analysed by western blotting. ERS: Energy Regeneration Solution. Ub: ubiquitin molecule

3.4.5 RING1 domain of ANKIB1 is very similar to the classical RING domain

It has been shown in several RBR-containing E3 ligases that the mutation of the catalytic cysteine in the RING2 totally impairs the ligase activity. Indeed, in a general manner, RING1 of the RBR-containing E3 ligases is not sufficient to perform a ligase activity by itself due to several missing cysteines or other important hydrophobic amino acids that are required to confer a classical RING activity. However, because we found out that ANKIB1ΔRING2 is still able to generate polyubiquitin chains with UBE2D3, a classical E2 involved in ubiquitination with RING E3 family, we decided to compare the RING1 of ANKIB1 and the RING1 of the other RBR-containing E3 ligases to a classical RING domain from cIAP1 and TRAF2 **(Figure3.18)**. First of all, six RBR E3s can only bind one Zn^{2+}. The binding of two Zn^{2+} is crucial to be able to act as a classical RING domain.

Classical RING motif: Cys-X2-Cys-X_{9-39}-Cys-X_{1-3}-His-X_{2-3}-Cys-X2-Cys-X_{4-48}-Cys-X2-Cys



<u>Figure 3.18.</u> RING1 comparison of the RBR-E3s and classical RING-E3s:
Sequence alignment of RING1 domains of the different RBR-containing E3 ligases and two classical RING domains of RING E3 ligases. Conserved cysteines are underlined in green and conserved histidine is underlined in blue. Conserved hydrophobic amino acids positions are marked with a blue triangle and conserved proline is marked with an asterisk. Substituted hydrophobic amino acids are marked in bold red. Additional amino acids from the original motif are marked as underlined black. Missing conserved histidine are marked with a black dot.

The binding of these Zn^{2+} ions are mediated by the different cysteines and histidine present in the RING motif (the seven essential cysteines are underlined in green and in blue for the histidine). Additionally, RING domain requires 3 hydrophobic residues (marked with a blue triangle) and proline that allow the binding of the E2, its conformational changes and consequently the transfer of the ubiquitin molecule to the substrate. The first hydrophobic residue is before the second cysteine of the motif. Parkin and RNF216 do not contain it. The second one is found 4 amino acids after the fifth cysteine. None of the RBR-E3s contain this latter except ANKIB1. Finally, the proline has to be just after the sixth cysteine and the last hydrophobic amino acid must be the following amino acid after the proline. Overall, despite two additional amino acids between the two last cysteines, ANKIB1 is the only RBR-E3s with a highly conserved RING1 domain, containing all the required amino acids in order to be similarly functional to a classical RING domain.

It could indicate that ANKIB1 can generate polyubiquitin chains independently of its RING2 and could explain why ANKIB1ΔRING2 is still able to contribute to TBK1 activation in TLR3 signalling pathway. Therefore, we hypothesised that ANKIB1 might be an E3 ligase between the RING family and the RBR family that has the ability to use both RING to generate polyubiquitin chains.

3.4.6 RING1 domain of ANKIB1 contributes to the phosphorylation of TBK1 independently of the RING2

In order to test the hypothesis of a ubiquitin ligase activity of ANKIB1 independently of its RING2, we have reexpressed HeLa and HT-29 ANKIB1 knock-out cells with a new construct called ANKIB1ΔRBR. It lacks the entire RBR domain, containing the RING1, the IBR and RING2 domains. Once the reexpression was successfully completed, we compared TBK1 activation in HeLa reconstituted with wild-type ANKIB1 and ANKIB1ΔRBR after poly(I:C) treatment.

Reexpression of the wild-type version of ANKIB1 rescues phosphorylation of TBK1 upon TLR3 activation. However, the ANKIB1ΔRBR reexpressed HeLa cells are not able to do so **(Figure 3.19)**.

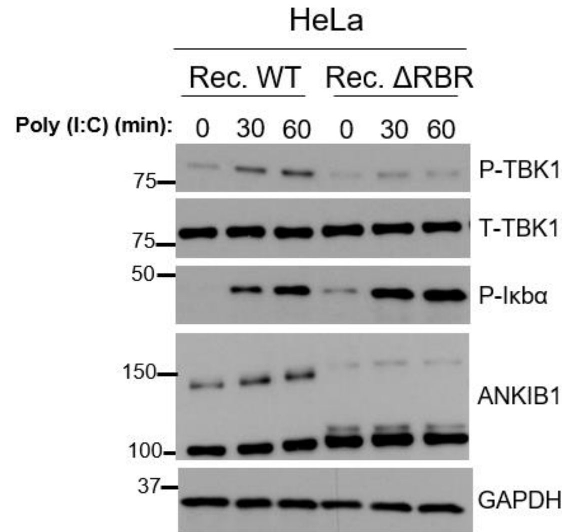


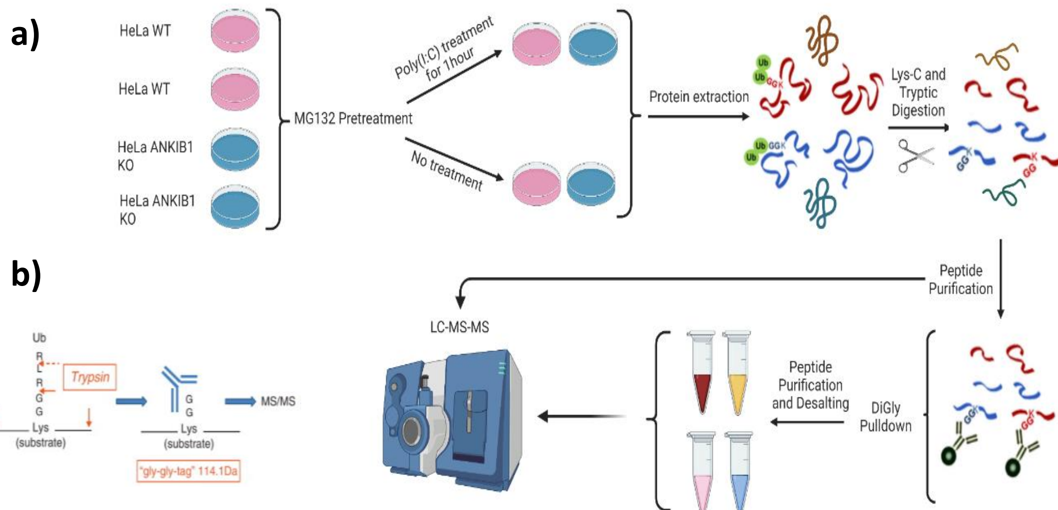
Figure 3.19. **ANKIB1 requires its catalytic activity to promote TBK1 phosphorylation:** *HeLa ANKIB1 knock-out stably reexpressed with moTAP ANKIB1 wild-type or ΔRBR. Cells were stimulated with 10µg/mL poly(I:C) for 30 and 60 minutes. Cells were lysed and proteins were analysed by western blotting.*

On the contrary, such as in knock-out cells, ANKIB1ΔRBR cells similarly induce the phosphorylation of Iκbα, read out of the activation of NF-κB. Together, this experiment confirms our hypothesis that ANKIB1 does not necessarily require the RING2 domain for its activity in TLR3 pathway regarding TBK1 phosphorylation and consequently IRF3 phosphorylation. Additionally, we can conclude that the ubiquitin chains generated by ANKIB1, and therefore its catalytic activity, is required for TBK1 activation in TLR3 signalling pathway.

3.4.7 Deciphering the substrate of ANKIB1 in TLR3 pathway

After we have demonstrated that the catalytic activity of ANKIB1 is crucial for TBK1 activation after TLR3 stimulation, we wanted to find out the substrate(s) of ANKIB1 following poly(I:C) treatment and understand more precisely its implication in this process. We, therefore, performed a di-gly in HeLa wild-type cells and Hela cells knock-out for ANKIB1. This approach allows us to enrich the ubiquitinated protein and analyse them by Mass spectrometry. Cells were pre-treated with MG-132, a proteasomal inhibitor in order to prevent the potential proteasomal degradation of ubiquitinated protein.

Indeed, K11 chains have been reported to induce both, proteasomal degradation and scaffold property. The workflow, as described in Figure 3.20, has been performed in order to generate and enrich the K-GG motif.



<u>Figure 3.20.</u> Workflow of the di-gly enrichment:
a) Work-flow performed in order to identify potential substrate for ANKIB1. HeLa wild-type cells were compared to HeLa ANKIB1 knock-out cells without treatment or treated with 10µg/mL poly(I:C). Lysates were digested with trypsin allowing the formation of the di-gly motifs. These latter were then pulled down by anti-di-gly pull-down. Samples were then analysed by Mass Spectrometry. b) detailed explanation of the generation of the di-gly motif after trypsin digestion of the ubiquitin chains.

The di-gly motif is generated after trypsin digestion of ubiquitinated proteins. Trypsin cleaves between the arginine and the glycine at the C-terminus of a ubiquitin molecule. The remaining GG from the molecule stays bound to the lysine targeted for the binding of the ubiquitin molecule to the substrate protein.

Therefore, di-gly enrichment method allows us to not only identify proteins which are potentially ubiquitinated by ANKIB1 but also the precise ubiquitination site of those potential targets. In parallel, we also performed a whole genome proteomic analysis in order to evaluate quantitatively the overall protein expression. It is used as a control to verify that potential ubiquitin differences found between wild-type and knock-out cells are not due to a difference in the total protein amount. The next step was the analysis of the samples by LC-MS/MS. From the analysis of our samples, we obtained a list of nearly 8,000 different peptides.

Due to the vast number of peptides, we decided to first separate the hits by categorising the peptides in terms of the gene ontology biology process (GOBP) **(Figure 3.21)**.

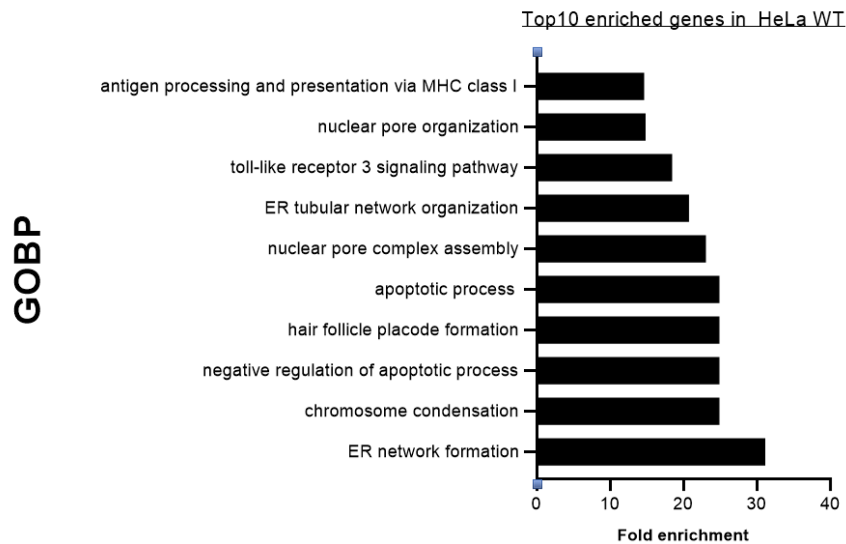
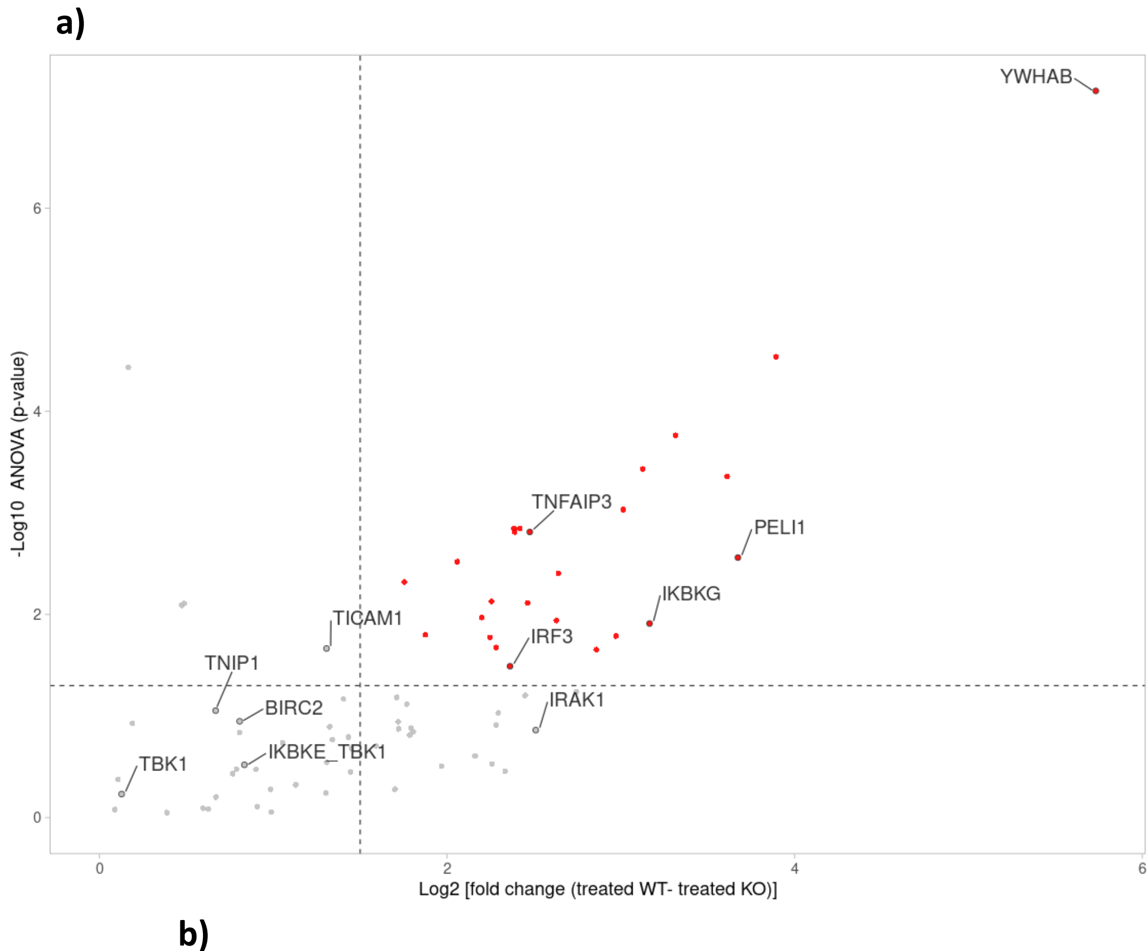


Figure 3.21. ANKIB1 deletion decreases the ubiquitination of proteins implicated in TLR3 signalling and cell death:
GOBP analysis of the di-gly enriched peptides detected by Mass spectrometry and absent in ANKIB1 knock-out cells following TLR3 activation.

We identified different subtypes of genes particularly upregulated in wild-type cells in comparison to ANKIB1 knock-out cells after TLR3 activation. Proteins implicated in the Endoplasmic reticulum network seem impacted by the absence of ANKIB1. Closer to immune signalling, proteins involved in TLR3 pathway are also strongly enriched compared to knock-out cell lines. Interestingly, it seems that ANKIB1 deletion also impacts the ubiquitination of proteins implicated in apoptosis or its regulation.

In order to analyse in depth and potentially find the substrate of ANKIB1, we decided to focus on the proteins classified as being implicated in TLR3 signalling pathway. To gain specificity, we kept only the peptides that had increased ubiquitination following poly(I:C) treatment and we analysed the intensity of the signal measured by Mass Spectrometry by volcano plot **(Figure 3.22.a)**. In parallel, the amount of ubiquitin peptides detected and the ubiquitination site have been summarised in Figure 3.22.b.



Number of di-gly enriched peptides detected (/4)					Ub site
	WT		ANKIB1 $^{-/-}$		
	UT	1h Poly(I:C)	UT	1h Poly(I:C)	
TBK1/IKBKE	0	3	0	0	137
IKBKG	2	4	0	0	344
IRF3	0	4	0	0	193
TICAM1	0	4	0	3	65
TNFAIP3	0	3	0	0	81
TNIP1	0	4	0	0	452
Peli1	0	4	0	1	172
YWHAB	0	4	0	0	13

Figure 3.22. di-gly enrichment has revealed several potential substrates for ANKIB1: *a) Volcano plot of the di-gly enriched peptide found after poly(I:C) treatment. b) Table recapitulating the main hits concerning proteins known to be involved in TLR3 signalling pathway. The number of peptides found between the four replicates and the ubiquitination site are summarised.*

TRIF was the most upstream component of TLR3 signalling, which was found in this analysis, however it does not seem to be affected by ANKIB1 absence as it was ubiquitinated to a similar extent in both WT and KO cells. This suggests that TRIF is probably not a substrate of ANKIB1. Interestingly, several proteins known to be implicated in TBK1 activation show differences in terms of ubiquitination profiles, notably NEMO and A20 which both have a p-value lower than 0,05. We also found that IRF3 was strongly ubiquitinated only in poly(I:C) treated wild-type cells. However, we think that this is only an indirect consequence of the absence of ANKIB1 due to our previous observation that the absence of ANKIB1 impacts more upstream proteins such as TBK1. Interestingly, TBK1 was also found to be ubiquitinated on the lysine 137, already described site, but not in absence of ANKIB1. However, this hit was not significant due to a low overall intensity. The di-gly enrichment analysis validated our previous results; however, it failed in answering the question whether TBK1 serves as a direct or rather indirect substrate. To address this question, we need to perform further experiments. Additionally, some hits were unexpected, notably Peli1 and YWHAB, both proteins already described to be implicated in the regulation of Toll-Like Receptor signalling pathways or cytosolic RNA sensing yet so far have not been linked to interferon production in TLR3 pathway. However, regarding their position in the volcano plot, they might be interesting candidates to investigate. To conclude, the di-gly enrichment analysis has proposed a few ANKIB1 substrate candidates. The next step will be to assess which of those proteins identified are actual substrates and which are not.

3.5 Relevance of ANKIB1 for the production of interferons by TRIF-mediated TLRs signalling

After characterisation of the role of ANKIB1 in TBK1 and IRF3 phosphorylation upon TLR3 activation, we aimed to find out if ANKIB1 plays a similar role in TLR4 signalling. TLR4 recognises gram-negative bacteria and then induces the formation of two signalling complexes very similar to the signalling complex mediated by TLR3. Indeed, TLR4 is the only Toll-Like Receptor that can involve both, Myd88 and TRIF.

3.5.1 ANKIB1 regulates also IRF3 phosphorylation in TLR4 pathway

To investigate the role of ANKIB1 in TLR4, we treated HT-29 wild-type cells and ANKIB1 knock-out with lipopolysaccharide (LPS) and studied the activation of the proteins involved in interferon production such as TBK1 and IRF3. Interestingly, in both wild-type and knock-out cell lines, TBK1 phosphorylation is similar upon LPS treatment (**Figure 3.23**). This confirms what has already been published regarding the dual signalling of TLR4 via both, Myd88 and TRIF. Indeed, both proteins are able to induce TBK1 phosphorylation. However, only TRIF signalling can recruit and activate IRF3. That is why, despite a similar overall activation of TBK1 following LPS treatment, IRF3 phosphorylation is strongly impaired in ANKIB1 knock-out cell line. On the contrary, p65 phosphorylation is not impacted by the deletion of ANKIB1.

This experiment proves that ANKIB1 does not only play a role in TLR3 signalling but rather contributes to TBK1 activation in other TRIF-dependent signalling pathways and more importantly contributes to IRF3 phosphorylation. Interestingly, this result shows that ANKIB1 is not involved in Myd88 dependant signalling, suggesting that both Myd88 and TRIF-induced complexes have some discrepancies, which could help in understanding the mechanism of action of ANKIB1.

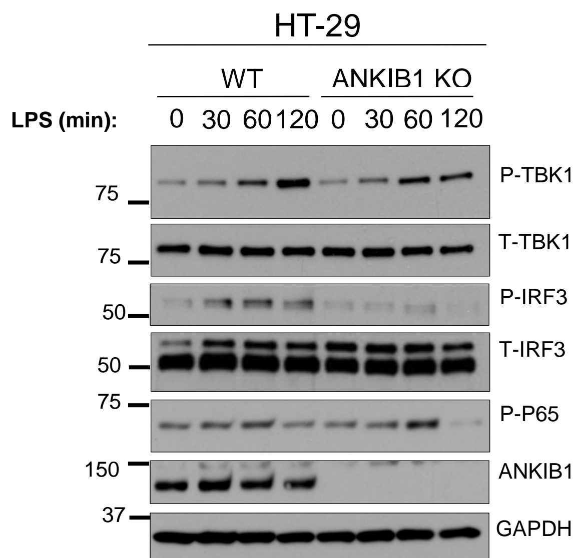
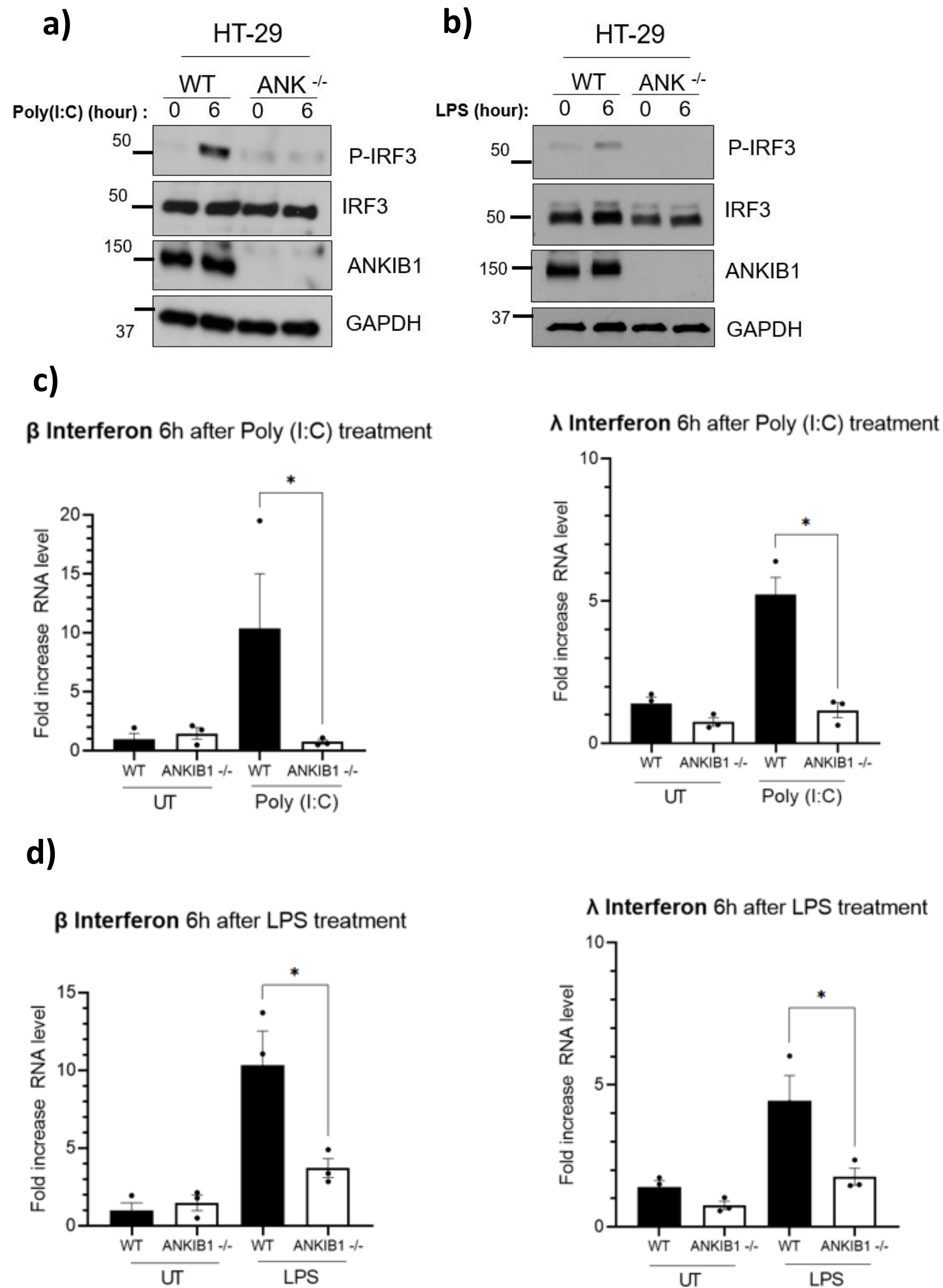


Figure 3.23. ANKIB1 deletion impairs IRF3 phosphorylation in TLR4 pathway:
HT-29 wild-type or ANKIB1 knock-out cells were stimulated with 1µg/mL of LPS for 30, 60 and 120 minutes. Cells were lysed and proteins were analysed by western blotting.

3.5.2 ANKIB1 deletion strongly impairs interferon production in TLR3/4 pathway

Once we have demonstrated that ANKIB1 is involved in TLR3 and TLR4-induced IRF3 activation, we wanted to study its implication in interferon production. To do so, we first wanted to assess the phosphorylation of IRF3 in a long-time point treatment to be sure that the activation is not only delayed in knock-out cell lines but really impaired. Thus, we treated the cells for 6h either with poly(I:C) or LPS and immunoblotted for phosphorylated IRF3 **(Figure 3.24, a and b)**. Despite the long-time point, IRF3 phosphorylation is strongly impaired in the ANKIB1 knock-out cell line as compared to the respective WT cell line in both, TLR3 and TLR4 signalling. This confirms that ANKIB1 deletion not only delays IRF3 activation but strongly disrupts it.

Next, we aimed to address whether, as a consequence of the impaired IRF3 phosphorylation, the production of type I and type III interferon is also impacted by the absence of ANKIB1 in both signalling pathways. We, therefore, performed RNA isolation followed by qPCR analysis of the gene induction of type I interferon (α and β) and type III interferon (λ) after poly(I:C) (Figure 3.24, c) or LPS treatment (Figure 3.24, d). After TLR3 activation, wild-type cells strongly induced the production of both types of interferons. However, knock-out HT-29 cells barely activated the production the latter. Similar results were observed in TLR4 signalling despite a milder induction of interferon production. Following TLR4 treatment, knock-out cells seem to still be capable of slightly inducing the production but to a lesser extent than wild-type cells. In conclusion, deletion of ANKIB1 strongly impacts the activation of TBK1 and the transcription factor IRF3 which consequently impairs the production of both type I and type III interferon following the activation of TLR3 and TLR4 signalling pathways.



<u>Figure 3.24</u>. ANKIB1 deletion impairs type I and type III interferon production mediated by TLR3 and TLR4:

HT-29 wild-type or ANKIB1 knock-out cells were stimulated for 6 hours with 10µg/mL of poly(I:C) in a) and c) or 1µg/mL of LPS in b) and d), respectively. a-b) Cells were lysed and proteins were analysed by western blotting. c-d) RNA isolation was performed, cDNA generated and qPCR performed with specific primers for the respective genes of interest.

4. Discussion

Ubiquitination is a crucial post-translational modification implicated in several cellular processes such as protein recycling, endocytosis, DNA repair, proteasomal degradation, and protein-protein interaction. Moreover, it plays a tremendous role in the regulation of immune/inflammatory signalling. In the last decades, more and more E3 ligases, with different types of activity, have been implicated in regulating the signalling of many immune receptors, notably the TNFR superfamily, TLRs, and other pathogen sensors. However, despite the redundant involvement of some proteins, they might not have the same activity and the same activation process. TBK1 is a crucial kinase that is involved in many signalling pathways, including TNF-signalling pathway where it prevents TNF-induced cell death, or TLR3 and other PRRs where it is essential to promote interferon response. However, our lab discovered that TBK1 is differently regulated in both pathways and that its recruitment and activation require different ubiquitin E3s in both signalling pathways (Lafont et al., 2018; Zinngrebe et al., 2016). In TNF signalling, our lab has demonstrated that TBK1 requires the catalytic activity of HOIP to be properly activated and to prevent TNF-induced cell death. However, HOIP deletion in TLR3 neither impairs its recruitment to the complex nor affect its activation. Due to the primordial role of ubiquitination required for TBK1 activation in the TNF signalling pathway, we aimed to address the potential role of a new E3 ligase in TLR3 signalling. Due to its primordial role in interferon production, it is necessary to understand in depth the modality of its recruitment and activation in this context.

4.1 ANKIB1 is required for TBK1 activation in TLR3 but not in TNF signalling pathway

After an siRNA screening of the different members of the RBR-containing E3 family that have not been reported to play a role in TBK1 activation, we identified ANKIB1, as a potential E3 implicated in this process **(Figure 3.1)**. We have shown that ANKIB1 is required for TBK1 phosphorylation upon TLR3 activation **(Figure 3.2)**. We have demonstrated the same requirement in different cancerous or non-cancerous cell lines, but also in primary macrophages isolated from the bone marrow of ANKIB1 knockout mice which we have generated **(Figure 3.4 and 3.6)**.

This suggests that ANKIB1 has a broad contribution in TLR3 signalling and that it does not act in a tissue-specific or organism-specific manner. This conclusion is also supported by the fact that ANKIB1 expression has been detected in many tissues, especially in tissues whose cells are an essential component of the immune system, e.g. spleen and Peyer's patches **(Fig. 3.5)**. ANKIB1 was strongly expressed in the lungs of the examined mice. The lung is an organ that recruits many immune cells, especially macrophages, due to the constant exposure to pathogens. The particularly high expression of ANKIB1 in this tissue was a first indication that this protein could play an important role in the immune system's first line of defence.

Furthermore, we showed that following poly(I:C) treatment, the activation of the transcription factor IRF3, which is essential for interferon production downstream of TBK1, is also impaired **(Figure 3.9)**. It is another indicator of the role of ANKIB1 in proper host defence. Nevertheless, regarding the gene-activatory pathways such as NF-κB and MAPK pathways, the absence of ANKIB1 does not have any consequences suggesting that ANKIB1 plays a unique role in the TRAF3-TBK1 axis of the signalling. Interestingly, in TNF signalling pathway, where HOIP is required for TBK1 phosphorylation, ANKIB1 deletion does not have any consequences **(Figure 3.2 and 3.4)**. This confirms our initial hypothesis that, despite being involved in both immune pathways, TBK1 activation requires different E3 ligases and does not exert the exact same role.

The fact that TBK1 activation differs in both pathways could be interpreted as a "safety mechanism". Indeed, considering that if a key protein such as TBK1, which plays a central role in so many different cellular responses such as autophagy, regulation of cell death, and most importantly in type I and type III interferon production were to depend on a unique E3 ligase, a dysregulation of this latter would have dramatic consequences. By being regulated and activated in different signalling pathways via different adaptor proteins and E3 ligases, the dysfunction of one of those proteins would not affect the contribution of TBK1 in all other cellular processes mentioned. Moreover, this differential modality of activation of TBK1 translates the fundamental role of the E3 ligases in organisms to initiate an appropriate response to a certain stimuli. Due to the activity of different E3 ligases, a unique protein such as TBK1 can be involved in a large variety of cellular processes.

In addition to its role in TNF and TLR3 signalling pathway where it depends on HOIP and ANKIB1 respectively, additional roles of TBK1 are regulated by other ubiquitin E3s. During mitophagy, PARKIN has been shown to ubiquitinate the damaged mitochondria ,thereby promoting the recruitment of the autophagic machinery via TBK1 recruitment and activation (Heo et al., 2015). During Salmonella infection, TBK1 also contributes to the degradation of the pathogen. Its recruitment to bacteria and its activation depends on K48 and K63 ubiquitin chains, suggesting again that another E3 is involved in this process (Thurston et al., 2016; Wild et al., 2011). Thus, TBK1 is an excellent example to illustrate the importance of the more than 600 E3 ligases encoded by the human genome.

4.2 ANKIB1 is recruited to TLR3-SC, potentially through its UIM domain

To gain some insight into the mechanism of action of ANKIB1, we aimed to compare the signalling complexes formed after TLR3 and TNFR1 activation via immunoprecipitation of the respective receptors. This highlighted a recruitment of ANKIB1 to TLR3-SC following poly(I:C) treatment but not to TNFR1-SC following TNF treatment **(Figures 3.7 and 3.8)**. The exclusive recruitment to TLR3-SC suggested that it depends on a protein that is present in TLR3 signalling but not in TNF signalling. Therefore, we extrapolated that ANKIB1 is recruited to TLR3 via TRIF, TRAF3, or Optineurin, proteins only present in TLR3- but not in TNF- signalling (Draber et al., 2015; Lafont et al., 2018). In fact, the pull-down of ANKIB1 showed that it interacts with phosphorylated TBK1, but also with optineurin and NEMO, both involved in TBK1 activation in TLR3 signalling pathway **(Figure 3.10 and 3.11, b)**. Interestingly, ANKIB1 does not seem to be able to interact with NAP1, which could suggest that ANKIB1 rather interact with a pull of TBK1 that colocalise with Optineurin exclusively. To understand the mode of interaction of ANKIB1 with its binding partners, we reexpressed a mutant version of ANKIB1 that lacks the Ubiquitin-Interacting-Motif (UIM) domain (ANKIB1ΔUIM). This domain confers the ability to interact with ubiquitinated proteins. Ubiquitination is extensively present in immune signalling, especially as polyubiquitin chains. It can promote the stability of the signalling complexes by promoting scaffolding activity, favouring the recruitment of other proteins. Oppositely, polyubiquitin chains are also present to control an appropriate activation of the different gene-activatory pathways by disrupting protein-protein interactions or leading to proteasomal degradation.

Overall, ubiquitination is found on many components of the different signalling complexes. Therefore, containing a UIM domain can confer a primordial advantage to be recruited to a signalling complex. Additionally, the study of the different UIM domains by Miller et al. has shown that the UIM domain of ANKIB1 interacts efficiently with polyubiquitin chains (three to six molecules of ubiquitin) (Miller et al., 2004). Interestingly, while the reexpression of a wild-type version of ANKIB1 rescued TBK1 phosphorylation, ANKIB1ΔUIM cells were not able to do so **(Figure 3.16)**. This result suggests that ANKIB1 requires the interaction via its UIM domain with a specific ubiquitinated protein within the complex to be able to promote TBK1 activation upon TLR3 stimulation. Additionally, preliminary data obtained from ANKIB1 pull-down with and without UIM domain suggests that ANKIB1ΔUIM is not able to interact with components of the TLR3-SC such as A20 or Optineurin. We therefore hypothesise that TLR3-SC contains a protein that is poly-ubiquitinated and which serves as a recruitment platform for ANKIB1 to the complex by interacting with its UIM domain. Further wild-type ANKIB1 pull-down assays need to be performed in cell lines depleted for specific components of the complex, such as TRAF3, TRIF and Optineurin thereby assessing the ability of ANKIB1 to interact with components of the TLR3-SC in order to identify the crucial protein for its recruitment to the complex. TRIF knock-out however would not be informative because, without TRIF, the entire complex formation would be compromised. However, reexpression of a mutated TRIF protein lacking the lysines and therefore cannot be ubiquitinated might be a solution to assess its role for the recruitment of ANKIB1 without disrupting TRAF3 and TRAF6 recruitment (Sasai et al., 2010). Because the deletion of the UIM domain disrupted the ability of ANKIB1 to promote TBK1 activation, we have to confirm that the deletion of this domain does not affect its proper conformation, which could lead to a disruption of its catalytic activity. Indeed, amino acids interplay with each other by forming different types of interactions such as disulphide, hydrogen or ionic bonds. Together, they are responsible for the tertiary structure of a protein. The deletion of a domain might therefore change the interaction between amino acid and the overall tertiary structure of the protein which can impair its activity.

4.3 Characterisation of the catalytic activity of ANKIB1

Because the RBR domain of ANKIB1 was not previously characterised, it was essential to assess its catalytic activity. An in-vitro ubiquitin assay showed that ANKIB1 is able to generate polyubiquitin chains in cooperation with UBE2D3 and to a less extent with UBE2L3 **(Figure 3.12)**. Mass Spectrometry analysis of the chains generated with both E2s mostly identified K11 ubiquitin chains. K63 was also found but in a low amount with UBE2D3 but close to 30% with UBE2L3 **(Figure 3.13)**. As described in the introduction, K11 represents only a minor part of the ubiquitin chains found in mammalian cells. K11 was thought for a long time to be only involved in DNA repair and proteasomal degradation. However, recent studies have found that K11-linkages mainly activate processes in cooperation with other ubiquitin linkage types, so-called mixed linkages. It has been shown that the presence of K11-linkages in mixed polyubiquitin chains, e.g. together with K48, can potentiate the proteasomal degradation of a targeted protein (French et al., 2021). Furthermore, the contribution of K11-linkages to scaffold activity in immune signalling was reported in 2010 by Dynek et al. Together with linear chains mediated by HOIP, cIAP1/2 have been implicated in the generation of K11- and K63-ubiquitin chains, which contribute to the recruitment of NEMO and TAK1 to TNFR1-SC (Dynek et al., 2010). In TLR3 signalling pathway, HOIP has been shown to be required to a similar extent for the recruitment of these proteins. However, cIAPs does not seem to be involved in the gene-activatory pathways mediated by TLR3 pathway. Indeed, the depletion of cIAPs has been reported to be involved in Myd88-mediated signalling pathways but not in the TRIF-mediated pathway (Tseng et al., 2010). Additionally, their absence does not impair interferon production following TLR3 or TLR4 activation. We, therefore, assume that the ubiquitin chains generated by cIAP1/2 in TLR3-signalling are not as detrimental as they are in the TNF signalling pathway where they are required for further recruitment of LUBAC, NEMO and TAK1 to the TNF-SC. Consequently, we further conclude that other E3s, similar to the cIAPs in TNF-SC, ubiquitinate further complex components in TLR3-SC thereby influencing its stability and inducing proper signal outcome. Overall, this suggests that ANKIB1 might exert a unique role in TLR3 signalling by the adding K11 (and K63) ubiquitin chains to the signalling complex.

In order to understand the relevance of the catalytic activity of ANKIB1 regarding the activation of TBK1, we generated different ANKIB1 versions with mutations in its

catalytic domains. The first mutant we reexpressed in ANKIB1 knock-out cells was ANKIB1ΔRING2, to prevent any activity of the RBR domain. As mentioned in the introduction, the deletion or mutation of the catalytic cysteine of the RING2 is sufficient to disrupt the catalytic activity of all RBRs described so far. Surprisingly, treatment with Poly(I:C) equally activated TBK1 and IRF3 in wild-type ANKIB1 and ANKIB1ΔRING2 while ANKIB1 knock-out cells were not **(Figure 3.16)**. As a control, we wanted to ensure that the catalytic activity of ANKIB1 was impaired by removing the RING2. In-vitro ubiquitin assay showed that indeed the deletion of RING2 totally impairs the formation of ubiquitin chains with UBE2L3. This was expected because UBE2L3 is an E2 that can only transfer the ubiquitin molecule to a cysteine and not a lysine, consequently contributing to the formation of ubiquitin chains via the transfer of the ubiquitin molecule to a HECT or RBR-E3 only. However, we were surprised to find that ANKIB1ΔRING2 was still able to generate polyubiquitin chains in cooperation with UBE2D3, albeit to a lesser extent than wild-type ANKIB1 **(Figure 3.17)**. Oppositely to UBE2L3, UBE2D3 is able to transfer ubiquitin molecules to lysines and cysteines, allowing cooperation with all types of E3 ligases. Interestingly, it has been reported that RBR-containing E3s cannot interact with UBE2D3 without IBR or RING2 which therefore seems to be different for ANKIB1 (Martino et al., 2018; Walden et al., 2018). In order to understand why ANKIB1 could work differently to all other members of the RBR family, we did a sequence alignment of the RING1 of each RBR-containing E3 in comparison to a classical RING domain. While every RBR-containing E3 presented a RING1 domain that was not conserved in terms of crucial residues, ANKIB1 was the only one that has a conserved RING1 domain very similar to the classical RING domain of all RING family members. This could suggest that ANKIB1 may be able to not only follow the RBR mechanism together with UBE2L3 but that, in addition, it could also act together with UBE2D3 as a classical RING E3 **(Figure 3.18)**. Indeed, ANKIB1 is the only RBR-E3 that contains all the required seven cysteines and one histidine that promote the binding of Zn^{2+} but also the four different hydrophobic residues essential for a stable interaction between the E2 and the RING domain of the E3 (Garcia-Barcena et al., 2020). Therefore, we could hypothesise that the interaction between ANKIB1 and UBE2D3 does not require the IBR and RING2, such as other members of the RBR family, explaining why we still observed a ligase activity in the absence of RING2.

Additionally, the IBR domain of ANKIB1 lacks a conserved cysteine that could therefore shift the preference of cooperating with UBE2L3 to UBE2D3. Because of this result and observation regarding the RING1, we generated another mutant that lacks the entire RBR domain to be certain that the catalytic activity of ANKIB1 is totally disrupted. Interestingly and in contrast to the mutant ANKIB1ΔRING2 that we tested first, ANKIB1ΔRBR reexpression in knock-out cells was not able to rescue the phosphorylation of TBK1 upon TLR3 activation **(Figure 3.19)**. This result suggests that the ubiquitin chains generated by ANKIB1 are indeed required for to activate TBK1 in TLR3 pathway and that the linkages are produced by ANKIB1 acting together with UBE2D3 as a classical RING E3. However, further experiments need to be done in order to verify that the absence of ubiquitination via ANKIB1 impairs the recruitment of TBK1 to the complex or only its activation at the complex. There is also the possibility that TBK1 is not a direct target for ANKIB1 but that ANKIB1 rather ubiquitinates and thereby activates other components of the signalling complex such as NEMO, Optineurin or TRAF3, which are required for TBK1 activation at the complex.

NEMO has been shown to be essential for TNF-induced TBK1 activation, dependent on the presence of linear chains in TNFR1-SC (Lafont et al., 2018). Although we showed that linear chains are not required for TBK1 phosphorylation in TLR3 signalling, NEMO has been shown to be involved in TBK1 activation, but most importantly, in interferon production (Audry et al., 2011). Moreover, as mentioned previously, it has already been shown and also been confirmed during this thesis (data not shown) that despite playing an essential role in the regulation of TLR3-induced cell death, cIAP1/2 are dispensable for TLR3-induced gene activation and interferon production. Thus, regarding the requirement of ubiquitin chains to recruit NEMO to the TNFR1-SC, we concluded that ANKIB1 might, via the addition of K11 and/or K63 chains, contributes to NEMO recruitment to the TLR3-SC and thereby contributes to TBK1 activation.

Because the UBAN domain of NEMO is very similar to the UBAN domain of Optineurin, we could also extrapolate that the chains generated by ANKIB1 might contribute to Optineurin recruitment to the TLR3 signalling complex and promote its role as an adaptor protein for TBK1. Indeed, it has been shown that the ubiquitin-binding domain of Optineurin is crucial to promote TBK1 phosphorylation and, therefore, interferon production (Gleason et al., 2011). Nevertheless, the ubiquitin E3 responsible for the ubiquitin chains remains unknown.

The study of the signalling complex in the presence and absence of ANKIB1 would be required to address these hypotheses and decipher the consequence of its deletion in the recruitment of the proteins involved in TBK1 activation. We could speculate that the absence of ANKIB1 and the ubiquitin linkage type that it can generate would impair the recruitment of TRAF3, NEMO or Optineurin to TLR3-SC and consequently the recruitment of TBK1.Another hypothesis involves A20 which was seen to interact with ANKIB1 following TLR3 activation. A20 is able to cleave K63 chains via its DUB activity. TRAF3 notably generated this type of chain to promote the recruitment of TBK1. We could therefore extrapolate that the deletion of ANKIB1 leads to an increase of A20 recruitment to the complex, therefore digesting in an uncontrolled manner the ubiquitin chains present in the signalling complex.

Additionally, we have to address the relevance of the cooperation between ANKIB1 and UBE2L3, which could have an important contribution for another role of ANKIB1, independent of TBK1 activation. Indeed, several E3 ligases have been shown to be implicated in different processes such as PARKIN, which is not only involved in regulating gene-activatory pathways in different immune signalling but also implicated in the induction of mitophagy.

4.4 ANKIB1 deletion impairs the ubiquitination of several proteins involved in TBK1-IRF3 activation

In order to find out a potential substrate for ANKIB1, we performed a di-gly enrichment in wild-type and knock-out cell lines. Regarding the classification of the enriched peptides found with the GOBP database, the deletion of ANKIB1 not only impairs the ubiquitination profile of the TLR3 signalling but also other processes such as chromosome condensation and regulation of apoptosis (Figure 3.21). It is known that ubiquitin chains, notably K11, regulate the transcription by modifying the accessibility of the DNA. For example, K11 chains are involved in inhibiting damaged DNA transcription. Ubiquitination is known to be crucial to control the balance between inflammatory signalling and cell death. For example, cIAP1/2 ubiquitinates caspase-3 and 7, consequently preventing their processing and activation (Burke et al., 2010; Kavanagh et al., 2014). ITCH, another E3 ligase, ubiquitinates $cFLIP_L$, which leads to its proteasomal degradation and therefore promotes apoptosis (L. Chang et al., 2006).

Overall, this could suggest that ANKIB1 might also be implicated in regulating cell death via its catalytic activity. Further work needs to be done in order to address this aspect. Regarding TLR3 pathway, many proteins were shown to be ubiquitinated in wild-type cells only **(Figure3.22)**. TRIF (TICAM1) ubiquitination was not impacted by the deletion of ANKIB1, suggesting that it rather ubiquitinates a protein downstream of TRIF. This goes in line with the absence of effect observed in ANKIB1 knock-out cells regarding the activation of the gene-activatory pathways NF-κB and MAPK. IRF3 peptides were found in wild-type only, suggesting that ANKIB1 deletion directly or indirectly impairs the ubiquitination of the Lysine 197. However, this ubiquitination site has already been described as a ubiquitin-like site for HERC5 that adds ISG15 molecule to IRF3. This ISGylation site ,together with two other sites on IRF3 (K360 and K366), are important to disrupt the interaction of IRF3 with Pin1, a K48 ubiquitin E3 that triggers its proteasomal degradation (Shi et al., 2010). Therefore, these ubiquitination are essential to promote IRF3 activation and interferon production. Moreover, because we observed that ANKIB1 is crucial for TBK1 activation, we think that our result identifying an absence of K197 ubiquitination on IRF3 in ANKIB1-depleted cells is rather a downstream consequence of the absence of TBK1 activation. Indeed as mentioned previously, TBK1 activation is essential for IRF3 recruitment to TLR3-SC but also its activation by direct phosphorylation. Interestingly, TBK1 has also been shown to be ubiquitinated on lysine 137. This ubiquitination site has already been identified to be essential for TBK1 activation and the mutation of this lysine disrupts the activation of the downstream transcription factor IRF3 completely (Wang et al., 2012). It is suggested that this lysine is key for its catalytic activity and ATP binding but ubiquitination may have a role in promoting its kinase activity. Additionally, it has been shown that ubiquitination of TBK1 is crucial for its proper activation. The degradation of these ubiquitin linkages by DUBs, such as CYLD, disrupts its activity (M. Zhang et al., 2008). K137 is localised on the kinase domain that can therefore contribute to its activity. The ddition of ubiquitin linkages to lysine residues within the kinase domain is possible and has already been reported for TBK1 but also other kinases such as RIP1. Tu D et al. have shown that a mutation of lysine K30 prevents TBK1 phosphorylation (Tu et al., 2013). Despite the low significance of the log2 intensity in the volcano plot representing the di-gly enrichment experiment **(Figure 3.22, a)**, the K137 ubiquitinated TBK1 peptide was found consistently in wild-type samples but not in ANKIB1 knock-out samples **(Figure 3.22,**

b). Therefore, TBK1 may serve as a direct substrate for ANKIB1, which needs to be further investigated. Other proteins found ubiquitinated in wild-type cells but not in ANKIB1 knock-out samples were A20 (TNFAIP3) and ABIN-1 (TNIP1). Both are part of a complex with other proteins such as TAX1BP1 and collaborate to control the inflammatory response by regulating the ubiquitination of components of the signalling complex such as NEMO. It has been shown that they interact with TBK1 in order to prevent its recruitment to ubiquitinated TRAF3 at the signalling complex and thereby inhibit its phosphorylation (Parvatiyar et al., 2010). One possible hypothesis could be that ANKIB1 ubiquitinates A20 and/or ABIN-1 by adding K11 ubiquitin linkages which leads to their proteasomal degradation, thereby controlling their activity and maintaining the phosphorylation status of TBK1. Furthermore, in the absence of ANKIB1, A20 activity might be unleashed, constantly preventing TBK1 activation or deubiquitinating TRAF3. Regarding Peli-1, its role in TLR3 signalling in interferon production remains very elusive and seems to be tissue-dependent. However, several reports have shown a contribution of Peli-1 to the promotion of gene-activatory pathways, which makes Peli-1 an unlikely target for ANKIB1 ubiquitination as the TLR3-triggered gene-activatory pathway is not affected when it is depleted. However, because Peli-1 is represented in the di-gly result with high significance, it cannot be completely excluded as an ANKIB1 target. Finally, the hit with the highest p-value was YWHAB, a member of the 14-3-3 complex. Very little is known about this protein complex but it has been claimed that it exerts a role in viral clearance and its inhibition results in an increased viral proliferation. It has been reported that the 14-3-3 complex facilitates the activation of TRIM25 in RLRs signalling, promoting interferon response. Its implication in interferon production mediated by TLR3 needs to be addressed in the future and YWHAB as a substrate for ANKIB1 cannot be excluded. Finally, NEMO (IKBKG) ubiquitination on the Lysine K344 located in its leucine zipper has also been identified as a possible substrate candidate of ANKIB1 **(Fig. 3.22)**. The leucine zipper of NEMO has been shown to be important to interact with other proteins and, therefore, could be involved in the interaction with adaptor proteins implicated in TBK1 recruitment to the signalling complex. In order to validate and interpret the di-gly enrichment results, different approaches will be required. One of the approaches would be in vitro ubiquitin assays with the different potential substrates listed in the table of the figure 3.22.

This experiment can possibly indicate which of those proteins identified in the di-gly enrichment can be directly ubiquitinated by ANKIB1 and which are not. However, because this experimental setup is in vitro only and represents very different conditions to the di-gly experiment which represented an in vivo situation, i.e. the endogenous conditions of the stimulated cell, the results we obtain from such an experiment might be misleading and the interpretation of it has to be done very carefully. Indeed, it is possible that in order to be ubiquitinated by ANKIB1, the substrate needs to be previously post-translationally modified or needs to interact with another protein in order to interact with ANKIB1, which is why another approach would be necessary. Then, TUBE pull-down following poly(I:C) treatment in cells expressing ANKIB1 versus those depleted for ANKIB1 will need to be also performed in order to confirm the potential result found with in vitro ubiquitin assay. This experiment allows us to assess the role of ANKIB1 in terms of ubiquitination for a specific protein endogenously and potentially validate a substrate protein found in the di-gly enrichment. It is important to mention that even if some very important proteins involved in TBK1 activation, such as TRAF3, have not been detected by MS; their ubiquitination profile will still be addressed. Indeed, several papers have shown that TRAF3 is essential for TBK1 activation in TLR3 pathway, while it is not required for TBK1 activation in Myd88-mediated TLR4 signalling(Hu et al., 2013). Regarding our data suggesting that TBK1 activation requires ANKIB1 only when it depends on TRAF3 such as in TRIF-mediated TLR signalling, TRAF3 as a substrate for ANKIB1 cannot be excluded. It is indeed important to consider that despite being a very valuable technique for the identification of proteins in an unbiased manner, proteomics comports some limitations regarding protein detection. A low-abundance protein, a non-charged peptide but also a protein that is poorly digested by trypsin, which leads to the generation of too big peptides, will not be detected.

4.5 ANKIB1 plays a role in TRIF-dependant TBK1 activation

Addressing the potential role of ANKIB1 in other interferon-producing TLRs such as TLR4 was very informative regarding the mechanism of action of this E3. TLR4 is the only TLR able to induce both Myd88 and TRIF-mediated signalling via two distinct arms. ANKIB1 deletion disrupted IRF3 activation but not TBK1 phosphorylation following LPS stimulation (**Figure 3.23**).

Because in TLR4 signalling, IRF3 is only activated through TRIF-mediated signalling, we can extrapolate that ANKIB1 is involved only in this latter but not in the Myd88-signalling arm of TLR4 pathway. Moreover, this result suggests that the mode of activation of TBK1 differs in TLR3 and TLR4 signalling pathway. Indeed, Clark et al. showed that TBK1 can be activated through two mechanisms. For the first mechanism, found in IL-1 and TLRs involving Myd88, respectively, a cross-talk between both canonical and non-canonical IKKs has been shown to exist. Clark et al. showed that IKKα/β phosphorylates TBK1. As a negative feedback mechanism, TBK1 phosphorylates these latter in order to prevent an excessive NF-κB response. The close proximity of the IKKs is mediated by the adaptor protein TANK that links NEMO to TBK1. This explains why TANK knock-out mice develop an excessive NF-κB activation, mediated through Myd88. Because TRIF-mediated NF-κB signalling is not involved in this pathology, it suggests another mechanism of activation of TBK1 when TRIF is involved. Additionally, despite the impact on NF-κB activity, they showed that the deletion of TANK did not impair interferon production following TLR3/4 activation. This confirms a previous work that reported a similar induction of the interferon production between wild-type and TANK knock-out mice after viral infection. This suggests that TANK has a unique role in TBK1 activation in Myd88 but not in TRIF-mediated signalling where TBK1 is required to phosphorylate IRF3 and therefore induces the production of type I and type III interferon. Another aspect confirming that TBK1 is differentially activated between TRIF and Myd88 is the discrepancy regarding the requirement of TRAF3. While TRAF3 is essential for TBK1 activation in TLR3 pathway, TRAF3 is rapidly degraded by cIAP1/2 in Myd88 pathways and therefore does not contribute to the activation of TBK1. Overall, TBK1 phosphorylation depends on different proteins depending on the signalling. In Myd88-dependent signalling, TBK1 is recruited to TRAF6-NEMO via TANK in order to be activated via canonical IKKs, independently of TRAF3. Oppositely, in TRIF-mediated TLR3 and TLR4 signalling, where ANKIB1 seems to be required, TBK1 is recruited and activated via TRAF3 by interacting with other adaptors such as NAP1 or Optineurin. To confirm this hypothesis, ANKIB1 pull-down analyses following LPS treatment are required. If our hypothesis is valid, we should see that upon TLR4 stimulation ANKIB1 interacts with TRIF, as found in TLR3 pathway, but does not with Myd88.

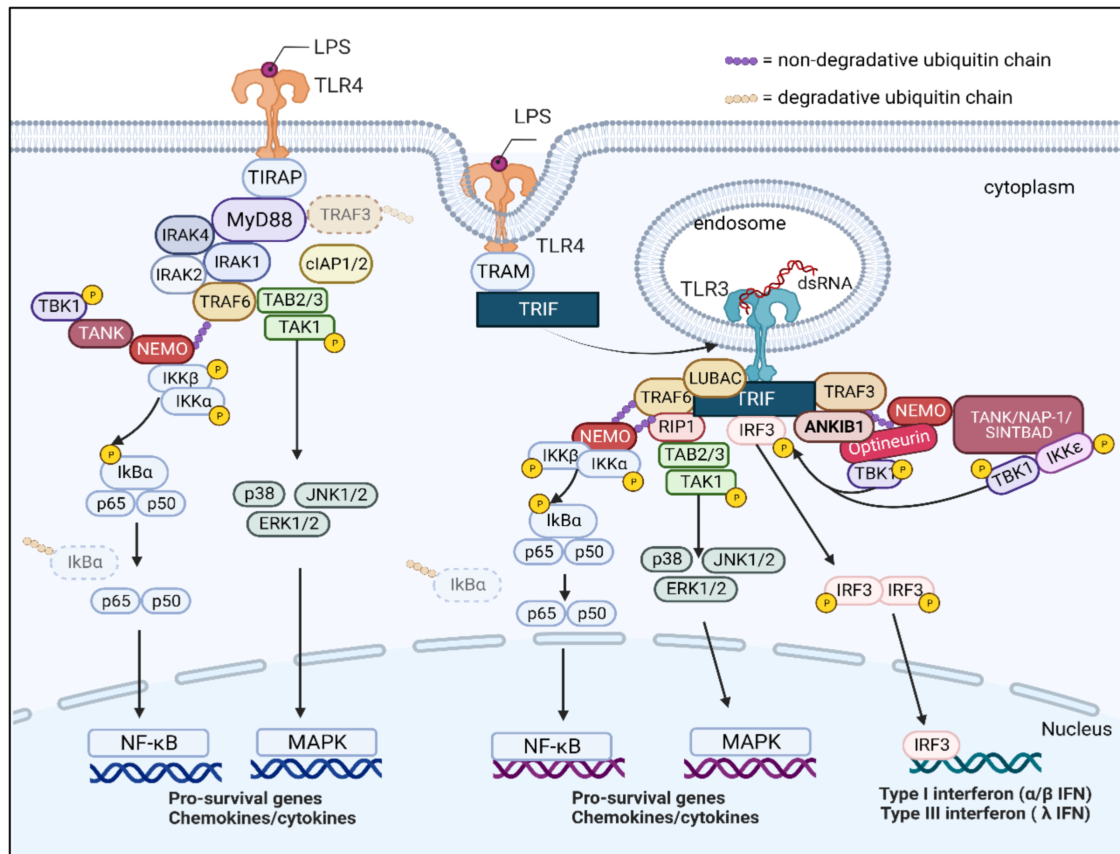


Figure 4.1 ANKIB1 in TRIF-mediated TLR3 and TLR4 signalling:
TLR4 is a unique TLR that mediates signalling complex formation via TRIF and Myd88. Both lead to the activation of similar effector proteins involved to the activation of different gene-activatory pathways such as NF-κB and MAPK pathways. However, the activation of TBK1 differs between both signalling. TRIF-mediated TLR4 signalling, similarly to TLR3 signalling involves TRAF3, a RING-E3 that allows the recruitment of different adaptor proteins such as TANK, NAP-1, SINTBAD and Optineurin, essential for TBK1 activation and recruitment to the complex. While TANK is the only adaptor protein for TBK1 in Myd88-mediated signalling pathway, its depletion does not impact the production of interferon mediated by IRF3, suggesting a differential recruitment and or activation of TBK1 between Myd88 and TRIF-mediated signalling pathway. The transcription factor IRF3 can only be activated via TBK1 through a TRIF-mediated signalling. Therefore, TBK1 is not only recruited to this signalling complex via TANK, such as in Myd88-pathway but rather involve also additional adaptor proteins. ANKIB1 has been shown in this thesis to contribute to this axis. It is recruited to the TLR3-SC, interacting with several components such as NEMO and Optineurin, but not NAP1, in order to induce TBK1 activation via TLR3 and TRIF-mediated TLR4 signalling. However, ANKIB1 is not required for TBK1 phosphorylation in Myd88-mediated TLR4 signalling.

4.6 ANKIB1 is required for an efficient interferon response following TLR3/4 activation

After observing that TBK1 and, most importantly IRF3 phosphorylation is strongly impaired in absence of ANKIB1 following TLR3 and TLR4 activation, we addressed its role in terms of interferon production via qPCR.

In both signalling pathways, ANKIB1 deletion disrupts the production of both, type I and type III interferons, confirming a major role of ANKIB1 in this process (Figure 3.24). However, oppositely to TLR3 pathway, LPS seems to be able to induce a slight production of interferons also in absence of ANKIB1. A Myd88-dependent interferon production or a residual TBK1 activation could potentially explain this. Indeed, it has been reported that TLR7, via IKKβ can directly phosphorylate IRF5 and induce interferon production. However, this remains controversial and seems to be tissue-dependent (Lopez-Pelaez et al., 2014).

4.7 Summary and future perspective

In this study, we have shown that ANKIB1 is a new regulator of TLR signalling. We have demonstrated that it is required for a proper TBK1 and IRF3 activation upon TLR3 activation. In parallel, its role in TNF signalling was addressed, but its deletion did not have any consequences in terms of gene-activatory pathways or TBK1 phosphorylation. We found that ANKIB1 is recruited to TLR3-SC but not TNFR1-SC suggesting that its recruitment depends on a protein specifically involved in a TRIF-mediated signalling complex. Following TLR3 activation, we reported a close interaction between ANKIB1 and several proteins involved in interferon production, such as TBK1, NEMO, or Optineurin. However, further work needs to be done in order to fully understand how ANKIB1 is recruited to TLR3-SC and to assess the potential role of the interacting proteins in this process. Moreover, we showed that the role of ANKIB1 depends on its UIM domain that confers an ability to bind to ubiquitinated proteins, probably at the signalling complex. Further experiments will allow us to determine the precise role of this domain. We also demonstrated that ANKIB1 has a ubiquitin ligase activity that preferentially forms K11 polyubiquitin chains in cooperation with UBE2D3. It can also generate K11 and K63 polyubiquitin chains with UBE2L3, but this interaction is less efficient than with UBE2D3. We showed that the RING2 domain of ANKIB1 was required for its activity with UBE2L3 but not with UBE2D3. Indeed, in an ANKIB1 version lacking the RING2 domain, ANKIB1 was still able to generate ubiquitin chains with UBE2D3 and to promote the activation of TBK1. Only the deletion of the entire RBR domain disrupts its activity and therefore impairs TBK1 phosphorylation.

This suggests that ANKIB1 might be able to act rather as a RING-E3 than an RBR-E3 in this process. However, the role of the ubiquitin linkages mediated by UBE2L3 and the RING2 of ANKIB1 remains unknown. Because K11 polyubiquitin chains have been shown to regulate both proteasomal degradation and protein stability, the real implication and consequences of these chains mediated by ANKIB1 require additional characterisation. The question regarding the substrate of ANKIB1 has also been addressed in this study but still requires further experiments. With the di-gly enrichment analysed by Mass Spectrometry, we found several interesting candidates such as A20, NEMO, and TBK1 but also other proteins, including Peli-1 or YWHAB which we did not consider before the MS analysis. These candidates, together with the interacting partners of ANKIB1 need to be tested and followed up in order to better understand how ANKIB1 contributes to TBK1 phosphorylation. In order to find out if ANKIB1 acts only in TLR3 pathway, its role in TLR4 signalling has also been addressed. However, absence of ANKIB1 in TLR4 triggering following LPS treatment had absolutely no effect on TBK1 phosphorylation, yet in contrast, impaired IRF3 activation. This result shows an involvement of ANKIB1 in TBK1 and IRF3 activation in TRIF-mediated TLR4 signalling but not in TLR4 signalling mediated via Myd88. Consequently, in both pathways, TLR3 and TLR4, the production of type I and type III interferon requires ANKIB1. A similar potential contribution needs to be addressed for other interferon-producing pathways involving TBK1 and IRF3 such as RIG-I, MDA5 but also the cGAS-STING pathway. Regarding the importance of interferon signalling during anti-viral immune responses, it is crucial to address in the future the relevance of ANKIB1 during infection, notably with influenza, where interferon and TLR3 have already been shown to increase the survival of the infected host (Hatesuer et al., 2017). To conclude, in this study, we contributed to a better understanding of the TLR3 signalling pathway and its mechanism of action in terms of interferon production via TBK1 activation, which involves a so far uncharacterised ubiquitin E3, named ANKIB1.

5. Appendix

5.1 Abbreviations

ABIN	A20 Binding Inhibitor of NF-κB
AIM2	Absent in Melanoma 2
ANKIB1	Ankyrin Repeat and IBR Domain-Containing Protein 1
APAF1	Apoptotic Protease Activating Factor 1
APC	Antigen Presenting Cell
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine Triphosphate
A20	TNF-A Induced Protein 3
BAFF	B-cell-Activating Factor
BAK	Bcl2 homologous Antagonist Killer
BAX	Bcl2-Associated X protein
Bcl-2	B cell Lymphoma 2
BID	BH3-Interacting Domain Death Agonist
CARD	Caspase Recruitment Domain
Caspase	Cysteinyl-Aspartate Specific Protease
cIAP 1/2	Cellular Inhibitor of The Apoptosis Proteins 1/2
cGAS	Cyclic GMP-AMP Synthase
c-FLIP	Cellular FLICE Inhibitory Protein
CLR	C-type Lectin-like Receptor
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
CYLD	Familial Cylindromatosis Protein
DAI	DNA-Induced Activator of the IFN
DAMP	Damage Associated Molecular Pattern
DD	Death Domain
DDM	n-Dodecyl-β-D-Maltopyranoside
DED	Death Effector Domain
DISC	Death-Inducing Signaling Complex
DIABLO	Direct Iap-Binding Protein with Low pI
DMEM	Dulbecco’s Modified Eagle’s Medium
DsRNA	Double stranded Ribonuclease Acid

DR	Death Receptor
DUB	Deubiquitinase
EDTA	Etilendiamine Tetraacetic Acid Endosomal Sorting
ERK	Extracelullar signal Related Kinase
ESCRT	Complexes Required for Transport
E1	Ubiquitin-Activating Enzyme
E2	Ubiquitin-Conjugating Enzyme
E3	Ubiquitin Protein Ligase
FADD	Fas-Associated DD
FasL	Fas cell surface death receptor Ligand
FBS	Fetal Bovine Serum
GM-CSF	Granulocyte Macrophages Colony-Stimulatory Factor
HECT	Homologous to The E6-AP Carboxyl Terminus
HOIL-1	Heme-Oxidized IRP2 Ubiquitin Ligase-1
HOIP	HOIL-1–Interacting Protein
HRP	Horseradish-Peroxidase
IBR	In-Between Ring
IFN	Interferon
IFNAR	IFN α/β Receptor
IL	Interleukine
IKK	IκB Kinase
IP	Immunoprecipitation
IRAK	IL-1R-Associated Kinase
IRF 3/7	IFN Regulatory Factor 3/7
ISG	IFN Stimulatory Gene
ISRE	IFN-Stimulated Response Element
IκB	Inhibitor of The Nuclear Factor κB
JAK	Janus Kinase
JAMM	JAB1/MPN/MOV34 metalloprotease
KDa	Kilo Dalton
KO	Knock-Out
LBP	LPS-binding Protein
LGP	Laboratory of Genetics and Physiology

LPS	Lipopolysaccharide
LUBAC	Linear Ubiquitin Chain-Assembly Complex
LRR	Leucine Rich Repeat
MAPK	Mitogen-Activated Protein Kinase
MDA-5	Melanoma Differentiation-Associated Protein 5
MHC	Major Histocompatibility Complex
MK2	MAPK-Activated Protein Kinase 2
MLKL	Mixed Lineage Kinase Domain-Like
MOMP	Mitochondrial Outer Membrane Permeabilization
mTOR	Mechanistic Target Of Rapamycin Kinase
MYD88	Myeloid Differentiation Primary Response 88
MS	Mass Spectrometry
MW	Molecular Weight
NALP	NACHT-, LRR- and PYD-Containing Protein
NAP1	Nucleosome Assembly Protein 1
NEMO	NF-kB Essential Modulator
NF-kB	Nuclear Factor κB
NG	n-Nonyl-β-D-Glucopyranoside
NIK	NF-κB Inducible Kinase
NK	Natural Killer
NLR	NOD-Like Receptor
NOD	Nucleotide-binding Oligomerisation Domain
OPTN	Optineurin
OTULIN	OTU Deubiquitinase With Linear Linkage Specificity
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PEI	Polyethylenimine
PKD1	Polycystin 1, transient receptor potential channel interacting
Poly(I:C)	Polyinosinic:polycytidylic acid
PRR	Pattern Recognition Receptor
PTM	Post Translational Modification
PYD	Pyrin Domain
RBR	Ring Between Ring Fingers

RIG-I	Retinoic Acid-Inducible Gene I
RHD	Rel homology domain
RHIM	RIPK Homotypic Interaction Motif
RIG	Retinoic acid-Inducible Gene
RING	Really Interesting New Gene
RIP	Receptor-Interacting Protein
RLR	RIG-I Like Receptor
RNF	RING Finger Protein
RPMI	Roswell Park Memorial Institute
SCF	Stem-Cell Factor
SCFβ-TrCP	Skp1, Cul1, Roc1 and the F-box protein β-TrCP
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel
SHARPIN	SHANK-Associated RH-Domain–Interacting Protein
siRNA	Small Interfering RNA
SMAC	Second Mitochondria-Derived Activator of Caspase
STAT1/2	Signal Transducer and Activator of the Transcription 1/2
STING	Stimulator of IFN Genes
TAB 2/3	TAK1-Binding Proteins 2/3
TAK1	Transforming Growth Factor-B-Activated Kinase 1
TANK	TRAF Family Member Associated NF-kB Activator
TAP	Tandem-Affinity Purification
TBK1	TANK-Binding Kinase 1
TCR	T Cell Receptor
TLR3/4	Toll-Like Receptor 3/4
TNF	Tumor Necrosis Factor
TIR	Toll-IL-1 Receptor
TIRAP	TIR Domain containing Adaptor Protein
TNFR	TNF Receptor
TRADD	TNFR1-Associated Via Death Domain
TRAF 2/3/5/6	TNF Receptor-Associated Factor 2/3/5/6
TRAIL1/2	TNF-Related Apoptosis-Inducing Ligand Receptor 1/2
TRAM	TRIF-Related Adaptor Molecule
TRIF	TIR-Domain-Containing Adapter-Inducing Interferon β

UB	Ubiquitin
UBAN	Ubiquitin-Binding domains found in ABINs and NEMO
UBD	Ubiquitin Binding Domain
UIM	Ubiquitin Interacting Motif
UNC93B1	Unc-93 Homolog B1
USP	Ubiquitin-Specific Proteases
XIAP	X-Linked Inhibitor of the Apoptosis
WT	Wild-Type

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