universität wien

DIPLOMARBEIT

Physical and functional characterization of the TBK1/IKKi core complex

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer.nat)

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Wien, im Oktober 2009

Acknowledgements

First of all I would like to thank my mum and my dad for the great support and encouragement during my whole studies. They gave me the opportunity to study whatever I wanted to and gave me the freedom to make my own decisions.

I would like to thank Prof. Dr. Giulio Superti-Furga who gave me the opportunity to work on this exciting project in the middle of this inspiring scientific environment.

I especially want to thank Adriana Goncalves for her constant supervision of my work, her motivation and encouragement at any time, and for reviewing my thesis.

Moreover I would like to thank Dr. Tilmann Bürckstümmer for his constant scientific advice and for reviewing my thesis.

I also want to thank Dr. Keiryn Bennett for the great support in all mass-spectrometry related issues.

Of course I would like to thank all the people at CeMM, I had an amazing year and I am so grateful I got to know them. They always gave a helping hand and provided a great working atmosphere.

Furthermore I would like to thank my whole family and friends, who were always there supporting me in every respect of my life.

THANK YOU!

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ABSTRACT

The proper functioning of the immune system is crucial for a human organism to defend against viral and bacterial infections. Consequently, it is extremely important to understand the complex interplay between molecules of the immune system. Upon infection, several human receptor types recognize conserved molecular patterns that are a feature of pathogens. A large number of proteins then participate in downstream signaling, resulting in expression of antimicrobial genes.

TBK1 (TANK-binding kinase 1) and IKKi (IκB kinase-I) are two related serine/threonine kinases that play an important role in innate immunity signaling. Upon bacterial and viral infection, TBK1 and IKKi activate the transcription factors IRF3/7 and NF-κB. TBK1 and IKKi have been found to interact with three adaptors: TBKBP1, TBKBP2 (TBK binding proteins 1 and 2) and TANK (TRAF family member associated NF-κB activator) (Bouwmeester, Bauch et al. 2004). Yet, the mechanism of TBK1 and IKKi activation and, correspondingly, the role of these proteins are still not fully understood.

The main focus of this study was to investigate the molecular architecture of this complex and to elucidate the role of the proteins concerning TBK1 activity. In order to achieve this we first performed a systematic TAP analysis of all the different components of the complex: the two kinases (TBK1 and IKKi) and the three adaptor proteins (TBKBP1, TBKBP2 and TANK). Even though we confirmed the interaction of the adaptor proteins to TBK1 and IKKi we didn't find any of the binding proteins interacting with each other, suggesting that TBK1 and IKKi are most likely forming independent sub-complexes with each of the adaptors.

In agreements with this hypothesis, immunoprecipitation experiments suggested that all three adaptor proteins bind to the same region of TBK1 (coiled coil 2). After analyzing the coiled coil 2 structure, we were able to identify single amino acids responsible for the interaction. Amino acids at the position M690 and E696 in TBK1 were important for its binding to all the adaptor proteins because mutation of these residues abolished binding to all of the TBK1 adaptors. On the other hand, mutation of L693 selectively abrogated binding of TANK without affecting binding of TBKBP1

or TBKBP2, indicating that the adaptor proteins bind to the same region but make contacts with different amino acids.

Additionally we found that upon overexpression conditions, TBK1 activity was independently of binding to the adaptor proteins. Altogether, these data suggest that each TBK1 adaptor forms a distinct sub-complex that is required for non-redundant functions of TBK1.

ZUSAMMENFASSUNG

Bei einer viralen oder bakteriellen Infektion, ist ein gut funktionierendes Immunsystem überlebenswichtig für den menschlichen Organismus. Ein gutes Verständnis für das Zusammenspiel der Bestandteile des Immunsystems ist daher entscheidend. Verschiedene Rezeptoren des angeborenen Immunsystems erkennen bestimmte molekulare Muster an Krankheitserregern, woraufhin eine große Anzahl von Proteinen an der Signalübertragung beteiligt ist und eine vermehrte Aktivierung antimikrobieller Gene hervorruft.

TBK1 (TANK-binding kinase 1) und IKKi (IκB kinase-I) sind zwei verwandte Serin/Threonin Kinasen und spielen eine wichtige Rolle bei der Signalübertragung im angeborenen Immunsystem. Nach bakterieller oder viraler Infektion aktivieren die beiden die Transkriptionsfaktoren IRF3/7 und NF-κB. Es wurde nachgewiesen, dass TBK1 und IKKi mit drei Bindungsproteinen, TBKBP1, TBKBP2 (TBK binding proteins 1 and 2) und TANK (TRAF family member associated NF-κB activator) interagieren (Bouwmeester, Bauch et al. 2004). Der Mechanismus zur Aktivierung von TBK1 und IKKi, und die Rolle der Bindungsproteine in diesem Prozess sind jedoch noch nicht vollkommen geklärt.

Das Hauptaugenmerk dieser Studie lag auf der Untersuchung der molekularen Architektur des Komplexes und der Bedeutung der Bindungsproteine bezüglich der Aktivität von TBK1 und IKKi. Um das zu untersuchen führten wir eine systematische TAP Analyse von den am Komplex beteiligten Komponenten, den beiden Kinasen TBK1 und IKKi und den Bindungsproteinen TBKBP1, TBKBP2 und TANK, durch .Obwohl wir die Interaktion zwischen den Bindungsproteinen und TBK1 und IKKi bestätigen konnten, haben wir festgestellt dass die Bindungsproteine selbst nicht aneinander binden. Dieses Ergebnis lässt vermuten dass TBK1 und IKKi wahrscheinlich unabhängige Komplexe mit den verschiedenen Bindungsproteinen formen.

Die Verwendung von verschiedenen TBK1 Mutanten und Immunoprezipitationsexperimente haben gezeigt, dass alle drei Bindungsproteine an dieselbe Region in TBK1, die so genannte coled coil 2 Region, binden.

Nach Analyse dieser Region konnten wir 2 Aminosäuren (M690 und E696) finden, welche wichtig für die Bindung der drei Bindungsproteine and TBK1/IKKi sind, da Mutationen in diesen Aminosäuren die Bindung von TBK1 an die 3 Bindungsproteine verhindern. Zusätzlich haben wir eine Aminosäure (an Position L693) entdeckt, die speziell die Bindung von TANK an TBK1/IKKi verhindert, ohne die Bindung von TBKBP1 und TBKBP2 an TBK1 zu beeinträchtigen. Das bedeutet dass die drei Bindungsproteine in der gleichen Region von TBK1 binden, allerdings die Interaktion von einzelnen verschiedenen Aminosäuren abhängt.

In unseren Überexpressionsexperimenten konnten wir außerdem sehen, dass die Aktivität von TBK1/IKKi nicht unbedingt mit der Bindung an die drei Bindungsproteine korreliert. Diese Daten sind ein Indikator dafür, dass die Bindungsproteine verschiedene Subkomplexe formen, welche sich in ihrer Funktion nicht ersetzen lassen.

1 Introduction

Immunity is defined as a biological defense mechanism that protects an organism against diseases. The immune system is established by the coordinated response of immune cell types and molecules to invaders (Abbas and Lichtman 2007). In higher vertebrates the immune system is divided into two parts: the late adaptive and the early innate immune system (Pasare and Medzhitov 2004).

1.1 The adaptive immune system

The adaptive immune system is also called the specific immune system because it has the ability to distinguish between very closely related microbes and molecules in an accurate way. It is capable of reacting to a large number of substances and has the ability to remember. It can therefore react more intensely upon repeated infections (Abbas and Lichtman 2007). The most important cell types in the adaptive immune response are B-lymphocytes and T-lymphocytes, which produce somatically generated receptors on their surface (Medzhitov and Janeway 2000).

There are two types of adaptive immune responses, humoral and cell-mediated immunity. The humoral immune response is conducted by B-lymphocytes which secrete antibodies into the blood and mucosa. The purpose of the humoral immune response is to defend against extracellular microbes and toxins. Cell-mediated immunity, on the other hand, fights against intracellular microbes with the help of T-lymphocytes (Abbas and Lichtman 2007).

An important feature of adaptive immunity is the *clonal selection* hypothesis, which purposes that antigen-specific lymphocytes develop receptors to antigens before exposure to specific antigens. This allows a large number of random lymphocyte clones to arise in the uninfected organism. Upon antigen detection *clonal expansion* results in an increase in the number of cells which express the receptor that recognizes the specific antigen originally detected (Abbas and Lichtman 2007).

It is also crucial for the organism that the immune components do not recognize self-antigens. This unresponsiveness to ‘self’ is called tolerance and is maintained by

several mechanisms. When tolerance fails it leads to disorders called autoimmune diseases (Abbas and Lichtman 2007).

1.2 The innate immune system

The innate immune system is defined as the initial response to microbes but it also stimulates the adaptive immune response. It consists of several barriers to prevent, control and defend against microbes. The physical and chemical barriers like epithelia and antimicrobial substances represent one of the first barriers of the innate immune system. If pathogens penetrate these initial barriers and invade an organism, phagocytic cells (e.g. macrophages and dendritic cells) and natural killer (NK) cells, the members of the complement system, cytokines and other blood proteins coordinate the innate immune response (Medzhitov and Janeway 2000).

The evolutionarily conserved innate immune system is the first line of defense against the microbial or viral invasion. It recognizes a set of highly conserved structures, which are specific to microbes. These structures are recognized by germ line encoded pattern-recognition receptors (PRRs) (Pasare and Medzhitov 2004). The microbial structures, generally referred to as PAMPs (pathogen-associated molecular patterns), are specifically produced by pathogens and not by their hosts. They are crucial for either survival or pathogenicity of the microbes and are usually a characteristic feature of a whole group of microorganisms. For instance, lipopolysaccharides (LPS) are always present in the membrane of gram negative bacteria (Medzhitov and Janeway 2000).

1.2.1 Signaling in innate immunity

The PRRs used by the innate immune system are divided into several groups. Their main function is to immediately protect the host from pathogens by activating the complement pathway and phagocytosis (Pasare and Medzhitov 2004). PRRs are mainly on the plasma membrane of effector cells such as macrophages, dendritic cells and B-lymphocytes but they can also been found in the host cytoplasm. The ability of effector cells to perform their functions immediately after recognizing the pathogen makes the innate immune system crucial for host survival (Medzhitov and Janeway 2000).

Examples of PRRs include Toll-like receptors (TLRs), (RIG-I)-like receptors (RLRs), C-type lectins, scavenger receptors, N-formyl Met-Leu-Phe receptors and (NOD)-like receptors (NLRs) (Abbas and Lichtman 2007). The signaling mediated by different receptors also leads to cross-talk further downstream to maintain effective immune response (Lee and Kim 2007).

1.2.1.1 NLR signaling

Nucleotide-binding oligomerization domain (NOD)-like receptors are a large family of receptors located in the intracellular part of the cell. NLRs typically contain N-terminal protein interactor domains, central nucleotide binding domains and C-terminal LRRs. The N-terminal structures help to divide the NLRs into 5 subfamilies. NLR signaling either activates NF-κB and MAPK, or certain NLRs also play a role in the caspase-1 mediated activation of the inflammasome (Kawai and Akira 2009).

1.2.1.2 RLR signaling

The retinoic acid-inducible gene-I (RIG-I)-like receptors are part of the cytosolic detection system belonging to the innate immune system. They are part of the RNA helicase family and specifically recognize RNA species from invading viruses in the cytosol. There are 3 family members: RIG-I itself, MDA5 and LGP2. Signaling via RLR results in the induction of Type-I Interferon and several inflammatory cytokines via IRF, MAPK and NF-κB pathways (Kawai and Akira 2009) (Nakhaei, Genin et al. 2009).

1.2.1.3 TLR signaling

The name Toll-like receptors originates from the protein Toll which is essential for the determination of the dorsoventral polarity during embryogenesis in *Drosophila*. It has been found that flies which have a mutation in the gene for *toll* are much more sensitive to fungal infections. These findings indicated that Toll is a receptor that detects fungal infections and leads to activation of the innate immune system (Lemaitre, Nicolas et al. 1996). Later a gene similar to Toll was detected in humans (which is now referred to as TLR4) and it was found that this gene can induce the expression of inflammatory cytokines (Kawai and Akira 2005). The mammalian

family of Toll-like receptors currently consists of 12 members (Kawai and Akira 2009). The different receptors recognize different pathogen associated molecular patterns (PAMPs): TLR2 detects bacterial lipoproteins and lipoteichoic acid, TLR5 detects flagellin, whereas TLR3 recognizes dsRNA, TLR4 recognizes LPS and TLR9 recognizes unmethylated CpG of bacteria and viruses (Iwasaki and Medzhitov 2004) (Aderem and Ulevitch 2000).

The TLRs can be divided in two groups based on their localization in the cell. TLR1, 2, 4, 5, 6 and 11 are only expressed on the cell surface and therefore detect membrane components of their respective pathogens such as lipids, proteins and lipoproteins. In contrast TLR3, 7, 8 and 9 are located in the endosome, lysosome or the endoplasmatic reticulum and recognize microbial nucleic acid (Kawai and Akira 2009). In human and *Drosophila* there are two characteristic Toll-like receptor domains: the leucine-rich repeats (LRR domain) and the Toll/interleukin-1 receptor domain (TIR domain). The TIR domain is present in all receptors and is responsible for downstream signaling. The adaptor molecules MyD88, TIRAP, TRIF and TRAM bind specifically to the TIR domain and recruit a downstream signaling cascade (Kawai and Akira 2005) (Fitzgerald, Rowe et al. 2003).

1.2.1.4 Activation of the NF-κB Pathway

The transcription factor NF-κB is crucial for the regulation of the development and maintenance of the immune system. It consists of homo- and heterodimers of five members of the Rel family which are NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB and c-Rel (Pomerantz and Baltimore 2002).

The TLRs and IL-1R (Interleukin-1 receptor) mediate the activation of NF-κB through the canonical IKKs (IKKα, IKKβ and IKKγ/NEMO). During this activation process the IKKs phosphorylate the NF-κB inhibitor IκB which leads to its degradation. NF-κB is then free and translocates into the nucleus to regulate the expression of certain target genes (Kawai and Akira 2005). This is the so-called canonical pathway of NF-κB activation.

In addition to the canonical activation of NF-κB, a non-canonical NF-κB pathway exists that is regulated by two kinases related to the IKKs, called TBK1 (TANK-binding kinase 1) and IKK-i (inhibitor of kappa B kinase epsilon or IKK-ε) which are also involved in the activation of NF-κB (Kawai and Akira 2005).

1.2.1.5 Activation of the IRF3/IRF7 Pathway

Another pathway, strongly regulated by the two kinases TBK1 and IKKi is the IRF3/7 pathway. Under non-stimulated conditions the transcription factor IFN regulatory factor (IRF)-3 is present in the cytoplasm. Upon stimulation IRF3 is phosphorylated at certain serine residues, homodimerizes and interacts with the co-activators CREB-binding protein (CBP) and p300 to enter the nucleus (Fitzgerald, McWhirter et al. 2003). Once in the nucleus, it activates promoters containing IRF3-binding sites and induces the expression of IFN-β and other target genes. It has been shown that TBK1/IKKi phosphorylate the serine sites on IRF3 responsible for activation (Kawai and Akira 2005). TBK1 or IKKi single knockout mice show moderate phenotypes regarding IRF3 activation: IKKi deficiency has no impairment on IRF3 activation and TBK1 deficient mice have only partial effects. However, mice deficient in both kinases show an almost complete abolishment of IRF3 activation. TBK1 and IKKi are therefore partly redundant for the activation of IRF3 (Ryzhakov and Randow 2007) (Yamamoto, Sato et al. 2003).

1.2.1.6 Crosstalk between NF-κB and IRF pathway

Crosstalk is a common feature in signal transduction and this is illustrated by the activation pathways of NF-κB and IRF (Figure 1). For instance the physical interaction between TANK (TRAF family member-associated NF-κB activator) and NEMO (inhibitor of kappa B kinase gamma) might be the basis for the formation of a p65-IRF3 complex which is required for the expression of several genes upon LPS stimulation. Another example is the A20 protein, which inhibits both the NF-κB and IRF pathway by polyubiquitination of the receptor interacting protein (RIP1) upon TNF-α stimulation. It directly prevents the IRF3 dimerization via binding to TBK1 and IKKi upon stimulation with dsRNA. Conversely, TRAF3 either negatively regulates

the alternative NF-κB pathway, or positively regulates TLR- and RIG-I-mediated IRF3 activation (Chau, Gioia et al. 2008).

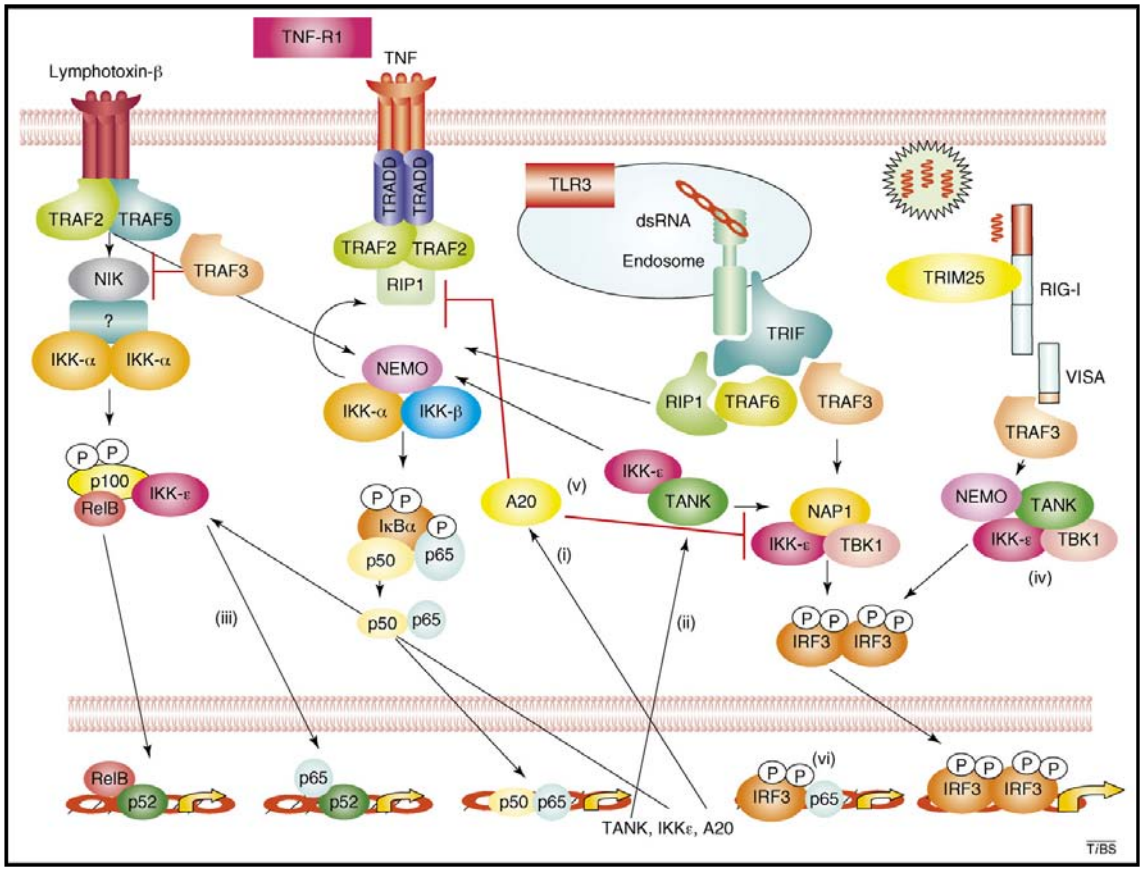

Figure 1: Crosstalk between the NF-κB and the IRF3 pathway (Chau, Gioia et al. 2008)

1.3 TBK1/IKKi

The non-canonical IκB kinase homologues TBK1 (TANK-binding kinase) also known as NAK (NF-κB activating kinase) or T2K (TNF-receptor associated factor 2 (TRAF2)-interacting kinase), and IKKi (IκB kinase-I also known as IKK-ε) are serine/threonine protein kinases. They are non-canonical members of the IKK family that have been shown to mediate innate immunity signaling during the process of NF-κB, IRF3 ad IRF7 activation (Chau, Gioia et al. 2008) (Guo and Cheng 2007).

1.3.1 Structure and function of TBK1 and IKKi

TBK1 was identified as a TANK-interacting protein using a two-hybrid screen. There is 94% analogy between murine and human TBK1. TBK1 is a protein composed of 729 amino acids which are divided into an N-terminal kinase domain, a ubiquitin-like domain (ULD) and two C-terminal coiled coil regions. (coiled coil 1: residues 603-650, coiled coil 2: residues 679-712). TBK1 has a 27% homology with IKKα and 45% homology with IKKβ within the residues 9-353 of TBK1. Immunoprecipitation experiments, however, showed that there was no binding of TBK to either IKKα, IKKβ or NIK (Pomerantz and Baltimore 1999).

IKKi was found in experiments that attempted to isolate genes that respond to immunological stimuli. IKKi is expressed mainly in immune cells in response to LPS or other inflammatory cytokines. The protein encodes 717 amino acids and consists of an N-terminal serine/threonine kinase domain, a ubiquitin-like domain (ULD) and a C-terminus leucine zipper and potential helix-loop-helix domain. Due to the shared 30% homology with IKKα and IKKβ after amino acid alignments it was called IKK-ε (or IKKi). The NF-κB stimulatory properties of IKKi were taken into consideration after it was shown that IKKi could phosphorylate IκB-α at Ser32 and Ser36 (Shimada, Kawai et al. 1999).

The two serine/threonine kinases TBK1 and IKKi share 64% sequence similarities and although they mediate the same signaling pathways there are several differences in downstream signaling (Ikeda, Hecker et al. 2007). For instance, during poly(I:C) stimulation in TBK1-deficient fibroblasts there is a decrease in IRF3 activation, whereas IRF3 activation occurs normally in IKKi-deficient fibroblasts (Hemmi, Takeuchi et al. 2004). It is also proposed that TBK1 and IKKi differ in DNA-virus mediated IFN response but operate redundantly in RNA-virus mediated IFN response (Miyahira, Shahangian et al. 2009).

Nevertheless, both kinases contain an ubiquitin-like domain (ULD). In mouse TBK1 the domain starts at amino acid 305 and is 79 aa long. The ULD in mouse IKKi shares 65% similarity with the TBK1 ULD. It has been shown that the ULD is required for kinase activation and IRF3 phosphorylation, because the binding of TBK1 on the IRF association domain (IAD) of IRF3 depends on the ULD domain. It

has also been found that deletion of the ULD in TBK1 and IKKi inhibits the phosphorylation on Ser172, which is a crucial phosphorylation site for kinase activity. These findings led to a model in which TBK1-ULD is transiently bound to IRF3-IAD but upon IRF3 phosphorylation it is freed, dimerizes and translocates to the nucleus (Ikeda, Hecker et al. 2007).

Besides the function in innate immunity signaling there have been several studies that propose a role for TBK1/IKKi in cell proliferation and tumor progression (Clement, Meloche et al. 2008). These findings further suggest that TBK1/IKKi must be tightly regulated.

Interestingly an alternative splicing form of TBK1, termed TBK1s has recently been found. TBK1s lacks exon 3-6 which leads to an in frame deletion of the kinase domain. TBK1 displays an inhibitory effect on virus-induced IFN-β induction (Deng, Shi et al. 2008).

1.3.2 The role of TBK1/IKKi in innate immunity signaling

Mice deficient in TBK1 die from liver apoptosis *in utero* in the same way as do mice deficient for p65, IKKβ or NEMO. It was therefore believed that, like the canonical IKKs, TBK1 is a NF-κB activating kinase (Bonnard, Mirtsos et al. 2000). During the characterization of TBK1-deficient cells, it was later found that TBK1 shows a significant role in type-I Interferon gene induction through the phosphorylation of IRF3 and IRF7 (Chau, Gioia et al. 2008).

Focusing on the cell-surface receptor TLR2, TLR3 and TLR4, there are 2 signaling pathways known (Figure 2). In the first MyD88 gets recruited and activates NF-κB via the canonical IKKs (IKKα, IKKβ and IKKγ/NEMO). In the second pathway TRIF gets recruited and either activates NF-κB as well via the canonical IKKs, or activates IRF3 with the help of TBK1/IKKi.

Some of the TLRs, like TLR3, which are located on the intracellular compartments and sensor foreign nucleic acids activate inflammatory cytokine production via the NF-κB pathway, but also trigger the TBK1/IKKi mediated activation of Type-I IFN (Kawai and Akira 2009).

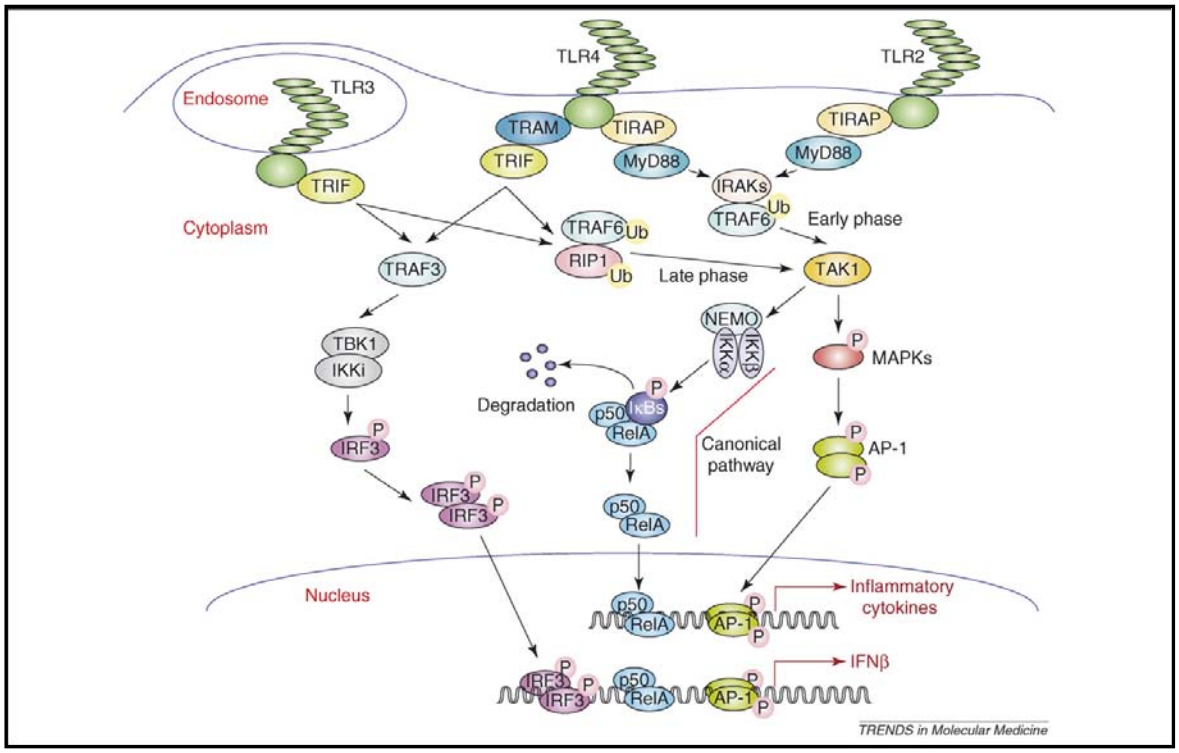

Figure 2: TLR2-, TLR3-, TLR4- mediated signaling (Kawai and Akira 2007)

Also, the intracellular RIG-I dependent receptor promotes TBK1/IKKi induced activation of IRF3 and IRF7. MAVS and further downstream TRAF3 mediate the binding of the receptor to TBK1/IKKi and downstream signaling might be accompanied by some of the scaffold proteins (Chau, Gioia et al. 2008) (Figure 3).

Additionally dsDNA is known to activate inflammatory cytokine and Type-I IFN production through the activation of IKKs and TBK1/IKKi (Kawai and Akira 2009). Therefore it is believed that in addition to TLR9, which is activated by hypomethylated DNA, another cytosolic DNA receptor is able to recognize DNA. Recently one candidate has been found which is called ZBP1 or DAI (DNA-dependent activator of IFN-regulatory factors). This protein successfully enhances DNA-mediated IFN induction in mouse fibroblasts. Via binding to double-stranded DNA it allows association with IRF3 and TBK1 (Takaoka, Wang et al. 2007) (Figure 3). The mechanism by which this occurs is not yet fully understood, although two kinases RIP1 and RIP3 have been identified which are crucial for DAI-induced NF-κB signaling (Rebsamen, Heinz et al. 2009).

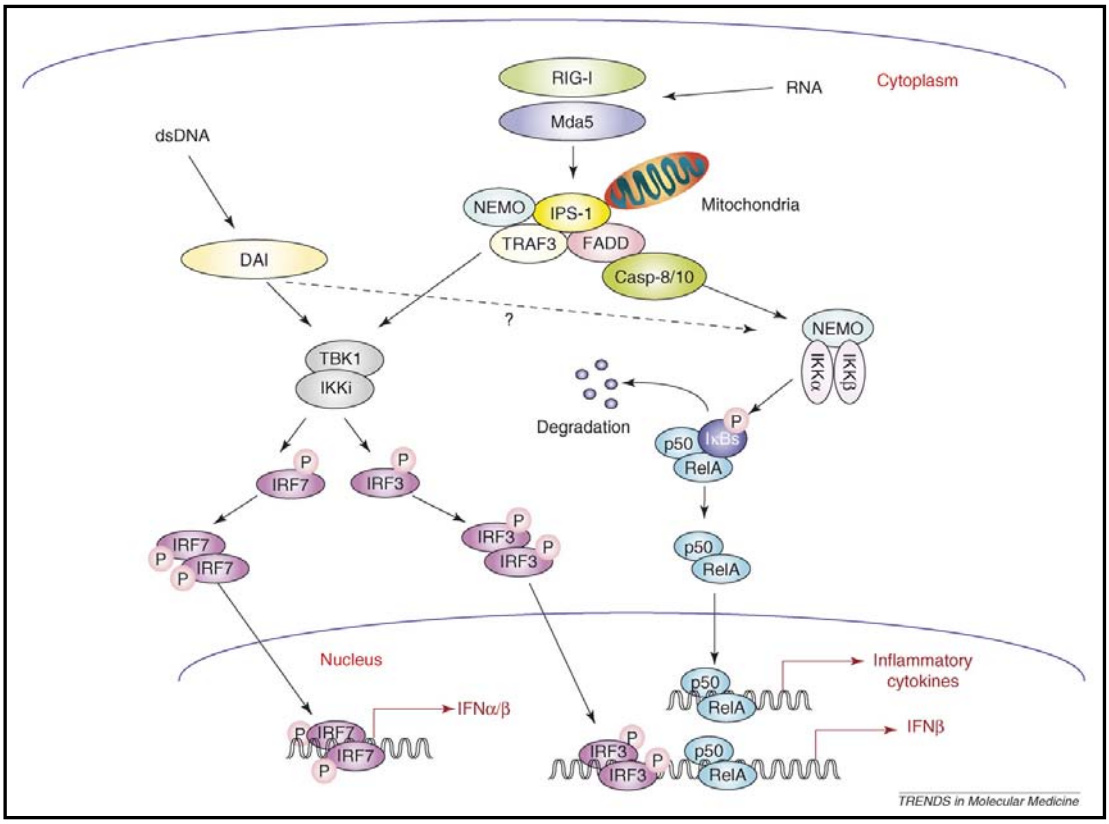

Figure 3: Antiviral signaling pathways mediated by IKK-related kinases (Kawai and Akira 2007)

Additionally it has been shown recently that another protein, called STING (stimulator of interferon genes), is required for the non-CpG intracellular DNA mediated induction of IFN. STING knockout mice were also shown to be sensitive to lethal infections after exposure to herpes simplex virus 1 (HSV-1). Interestingly STING also relocalized with TBK1 from the endoplasmic reticulum to perinuclear vesicles (Ishikawa, Ma et al. 2009).

Taken together TBK1 and IKKi are crucial for the activation of NF-κB and IRF3/7 pathways, as described above but in all the cases need the support of certain adaptor proteins for proper signaling.

1.3.3 TBK1/IKKi interaction partners

TBKBP1 (also referred to as SINTBAD), TBKBP2 (also referred to as NAP1, NAK-associated protein1 or Azi 2) and TANK (also referred to as I-TRAF) are three described interaction partners, which may support the activation of inflammatory cytokines of Type-I interferon (Bouwmeester, Bauch et al. 2004). Recent findings

indicate that the distinct interactors might act in a pathway specific manner. TBKBP2 is required for the TLR-mediated IRF3 activation, whereas TANK has a specific role in IRF3 activation via the LPS-induced TLR4 but not via the ds-RNA-mediated TLR3 (Gatot, Gioia et al. 2007). Interestingly TANK, TBKBP1 and TBKBP2 signaling pathways do not only mediate TLR-induced signaling but also RIG-I dependent signaling to trigger TBK1 and IKKi activation.

Knockdown of either TBKBP1, TBKBP2 or TANK inhibited IRF reporter construct activation upon Sendai virus infection. This was also observed by knocking down MAVS but not by knocking down TRIF. Therefore an activation mechanism of IRF3 via TBK1 after Sendai virus infection seems to be mediated via MAVS instead of TRIF. On the other hand TBK1, TRIF and also MAVS are required for IRF activation upon poly(I:C) stimulation. Here the knock-down of TBKBP1, TBKBP2 or TANK also reduce the IRF reporter activity. Concerning the NF-κB pathway neither TBK1, MAVS, TRIF nor TBKBP1, TBKBP2 or TANK knock-down impaired the signaling upon TNF-α, peptidoglycan or PMA stimulation (Chau, Gioia et al. 2008) (Kawagoe, Takeuchi et al. 2009).

In additional to the signaling properties of these proteins it is also believed that the adaptors are involved in post-translational modifications. For instance TANK gets phosphorylated in macrophages by TBK1 and IKKi upon LPS stimulation and is independent of their kinase activity. The kinases are also responsible for Lys63-linked polyubiquitination of TANK, independently of phosphorylation (Gatot, Gioia et al. 2007). This process is related to the polyubiquitination of the IKK adaptor protein NEMO (Tang, Wang et al. 2003).

In contrast to the adaptor protein of the canonical IKKs NEMO, very little is known about the adaptors of TBK1/IKKi. Nevertheless it has been shown that TANK, TBKBP1, and TBKBP2 share a so-called TBK1/IKKi binding domain (TBD) with which, as the name implies, they bind to these kinases. Structurally the three binding proteins share distinct regions of homology, like the coiled coil regions, the TBD and zinc finger structures, in a pair wise fashion as described below (Ryzhakov and Randow 2007).

1.4 TANK

TANK was discovered to be a TRAF-interacting protein which helps to induce NF-κB reporter gene expression with TRAF2 (Cheng and Baltimore 1996). It was later found to bind to the canonical IKK subunit IKKγ/NEMO, but its role remained unclear due to the fact that TANK knock-down cells didn't show any effect on TNF-α or LPS-induced IKK activation (Chariot, Leonardi et al. 2002) (Chau, Gioia et al. 2008). TANK has both stimulatory and inhibitory effects on activation of the transcription factor NF-κB. With the use of the N-terminal domain (residue 1-168) and a central region (residue 169-190) which is required for TRAF2 interaction, TANK acts as a stimulatory protein on NF-κB activation in the presence of low amounts of TRAF2. However, the C-terminal domain of TANK (residue 190-413) appeared to have an inhibitory effect on the N-terminal domain *in trans* during co-expression of high amounts of TRAF2, leading to inhibition of NF-κB activation (Cheng and Baltimore 1996).

TANK is probably required for the upstream action on TBK1/IKKi, leading to an IKK independent activation of NF-κB. This theory is supported by fact that TANK constitutively binds TBK1 and IKKi (Bouwmeester, Bauch et al. 2004) (Pomerantz and Baltimore 1999) (Nomura, Kawai et al. 2000). Apparently TBK1, TRAF2 and TANK form a ternary complex that leads to the NIK-IKK cascade but this signaling pathway is not required for the NF-κB activation via TNF-α, IL-1 or CD40. Therefore TANK is believed to have two functions in NF-κB activation, one inhibitory function in a TBK1-independent pathway and a contrary stimulatory function which depends on TBK1 (Pomerantz and Baltimore 1999).

Additionally TANK might play a role in RIG-I dependent signaling pathway against viral infection, due to the fact that TANK knockdown MEFs show much higher viral titer and inhibit the production of IFN. The interaction of TANK with the adaptor protein MAVS upon Sendai virus infection supports this suggestion (Kawagoe, Takeuchi et al. 2009) (Guo and Cheng 2007).

To compare the structures of the 3 binding proteins the domain composition of each of the three adaptors was published (Figure 4). In the case of TANK, a N-terminal CC domain is followed by the so-called TBK1/IKKi binding domain (TBD). In the case of TANK the TBD is also required for TRAF2 binding. The C-terminal part, which is known to have an inhibitory effect on NF-κB activation includes one zinc finger domain on the very C-terminus (Ryzhakov and Randow 2007).

1.5 TBKBP1

TBKBP1 is required for IRF3 activation upon Sendai virus infection just like TANK and TBKBP2. TBKBP1 was shown to specifically interact with TBK1/IKKi but not with the canonical IKKs (Ryzhakov and Randow 2007).

Similarly to TANK it is composed of 3 coiled coil regions in the N-terminus and the TBK1/IKKi binding domain. The C-terminal part of TBKBP1 contains a proline-rich region and two C2H2 type zinc finger domains similar to that found in TANK (Figure 4). It was also found that TBKBP1 preferably forms homo-oligomers and to a lesser extent heterocomplexes with TBKBP2. No interaction was identified with TANK (Ryzhakov and Randow 2007).

1.6 TBKBP2

Originally TBKBP2 was discovered to be an activator of IKK-related kinases. It was also believed that TBKBP2 protects cells from apoptosis, induced via TNF-α. According to this, TBK1 and IKKi recruit the subunit TBKBP2 and activate NF-κB upon virus stimulation. TBKBP2 was shown to bind to RIG-I and MDA5 on a different site than it binds to TBK1. Additionally TBKBP2 was found to co-precipitate with TRIF and MAVS insinuating a role of TBKBP2 in TLR3 and RIG-I/MDA5 dependent activation of IRF3 and in RIG-I/MDA5 dependent activation of NF-κB upon viral infection (Sasai, Shingai et al. 2006).

TBKBP2 shares structural similarities with TBKBP1 on their first 80 amino acids in the N-terminal coiled coil regions, whereas the coiled coil region 2 is more similar to the single coiled coil in TANK. Similar to the two other adaptor proteins of TBK1/IKKi, TBKBP2 also contains the TBD (Ryzhakov and Randow 2007) .

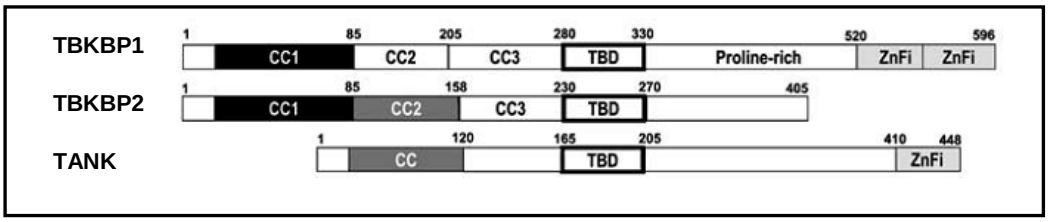

Figure 4: Domain composition of TBKBP1, TBKBP2 and TANK modified from (Ryzhakov and Randow 2007)

1.7 Aim of the study

TBK1 and IKKi are important kinases in innate immunity signaling and have been found to occur in a complex with the adaptor proteins TBKBP1, TBKBP2 and TANK. The aim of this thesis is to investigate the architecture of this core complex and to define the relationship between the assembly of the complex and the activity of TBK1.

The approach we used in this study was tandem affinity purification (TAP) followed by mass-spectrometry analysis in order to further define the structure and binding properties of TBK1 with the adaptors. The creation of several TBK1 mutants for use in co-immunoprecipitation experiments helped to determine the exact binding region to TBKBP1, TBKBP2 and TANK. In addition, autophosphorylation studies, reporter gene assays and kinase assays were performed to investigate the impact of binding between TBK1 and the adaptors, on TBK1 activity. Finally we performed immunoflourescence staining in order to investigate the subcellular localization of TBK1 and its binding proteins.

This study therefore helps to understand the molecular architecture of the TBK1 complex, the assembly of which is needed for TBK1 to perform downstream signaling.

2 Materials and Methods

2.1 Cell culture and transfection

HEK293

HEK (Human embryonic kidney) 293 cells were used for reporter gene assay and kinase assay. The cells were cultured in DMEM media (PAA) containing 10% FCS (Gibco) and 1% Penicillin/Streptomycin (PAA) at 37°C with 5% $CO_{2.}$ To detach the cells from the surface of the plate 0.05% Trypsin-EDTA (PAA) was used.

HEK293T

HEK293T (where T stands for large T-antigen of SV40 (simian virus 40)) cells were used for co-immunoprecipitation and Western Blots (P-TBK1, TBK1, myc, V5) because of their high transfection efficiency. The cells were cultured in DMEM media (PAA) containing 10% FCS (Gibco) and 1% Penicillin/Streptomycin (PAA) at 37°C with 5% $CO_{2.}$ To detach the cells from the surface of the plate 0.05% Trypsin-EDTA (PAA) was used.

HeLa

HeLa cells were used for Immunofluorescence experiments. The cells were cultured in DMEM media (PAA) containing 10% FCS (Gibco) and 1% Penicillin/Streptomycin (PAA) at 37°C with 5% $CO_{2.}$ To detach the cells from the surface of the plate 0.05% Trypsin-EDTA (PAA) was used.

RAW264.7 Macrophages

RAW264.7 cells were taken to perform tandem affinity purification. The cells were cultured in DMEM media (PAA) containing 10% FCS (Gibco) and 1% Penicillin/Streptomycin (PAA) at 37°C with 5% $CO_{2.}$ To detach the cells from the surface of the plate RAW dissociation buffer was used.

Freezing cells

To freeze the cells usually one 10 cm dish was used as one aliquot. The cells were detached from the surface and centrifuged at 300 x g at RT for 5 min. Then the pellet was resuspended in DMEM (PAA) containing 20%FCS (Gibco) and 20%DMSO (Merck). The cells were immediately put on ice then transferred into a cryo-box at -80°C. On the next day the cells were transferred to the liquid nitrogen tank.

Thawing cells

For thawing a new aliquot of cells, one tube was taken out of the liquid nitrogen tank and quickly thawn in a water bath at 37°C. Then the cells were immediately transferred into 10 ml of culture medium and centrifuged at 300 x g at RT for 5 min. The cells were resuspended and seeded on a 10 cm dish.

Transient Transfection

For a transient transfection the cells were seeded 1 day before transfecting (table 1). Lipid based transfection was performed using Polyfect (QIAGEN) Therefore the DNA was diluted into serum-free DMEM (PAA) and then Polyfect was added. After incubation of 5-10 min at RT the appropriate amount of FCS containing culture media was added. Finally the mixture was put drop-wise onto the cells. Then the cells were incubated at 37°C for 24h to 48h.

Plate format	**Cells per well**	**DMEM (w/o FCS)**	**DNA**	**Polyfect**	**DMEM (with FCS)**
24 well plate	1-1.5 x 10^5	15 µl	0.3 – 1 µg	5 µl	85 µl
10 cm plate	6 x 10^6	300 µl	6 +6 µg (double-transfection) or 10 µg	60 µl	700µl

Table 1: Transient transfection schedule

Cell Lysis

All the steps were performed at 4°C. To lyse the cells the media was sucked off and the cells were washed with ice-cold 1xPBS (PAA). Then the appropriate amount of ice cold lysis buffer (table 2) was added to the cells. For Immunoprecipitation the cells were lysed in Frackelton buffer whereas for reporter gene assays the cells were lysed in IP-Buffer. Using a cell scraper (10 cm dish) or gently pipetting up and down (24-well plate) the cells were detached from the surface and collected in a 1.5 ml Eppendorf tube. Then the lysates were centrifuged at 14000 x g at 4°C for 15 min, the supernatant was collected and protein concentration was measured using Bradford reagent (BioRad).

Plate format	**Amount of buffer**
24 well	100 µl
10 cm plate	750 µl

Table 2: Amount of lyses buffer

Used Reagents

- RAW-dissociation buffer: 135mM KCl, 15mM sodium citrate
- Frackelton buffer: 10mM Tris/HCl pH7.4, 50mM NaCl, 30mM Na-pyrophosphate, 1% Triton X-100 → before use 1mM PMSF, 1mM DTT, 100µM Na_3VO_4 and 100µM NaF was added
- IP Buffer: 50mM Tris/HCl pH 7.5, 150mM NaCl, 1% NP-40, 5mM EDTA, 5mM EGTA, → before use 1mM PMSF, 1mM DTT, 100µM Na_3VO_4 and 100µM NaF was added

2.2 Cloning strategy

For the different mutations of TBK1 the primers taken are listed below (table 3). For the adaptors TBKBP1, TBKBP2 and TANK, the mouse sequence (PubMed) was taken and cloned into gateway compatible vectors.

Primer name	Primer sequence
TBK1 M690A fw	GATGACTCTTGGTGCGAAGAAGTTAAAGG
TBK1 M690A rv	CCTTTAACTTCTTCGCACCAAGAGTCATC
TBK L693A fw	GGTATGAAGAAGGCAAAGGAGGAGATGG
TBK L693A rv	CCATCTCCTCCTTTGCCTTCTTCATACC
TBK1 K696E fw	GAAGTTAAAGGAGAAGATGGAAGGCGTGGTTAAG
TBK1 K696E rv	CTTAACCACGCCTTCCATCTtCTCCTTTAACTTC
TBK1 N707A fw	GGAGCTGGCCGAGGCCAATCATATTTTAG
TBK1 N707A rv	CTAAAATATGATTGGCCTCGGCCAGCTCC
TBK1 Δ CC1 fw	GATAAGTCGGAAGAGCTGCCTCAGAAAATGC
TBK1 Δ CC1 rv	GCATTTTCTGAGGCAGCTCTTCCGACTTATC
TBK1 ΔCC2 fw	CGAGTTACAAGAAACTTGACCTCAGAAAATGCTCG
TBK1 ΔCC2 rv	CGAGCATTTTCTGAGGTCAAGTTTCTTGTAACTCG
TBK1 Δ ULD fw	CTTTGCAGAGACCAGTCGGGAACAACTCAATAC
TBK1 Δ ULD rv	GTATTGAGTTGTTCCCGACTGGTCTCTGCAAAG
TBK1 Δ C-terminus fw	CCTATCTTTGTCACGTGACGGGAACAACTCAATACC
TBK1 Δ C-terminus rv	GGTATTGAGTTGTTCCCGTCACGTGACAAAGATAGG

Table 3: List of primers used in this study

2.2.1 PCR

To amplify the constructs and to clone them into appropriate vectors, PCR was used (table 4).

Template	1 µl
10 µM Primer mix	2 µl
10 x Advantage Buffer	5 µl
TAQ advantage Polymerase	1 µl
dNTPs	2 µl
ddH_2O	39 µl
total	**50µl**

Table 4: PCR composition

The amplification process was performed as following:

Primery Denaturation	98°C	2 min	
Denaturation	98°C	30 sec	25x
Annealing	55°C	1 min	
Elongation	68°C	1min/kb	
Final Elongation	68°C	10 min	

The PCR was then loaded on a 1% Agarose (InvitroGen) gel and the bands at the right size were cut out and purified using a QIAquick gel extraction kit (QIAGEN).

2.2.2 Mutagenesis PCR

To insert point mutations or deletions, Mutagenesis PCR was performed. Therefore the following reagents were mixed (table 5).

Template	1 µl
10 µM Primer mix	2.4 µl
10 x Buffer	5 µl
Pfu Polymerase	1 µl
dNTPs	1 µl
ddH_2O	39.6 µl
total	**50µl**

Table 5: Mutagenesis PCR composition

The amplification process was performed as following:

Primary Denaturation	95°C	30 sec	
Denaturation	95°C	30 sec	20x pointmutation
Annealing	55°C	1 min	18x deletion
Elongation	68°C	5min	

After PCR reaction the unmethylated plasmid was digested with Dpn1 to obtain only the mutated version of the plasmid. Therefore 1 µl DpnI (New England BioLabs) was mixed to each PCR tube and incubated for 1h at 37°C. Afterwards 2-3 µl of the mix were used for Transfection.

2.2.3 Gateway cloning

For easier cloning procedure gateway cloning was used. Therefore gateway compatible primers were prepared in order to clone the construct into the pDonor vector.

N-terminal fusions

Sense attB1 primer: 5‘ - **Gggg aca agt ttg tac aaa aaa gca ggc t**cc $(NNN)_{5-10}$ –3‘

Antisense attB1 primer: 5‘ – **gggg ac cac ttt gta caa gaa agc tgg gt** STOP $(NNN)_{5-10}$ –3‘

C- terminal fusions

Sense attB1 primer:5‘ - **Gggg aca agt ttg tac aaa aaa gca ggc t***ag act gcc* atg $(NNN)_{5-10}$ –3‘

Antisense attB1 primer: 5‘ – **gggg ac cac ttt gta caa gaa agc tgg gt***NOSTOP* $(NNN)_{10-15}$ –3‘

BP reaction

A BP reaction was performed in order to clone the obtained PCR fragment into the pDONR vector (Figure 5). The reaction was performed at RT for 1h (table 6) and stopped by adding 1 µl of proteinase K.

Template (PCR fragment)	3 µl
pDONR	1 µl
BP clonase	2 µl
ddH_20	4 µl
total	**10 µl**

Table 6: Composition of the BP reaction

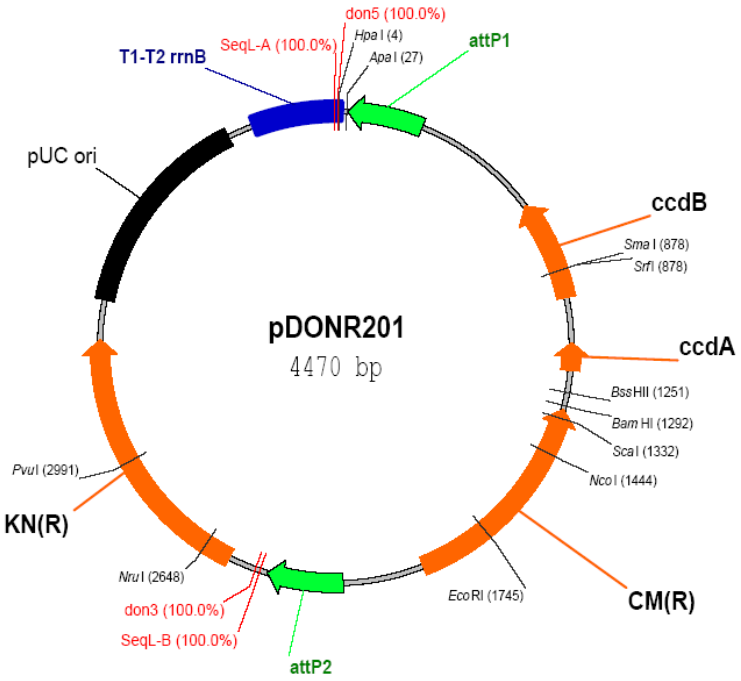

Figure 5: Vector map of pDONR201

LR reaction

The plasmid preparation obtained from the BP reaction was further used to clone the gene of interest into a compatible gateway destination vector containing the preferred tag (table 7).

backbone	tag	resistance
pCS2-Nterm-6myc	6myc	Amp
pTRACER-Cterm-V5	V5	Amp
pSG-Nterm-4HA	4HA	Amp

Table 7: List of destination vectors

The LR reaction was carried out for 1 h at 37°C 1h (table 8) and stopped by adding 1 µl proteinase K.

Destination vector	1 µl
Entry Clone	3 µl
LR clonase	2 µl
ddH_20	4 µl
total	**10 µl**

Table 8: Composition of the LR reaction

Transformation DH5α

2µl of the BP or LR reaction were transformed into subcloning Efficiency DH5α bacteria (InvitroGen). After incubation on ice for 30 min the bacteria were heat-shocked at 42°C for 30 seconds. After incubation at 4°C for 2 min 750 µl of SOC media was added and the bacteria were shaken on 37°C for 1 hour. Then the cells were plated on an agar plate containing the appropriate antibiotic for selection.

BsrG1 digestion

To confirm the correct cloning during the BP or LR reaction a BsRG1 digestion was performed for 1h at 37°C (table 9).

Plasmid to test	8 µl
Buffer (New England Biolabs)	1 µl
BsRG1 (New England Biolabs)	1 µl
total	**10 µl**

Table 9: BsrGI digestion

To analyze the digestion, the mix was loaded on a 1% agarose gel (InvitroGen). The right clones were further analyzed by sequencing.

Miniprep

3 clones were picked and inoculated in 3 ml media containing the respective selection antibiotics. They were grown o/n shaking at 37°C. On the next day the plasmid was isolated using a QIAprep spin miniprep kit (QIAGEN).

Maxiprep

After analyzing the sequence by microsynth the remaining Miniprep-culture was used to inoculate 250ml LB media with the correct antibiotic for selection. After growing the culture o/n at 37°C maxiprep was performed using a HiSpeed Plasmid Maxi Kit (QIAGEN).

2.3 Tandem Affinity Purification

For TBK1, IKKi and each of the adaptor proteins two TAP-pulldowns were performed. Stable RAW264.7 macrophage cell lines expressing the respective bait protein with the GS-TAP tag (existing in the lab, Figure 6) were taken.

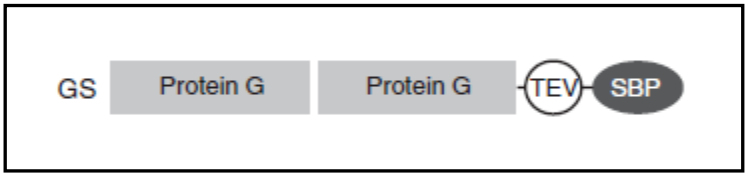

Figure 6: Composition of the GS TAP tag (Burckstummer, Bennett et al. 2006)

The cells were collected, lysed in lysis buffer and incubated on ice for 20-30 minutes. Then the cell suspension was centrifuged 15 min at 15000 x g. The supernatant was taken and again centrifuged with an Ultracentrifuge for 1h at 100000 x g. 90µl sample was collected for Western Blot analysis. In the meantime the rabbit IgG Agarose (Sigma) was washed 2-3x (1000rpm) with lysis buffer. Then 200µl of beads were added to the lysates and incubated at 4°C shaking for 2h. The beads were than collected by centrifugation at 600rpm, 1 min, 4°C whereas an aliquot of the

supernatant was kept, transferred into a small column (0.8 ml MoBiColM1002) and washed with 10ml lysis buffer. Then the sample was washed with 5ml TEV cleavage buffer (0.2% detergent, no protease inhibitors!) and afterwards 360µl TEV cleavage buffer with 40µl TEV protease was added. The mixture was incubated for 1h at 16°C shaking at 800rpm.

In the meantime the Streptavidin beads were washed 3x with TEV cleavage buffer (0.2% detergent). After incubation the TEV eluate was dropped out by gravity flow into an Eppendorf tube whereas another 400 µl TEV cleavage buffer (0.2% detergent) was added and the column was emptied by applying pressure (syringe). An aliquot of this eluate was saved and the rest was mixed with the Streptavidin beads and incubated for 1h at 4°C rotating. Then the suspension was centrifuged and the beads were transferred to a new column where the beads were washed with 10ml TEV cleavage buffer (0.2% detergent). After preparation of a saturated biotin solution a spatula tip was added in 5ml biotin buffer. Then the Streptavidin beads were incubated with 400µl saturated biotin solution for 5 min at 16°C. After incubation the eluate was dropped out by gravity flow into an Eppendorf tube whereas afterwards another 400 µl saturated biotin solution was added and incubated for another 5 min. Then the column was emptied by applying pressure (syringe). Then the remaining beads are boiled in 50µl SDS sample buffer and lyophilized over night. On the next day the dried biotin eluate were dissolved in 50µl SDS sample buffer.

Used Reagents

- Lysis Buffer: 50mM Tris/HCl, pH 7.5, 5% glycerol, 0.2% NP-40, 1.5mM $MgCl_2$, 100mM NaCl → add freshly: 1mM Na_3VO_4, protease inhibitors, and 25mM NaF
- TEV cleavage buffer: 10mM Tris/HCl, pH 7.5, 100mM NaCl, 0.5mM EDTA
- 4x sample buffer: 200mM Tris/HCl pH6.8, 40%glycerol, 8% SDS, 0.004% bromphenolblue, before use 10% β-mercaptoethanol is added

2.4 Mass spectrometry

After purification the TAP eluates were given to the mass-spectrometry department at CeMM (Head: Dr. Keiryn Bennett) where mass-spectrometry analysis was carried out.

2.4.1 One-dimensional SDS-Page and silver staining

First the eluted samples were separated by 1 Dimensional SDS-Page on a bis-Tris gel (NuPAGE, Invitrogen, CA). Further the gel was silver stained and separate lanes were cut out and residual SDS was removed.

2.4.2 *In situ* tryptic digestion

Proteins were reduced with dithiothreitol, alkylated via incubation with iodoacetamide and digested with modified porcine trypsin (Promega Corp., Madison WI). The mixture of peptides was then extracted from the gel slices and desalted. Then the volume of the sample was reduced to 2 µl in a vacuum centrifuge and reconstituted to 10µl with 5% formic acid prior to LCMS analysis.

2.4.3 Liquid Chromatography and Mass Spectrometry

Mass spectrometry analysis was conducted on a hybrid LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific, Massachusetts, USA) using the Xcalibur version 2.0.6. It was coupled to an Agilent 1200 HPLC nanoflow system by a nanoelectrospray ion source using liquid junction (Proxeon, Odense, DK). The solvents of the LCMS were used in two phases. The phase A contains 0.4% acetic acid, 0.005% HFBA in water and the phase B contains 0.4% acetic acid and 0.005% HFBA in 90% acetonitril. A thermostatted micro-autosampler was used to load automatically 8µl of the tryptic peptide mixture onto a trapping pre-column (Zorbax 300SB-C18 5µm, 5x0.3 mm, Agilent) with a binary pump at a flow rate of 40µl/min. For loading and for washing the pre-column 100% Phase A was used. The peptides were then eluted by back flushing onto a 16 cm fused silica analytical column (inner diameter of 50µm) packed with C18 reversed phase material (ReproSil-Pur 120 C18-AQ, 3µm, Dr.Maisch GmbH). With a gradient (from 3-13% B within 4 min, 13-35% B within 35 min, 35-50% B within 11 min and 50-100& B within 6 min and hold at a

constant flow rate of 100nL/min for 15 min) the peptides were eluted. Certain background ions were used as lock masses for internal calibration.

Data analysis was carried out in a data-dependent acquisition mode by the use of a top 10 collision-induced dissociation (CID) method and a dynamic exclusion for selected ions of 60 sec. The maximal accumulation time of ions on the LTQ Orbitrap was 150ms on the MS^n in the LTQ and 1000ms in the C-trap. To prevent an overfilling of the ion traps an automatic gain control was used. In both LTQ and Orbitrap injection waveforms were activated. In the Orbitrap intact peptides were detected at a resolution of 60000.

2.4.4 Data analysis

The obtained data were processed by the use of Bioworks V3.3.1 SP1 (ThermoFisher, Manchaster, UK). The data files were therefore merged with an internally-developed program and searched against the murine IPI database version v3.41 with the search engine MASCOT. Perl script, which performs an initial search with a relatively broad mass tolerance on both precursor and fragment ions (+/-10 ppm and +/-0.6 Da) was used for submission to MASCOT. After this, peptides with a high confidence are used to recalibrate all precursor and fragment ion masses before a second search with narrower mass tolerances (+/-4 ppm and +/- 0.3 Da). One missed tryptic cleavage site was allowed. Carbamidomethyl cystein was set as fixed and oxidized methionine was set as variable modification. At least two unique peptides with a MASCOT peptide ion score greater than one, or equal to, were required for unambiguous protein identification.

2.5 Co-Immunoprecipitation

For co-immunoprecipitation $6x10^6$ HEK293T cells were seeded into 10 cm dishes. After 24h 6µg of each construct was transiently transfected according to the protocol. After 24h of incubation the cells were lysed with Frackelton Buffer and then protein concentration was calculated using Bradford reagent.

For the IP 2.5 mg of protein were diluted with Frackelton Buffer to a final volume of 300µl. The appropriate beads (myc, V5, or HA tagged) were used in a 1:1

suspension. Before use the beads were washed at least 3 times with Frackelton buffer. Therefore the beads were centrifuged at 150 x g for 1 minute. Then the supernatant was discarded and one volume of Frackelton Buffer was added. Finally 50µl of beads were added to the samples which were than incubated for 1-2 h rotating on a wheel at 4°C. After the incubation time the beads were washed 4 times the same way as described above with Frackelton buffer. When the last supernatant was discarded 60 µl of 4 x sample buffer was added and the beads were boiled for 5 min at 95°C. After one more centrifugation step the supernatant was taken and used for Western Blot analysis.

Used Reagents

- Frackelton Buffer: 10mM Tris/HCl pH7.4, 50mM NaCl, 30mM Na-pyrophosphate, 1% Triton X-100 → before use 1mM PMSF, 1mM DTT, 100µM Na_3VO_4 and 100µM NaF was added
- 4x sample buffer: 200mM Tris/HCl pH6.8, 40%glycerol, 8% SDS, 0.004% bromphenolblue, before use 10% β-mercaptoethanol is added

2.6 Western Blot

The mini protean 3 system (BioRad) was used to prepare polyacrylamide gels for electrophoresis. In general 9 % gels were prepared according to the following table whereas one minigel was prepared the following (table 10).

Separation gel	**9 % gel**
30% Acrylamide/Bis (Bio-Rad)	1.5 ml
Running gel Buffer	1.25 ml
10% APS	50 µl
TEMED (Merck)	5 µl
water	2.25 ml

Table 10: Separation gel

The separation gel was poured quickly into the system and filled up to a height of 7 cm. It was the covered with 700µl of Isopropanol and polymerized for approximately 15 min. Then the Isopropanol was discarded and the stacking gel was prepared as following (table 11).

Stacking gel	
30% Acrylamide/Bis (Bio-Rad)	0.334ml
Stacking gel Buffer	0.5ml
10% APS	20µl
TEMED (Merck)	3µl
water	1.166ml

Table 11: Stacking gel

It was filled until the edge of the glass plate and a 15-well comb was immediately inserted into the gel. After approximately 10 min the stacking gel was polymerized, the comb was removed and the gel slots were rinsed with water.

The gel was put into a running chamber and filled up with 1 x SDS running buffer. 5µl of pre-stained protein ladder (Fermentas) was used as a marker. In general 100µg of protein diluted into 4 x sample buffer were loaded in one gel slot. The electrophoresis was than performed at 120 V until the bromphenolblue reached the bottom of the gel.

For immunoblot the sandwich was prepared as following: 3 Whatman papers were soaked in 1 x Western Blot buffer and put in the transfer chamber. The membrane (Whatman) was also soaked in Buffer and put onto the papers (for a PVDF membrane, the membrane has to be pre-incubated in methanol according to the instructions). Then another soaked staple of 3 Whatman papers was put on top of the staple. Using a 10 ml pipette the air bubbles were removed from the sandwich. To transfer the proteins on the membrane the sandwich was orientated in that direction that the gel was at the cathode and the membrane at the anode and the blot was performed for 1h with 1 mA/cm$^{2.}$

After blotting the membrane was blocked in 5 % milk (BioRad) in PBS-Tween (PAA-Sigma) for 30 min. The primary antibody was diluted in 5 % milk PBS-Tween and

incubated either at RT for 1 h or at 4°C o/n. After 3 times washing for 5 min with PBS-Tween the secondary antibody (diluted in 5% milk in PBS-Tween) was incubated on the membrane at RT for 1 h. (When phosphorylated proteins were supposed to be detected TBS-Tween was always used instead of PBS-Tween). After another 3 washing steps the membrane was either analyzed using an Odyssey Li-Cor machine (for fluorescence labeled antibodies) or developed with HRP-peroxidase.

Used Reagents

- Gel buffer (4x): 1.5M Tris-HCl pH 8.8 (Sigma), 10% SDS (Serva) in deionized water
- Stacking gel buffer (4 x): 0.5M Tris-HCl pH 6.8 (Sigma), 10% SDS (Serva) in deionized water
- APS: 10% Ammonium persulfate (Merck) in deionized water
- Sample buffer (4x): 0.2M Tris-HCl pH 6.8, 40% glycerol (Serva), 8% SDS (Serva), bromphenolblue (Sigma) in deionized water
- SDS running buffer (5x): 250mM Tris (Sigma), 1.9M Glycin (Serva), 35mM SDS (Serva) in deionized water
- Western Blot Buffer (1x):1xPBS-Tween: 500ml 10xPBS(PAA) diluted in4.5l deionized water, add 5 ml Tween 20 (Sigma)
- 10xTBS: 100mM Tris/HCl pH7.4 1.5 M NaCl

The following antibodies were used in this study (table 12).

Antibodies	**Dilution**
TBK1 rb	1:1000 in 5% milk in PBS-T
P-TBK1 rb	1:1000 in 5% milk in TBS-T
V5 mo (InvivoGen)	1:5000 in 5% milk in PBS-T
HA-11 mo	1:3000 in 5% milk in PBS-T
2nd Anti-rb-HRP	1:5000 in 5% milk in PBS-T
2nd Goat anti mouse 700 (InvitroGen)	1:7000 in 5% milk in PBS-T
2nd anti rabbit 800 (InvitroGen)	1:7000 in 5% milk in PBS-T
2nd rb Myc-800 (Rockland)	1:7000 in 5% milk in PBS-T

Table 12: Used antibodies and respective dilution

2.7 Immunofluorescence

For Immunofluorescence experiments HeLa cells were seeded in cover slips with a density of $1x10^4$. On the following day the cells were transfected with 150ng of the respective construct per well. After 3h the media was changed and the cells were incubated at 37°C for another 24h. On the third day the cells were fixed after washing them one time with 1x PBS (PAA). The fixation procedure requires 4% formaldehyde in PTEMF solution and was out on the cells for 10 min. After washing the cells again 3 times they were blocked in a 3% BSA (Sigma) PBS solution (blocking solution) for 30 min. Then the first antibody was incubated for one hour and after again 3 washing steps also the secondary antibody was incubated for 1h in the dark (table 13). The cells were again washed 2 times with PBS-T and then stained with DAPI (1:100) for 10 minutes. After another 3 washing steps the chambers were sealed with MOWIOL, dried over night and kept in the dark.

Antibodies	**Dilution**
Anti-Myc (9E10), mo monoclonal (BD)	1:1000 in blocking solution
Anti V5 mo monoclonal(InvivoGen)	1:200 in blocking solution
2^{nd} Anti mouse Alexa Fluor 594 (Invitrogen)	1:3000 in blocking solution

Table 13: used antibodies and respective dilution

- PTEMF-buffer: 0.2% Triton X-100 (Sigma), 20mM pipes-buffer pH 6.8 (Sigma), 1mM $MgCl_2$ (Merck), 10mM EGTA (Fulka), 4% Formaldehyde (Merck)

2.8 Reporter gene assay

HEK293 cells were seeded in 24-well plates and on the next day transfected with 0.4µg of plasmid, 48ng renilla reporter and either 72ng of IFN-β luciferase reporter or NF-κB luciferase reporter. After 24h incubation the cells were lysed in 100µl of IP-buffer and 20µl of each sample was used for reporter gene assay. Luminescence of each sample was measured in biological duplicates. Western Blot was performed to measure the expression levels of the respective transfected plasmids.

2.9 Kinase assay

HEH293T cells were seeded in 10 cm dishes and transfected the following day with 8µg of the respective plasmids. After 24h of incubation the cells were lysed in 700µl of Frackelton buffer. Then the tagged beads were washed 3 times with Frackelton buffer and 2mg of the lysate was incubated with 40µl of the beads for 1-2h rotating at 4°C. Afterwards the beads were washed 3 times with Frackelton buffer and 1 time with kinase buffer. Finally the beads were resuspended in 350µl of kinase buffer. 60µl of this suspension was added to 500µl Eppendorf tubes (1 tube per reaction) whereas duplicates were measured for each construct. The beads were then resuspended in 10µl of Kinase buffer and 10µl of hot-mix (hot-laboratory) was added table 14).

Hot master mix ATP	
Cold ATP (2mM)	0.5µl
[γ^{32}P] ATP	0.5µl
IRF3 peptide (50µM)	0.4µl
Mg kinase buffer	8.6µl
total	**10µl**

Table 14: Composition of Hot-ATP master mix

The reaction was further incubated for 30 min at 30°C and stopped by adding 12.5µl 7.5M guanidinium chloride. Then the mixture was spotted on a SAM2 Biotin Captire Membaren (Promega) and washed as following.

30 sec with 2M NaCl

3 x 2 min with 2M NaCl

4 x 2 min with 2M NaCl + 1% H_3PO_4

2 x 30 sec with H_2O

15 sec with Ethanol

Kinase activity was then measured on a Scintillation counter.

- Kinase buffer: 40mM Tris/HCl pH 7.5, 10mM $MgCl_2$, 1mM DTT

3 Results

TANK, TBKBP1 and TBKBP2 are believed to be cofactors for TBK1/IKKi in signaling cascades that activate upon viral infection (Ryzhakov and Randow 2007) (Sasai, Shingai et al. 2006) (Guo and Cheng 2007).

The first evidence for this was provided in 2004 when the entire human TNF-α pathway was mapped in one go and TBK1/IKKi was found to be in a complex with TBKBP1, TBKBP2 and TANK (Bouwmeester, Bauch et al. 2004).

In order to follow up these findings and specify the architecture of the core complex, we performed TAP pulldowns and co-immunoprecipitations. Further, the activity of TBK1 in relation to the core complex was examined by the use of autophosphorylation studies, kinase assays and reporter gene assays.

3.1 Pulldown of TBK1/IKKi and the adaptor proteins TBKBP1, TBKBP2 and TANK

Stable cell lines that efficiently express the GS-TAP-tagged version of the respective proteins were generated according to the protocol of Buerckstuemmer et al 2006. The lysates of these cells were used for tandem affinity purification (TAP), which is a two-step affinity purification that allows the isolation of protein complexes close to physiological conditions. The purified eluates were then sent for mass-spectrometry analysis (Burckstummer, Bennett et al. 2006).

3.1.1 Tandem Affinity Purification

The binding of the adaptors to TBK1 has reproducibly been shown by using the method of Tandem affinity purification (TAP). In TAP, TBK1 is used as a bait to ‘fish out’ the co-operating factors TBKBP1, TBKBP2 and TANK (further referred to as TBK1 adaptor proteins).

In order to study the exact structure of the core complex we first performed reverse TAP pulldowns of the binding proteins side by side to TBK1 and IKKi pulldowns. We wanted to find out whether the adaptors TBKBP1, TBKBP2 and TANK do not only bind to TBK1 and IKKi but also to one another in order to form

the proposed stable complex. As a negative control we used an unrelated protein, which we purified in parallel and was also expressed in RAW264.7 macrophages.

To see whether the baits are expressed in the lysates, one can use any rabbit-derived antibody for western blotting (here: anti-TBK1) as the protein G moiety of the tag binds to virtually all antibodies. Binding of TBK1 was monitored in the final eluates (also referred to as 'boiled beads') using anti-TBK1 as well.

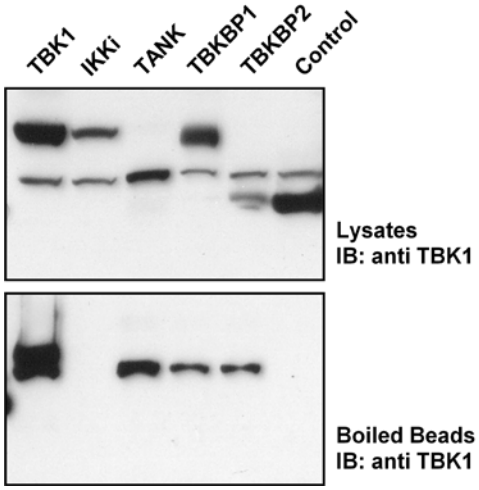

Figure 7: The final TAP eluated samples and the corresponding cell lysates were used for Western Blot analysis with anti-TBK1 antibody. All of the bait proteins can be seen expressed in the lysates at the right size. In the final eluates the binding of TANK, TBKBP1 and TBKBP2 but not the control to TBK1 is visible.

The anti-TBK1 antibody could detect the expression of all the bait proteins in the lysates. By performing a TBK1 Western Blot of the eluates, which were further analyzed by mass spectrometry, we could confirm the specific binding of TBK1 to TBKBP1, TBKBP2 and TANK but not to the control. As previously reported we also found endogenous TBK1 bound to the tagged TBK1, which suggests that TBK1 may form homooligomers.

3.1.2 Silver stained gels of TAP eluates

The final eluates were separated on an SDS-PAGE and bound proteins were visualized by silver staining. Next, each lane was sliced into 20 slices, digested with trypsin and submitted to mass-spectrometric analysis.

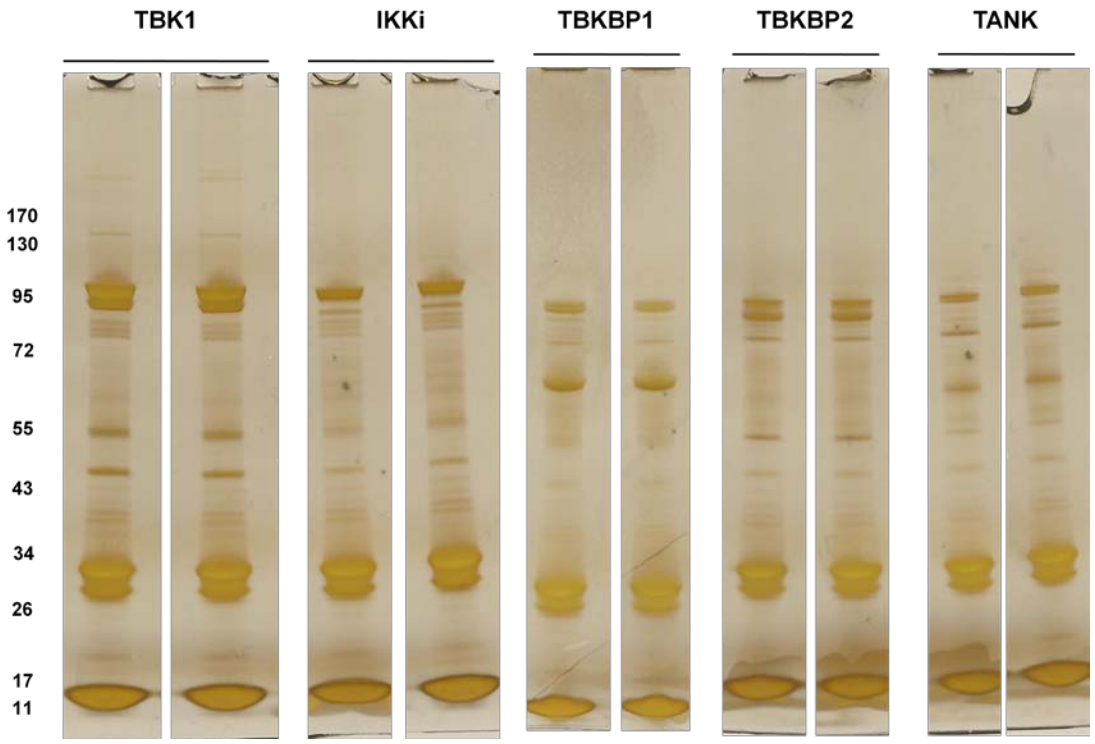

Figure 8: The final TAP eluates of the different bait proteins (in duplicates) were separated on an SDS-PAGE and visualized by silver staining.

3.1.3 Mass-spectrometry and Bioinformatical analysis

We use mass-spectrometry peptide counts as a read out for protein abundance. The peptide counts for a given protein, equates to the number of unique tryptic peptides that were identified by mass-spectrometry analysis. It partially correlates with the amount of protein that was present in the final eluate and hence with the strength of the interaction.

Bioinformatic analysis of the pulldown results is required in order to eliminate non-specific proteins and to get a reliable result with as little false positives as possible. Two major criteria must be considered in the data analysis. The first is the use of an appropriate negative control. Only proteins which are enriched in the

specific pulldowns and not in the negative control pulldown are considered to be real interactors. Proteins found in both pulldowns are considered as background. The second criterion concerns the abundance of proteins identified in any pulldown performed at CeMM mass-spectrometry facility. Software designed at CeMM (ProtFollow) can be used to compare the appearance of a specific protein in different mass spectrometry data.

The resulting data for the interaction properties of the TBK1 core complex are depicted in a table which displays peptide counts (Figure 9).

		Peptide counts				
#	Pulldown	TBK1	IKKi	TBKBP1	TBKBP2	TANK
1035	TBK1	52		10	18	21
1036		56		13	15	24
1037	IKKi		43		2	21
1038			41	2	2	8
1041	TBKBP1	15		23		
1042		20	4	14		
1043	TBKBP2	19	10		11	
1044		18	7		10	
1039	TANK	27	22			25
1040		28	25			27

Figure 9: Matrix of the peptide counts of the TBK1/IKKi and adaptor pulldowns. The blue labeled caskets indicate the abundance of the bait protein whereas the white labeled caskets show the amount of peptides detected.

The table describes the specific peptide counts for each pulldown in duplicates. The amount of bait protein is depicted in blue. High amounts of detected bait protein reflect the quality of the respective pulldown. TBK1 has high peptide counts for all the three adaptor proteins, whereas in comparison IKKi shows low

peptide counts. For instance, TBKBP1 was found as an interactor of IKKi in only one pulldown.

The reverse pulldowns of the adaptor proteins TBKBP1, TBKBP2 and TANK, showed high peptide counts of the respective kinases TBK1 and IKKi, confirming the previously described interactions. Surprisingly TBKBP1, TBKBP2 and TANK did not show any binding to one another. These data suggest that the adaptor proteins do not form a single core complex but instead participate in distinct sub-complexes. In line with this argument, there is very little overlap between the proteins found in each adaptor protein pulldown (data not shown).

Our TAP pulldowns supported the previous findings that TANK binds to NEMO, Traf1 and Traf2 (data not shown) (Cheng and Baltimore 1996; Kaye, Devergne et al. 1996; Rothe, Xiong et al. 1996; Chariot, Leonardi et al. 2002). This confirms the reliability of the TAP-MS method employed at CeMM and indicates any newly identified proteins are likely to be true interactors.

We also found several new proteins involved in innate immunity signaling interacting with either one of the adaptors, but these findings have not been followed up in this study.

3.2 Interaction of TBK1 or TBK1 mutants with the adaptors

After the pulldown results showed that none of the adaptors interact with themselves we focused our interest on the interaction between TBK1/IKKi and TBKBP1, TBKBP2 and TANK.

To specify the binding properties we performed immunoprecipitation studies with several TBK1 mutants.

3.2.1 Confirmation of the interaction via Co-Immunoprecipitation

The results obtained by TAP pulldowns were first verified with co-immunoprecipitation (co-IP). HEK293T cells were co-transfected with Myc-tagged TBK1 and HA-tagged adaptors or kinases. The cell lysates were used for immunoprecipitation with anti-Myc-agarose.

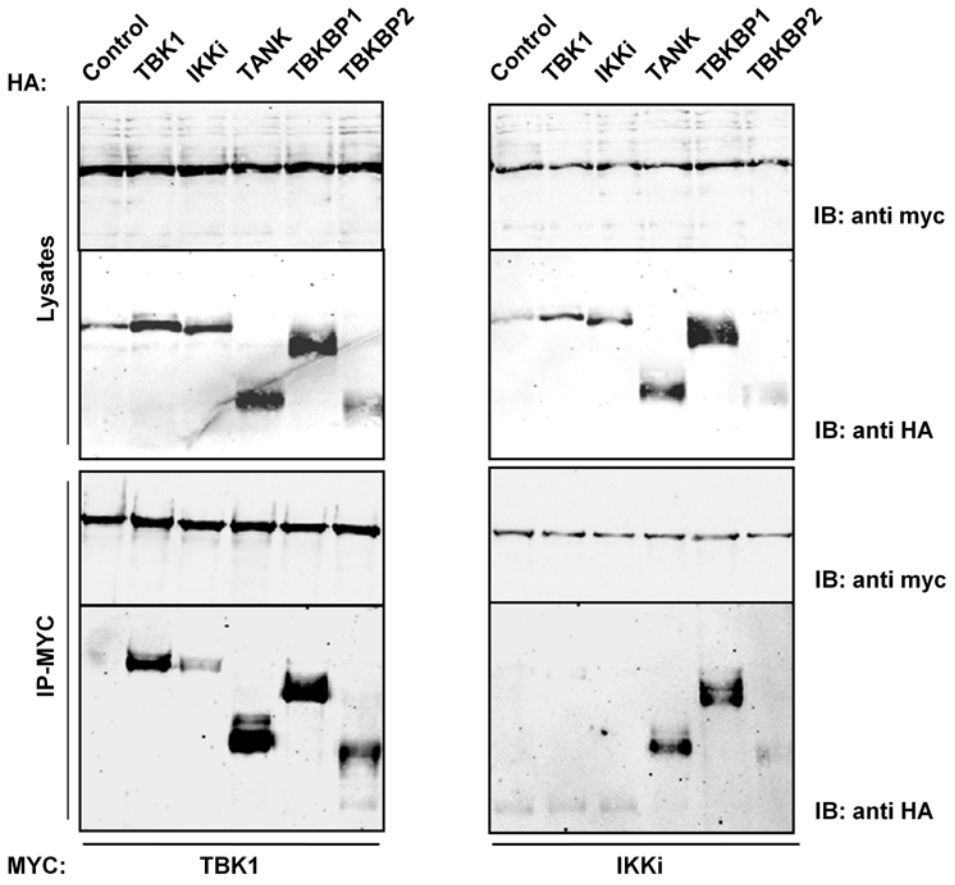

Figure 10: HEK293T cells were cotransfected with Myc-tagged TBK1 or IKKi and HA-tagged adaptor proteins or kinases. The cell lysates were co-immunoprecipitated with anti-Myc-agarose. On the Western Blot it can be seen that TANK, TBKBP1 and TBKBP2 but not the control bind to TBK1 and IKKi by using an anti-HA antibody.

As expected from the TAP results, both TBK1 and IKKi interact with all the three adaptor proteins. TBKBP2 is expressed at lower levels than the other adaptors and therefore, the interaction can only be weakly seen. Interestingly TBK1 oligomerizes with TBK1 and to a lesser extent with IKKi whereas IKKi doesn't show any sign of oligomerization although TBK1 and IKKi are almost equally expressed.

3.2.2 Creation of TBK1 deletion mutants

It was found that 43 residues in the C-terminus of TBK1 are sufficient for binding to TANK (Pomerantz and Baltimore 1999). Until now it was not known at which sites TBKBP1 and TBKBP2 interact with TBK1 and IKKi.

TBK1 is classified into several functional domains: the kinase domain, the ubiquitin-like domain (Ikeda, Hecker et al. 2007), and two coiled coil regions. In order to investigate the interaction between TBK1/IKKi and the adaptor proteins TBKBP1, TBKBP2 and TANK we used a systematical approach where we deleted those domains separately. This resulted in 5 different mutants and the wild type control (Figure 11).

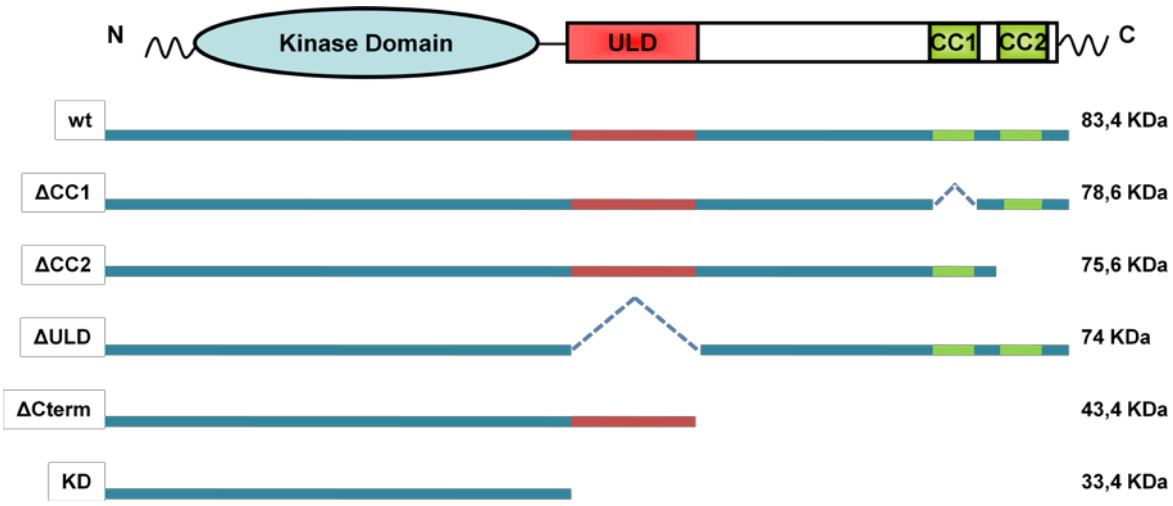

Figure 11: Map of the created deletion mutants of TBK1

3.2.3 Binding of TBK1 deletion mutants to the adaptors

With these deletion mutants we performed co-immunoprecipitation in order to define the region of TBK1 that is needed to bind each of the adaptor proteins. HEK293T cells were co-transfected with Myc-tagged TBK1wt or Myc-tagged TBK1 deletion mutants, and with either V5-tagged TBKBP1, TBKBP2 or TANK. Immunoprecipitations were performed in both directions, using either the Myc or the V5 tag. The respective Western Blots of the lysates and eluates are shown below.

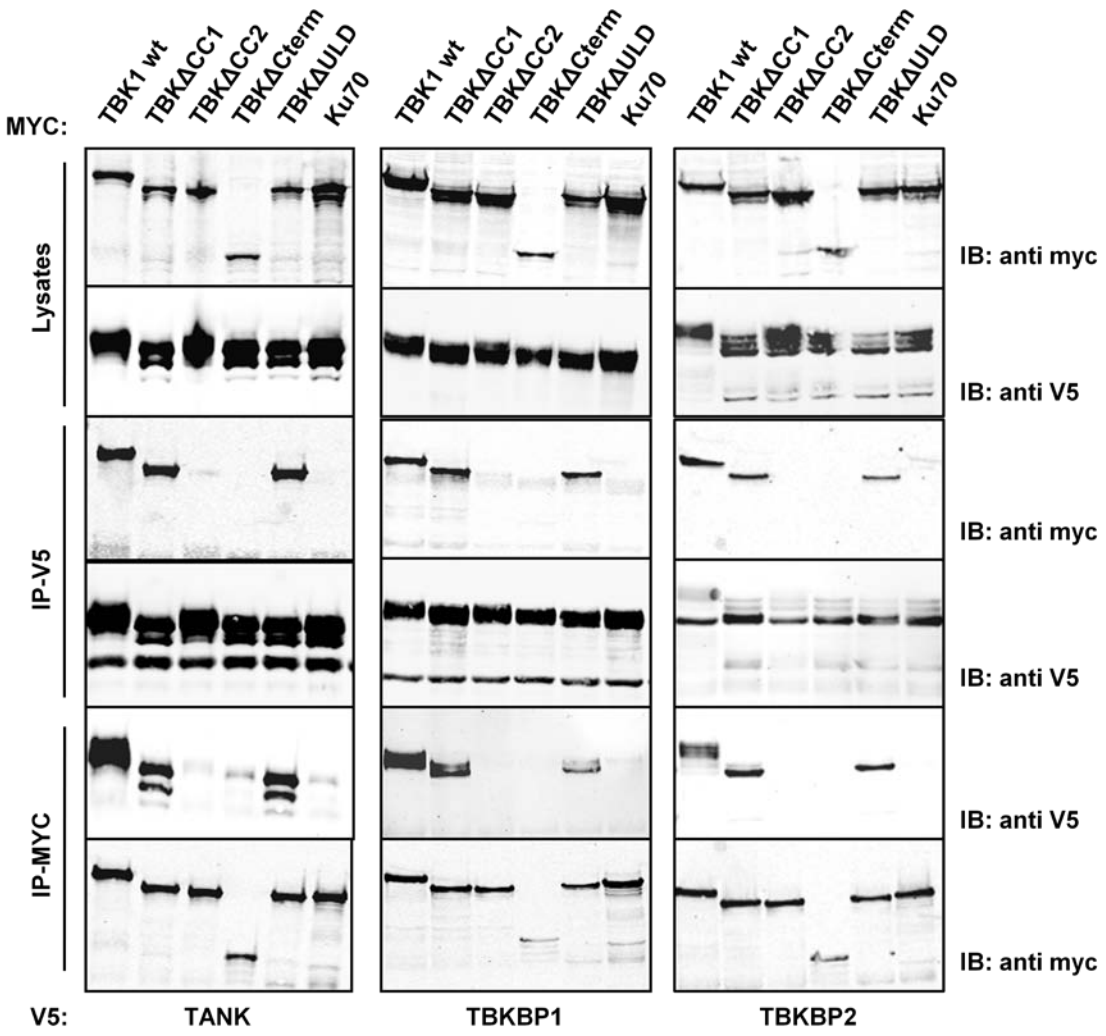

Figure 12: HEK293T cells were co-transfected with Myc-tagged TBK1wt or TBK1 deletion mutants and V5-tagged adaptors. After co-immunoprecipitation with the use of anti-Myc-agarose and anti-V5-agarose, the anti-Myc Western Blot of the V5-IP and vice versa show the binding results. When coiled coil 2 or the whole C-terminus is deleted the binding to the three adaptors is abolished, whereas when deleting coiled coil 1 or ULD the adaptors can still bind.

The anti-Myc and anti-V5 Western Blot of the lysates show that TBK1 and the different TBK1 mutants as well as the adaptors are all expressed. The result of the co-IP shows that the adaptor proteins TBKBP1, TBKBP2 and TANK are all able to bind to the deletion mutants missing coiled coil region 1 or the ubiquitin-like domain as stably as the TBK1wt.

In contrast, upon deletion of the coiled coil region 2 or the whole C-terminus, the binding to each one of the three adaptor proteins was completely abolished. This

suggests that the binding of the adaptors TBKBP1, TBKBP2 and TANK occurs in the coiled coil 2 region (amino acid 679-712) of TBK1.

3.2.4 Creation of TBK1 point mutants

To further detail the binding properties and to exclude an artifact of the TBK1 mutant missing the coiled coil 2 region we created several point mutants in this region. Therefore it was necessary to understand the structure and behavior of coiled coil motifs.

The first to discover the structural existence of a coiled coil was Crick in 1953. In general coiled coil is a very common structural motif which usually contains 2-5 α-helices wrapped around each other. In contrary to regular α-helices, which contain 3.6 residues per complete turn, the most common left –handed coiled-coil structure lowers this value to 3.5 (Mason and Arndt 2004). The helices consist of 2-200 heptad repeats (a-b-c-d-e-f-g). According to the PV ('Peptide Velcro') hypothesis each position is associated with special properties of the very amino acid located there. Therefore position a and d must be hydrophobic and non-polar residues (e.g. leucine, valine or isoleucine), which appear at the interface of two helices. But then position e and g are normally exposed to the solvent and therefore polar and charged to form interhelical electrostatic interactions (e.g. glutamate or lysine) (Mason and Arndt 2004).

The coiled coil architecture is crucial for the function in oligomerization and molecular recognition of proteins (Burkhard, Stetefeld et al. 2001).

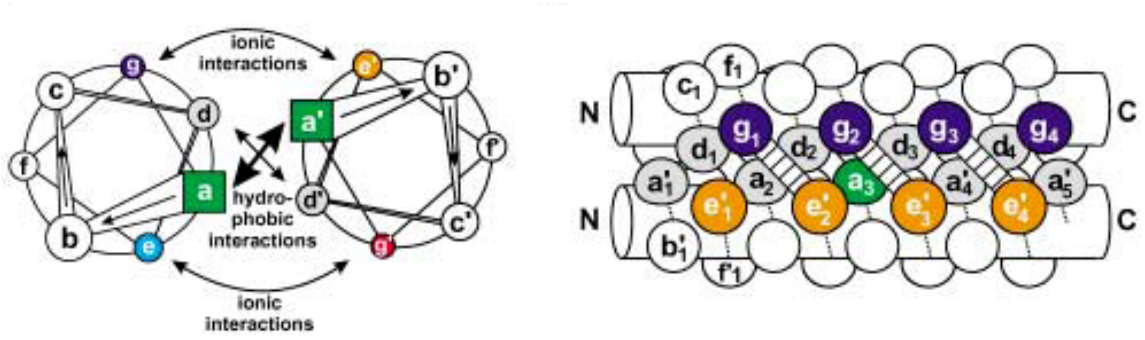

Figure 13: Interaction pattern of a parallel dimeric coiled coil with the specific residues in two different views (Mason and Arndt 2004)

Due to the fact that TBK1 and IKKi act in a similar way we identified the evolutionary conserved regions within their respective coiled coil 2 domains (conserved residues are indicated in red).

CC2 domain of TBK1 and IKKi

TBK1: SNTLVEMTLGMKKLKEEMEGVVKELAENNHILERFGS

IKKi: SPTRKDLLLHMQELCEGMKLLASDLLDNNRIIERLNR

According to the properties of coiled coils we predicted the structure of the coiled coil in the TBK1CC2 region and the amino acids crucial for coiled coil formation.

LVEMTLGMKKLKEEMEGVVKELAENNHILER

Based on the conserved residues between TBK1 and IKKi and the amino acids crucial for coiled coil formation we selected several residues to mutate. We took 4 amino acids (M690, L693, E696 and N707) out of those candidates and mutated them either neutrally to alanine or to the opposite charge (M690A, L693A, E696K, N707A). Those mutants were then used for interaction and activity analysis.

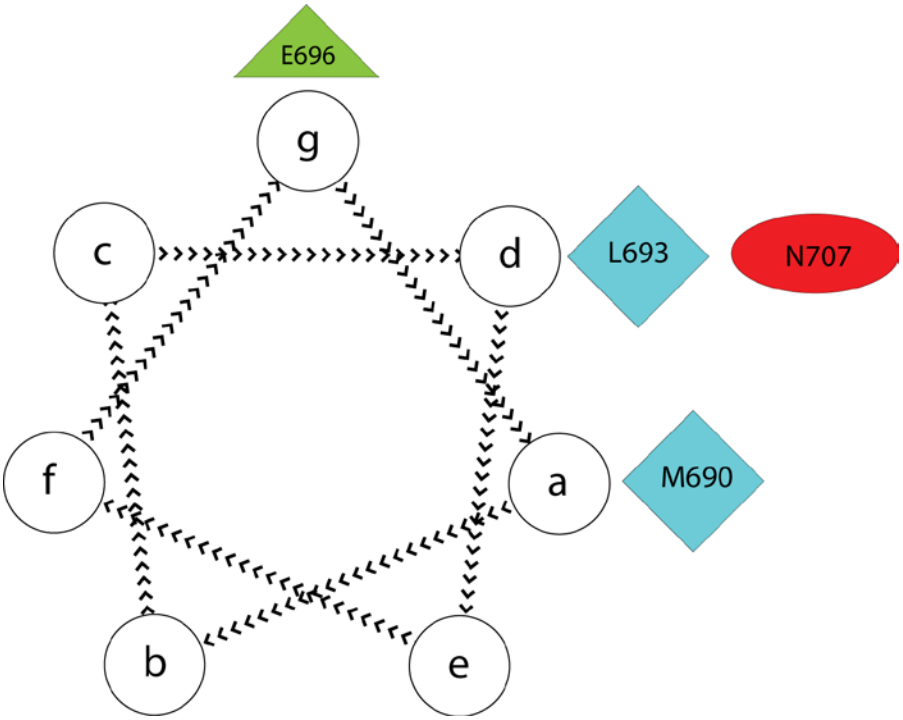

Figure 14: Coiled coil wheel structure of TBK1CC2 indicating the position of the mutated amino acids

3.2.5 Binding of TBK1CC2 point mutants to the adaptors

After successful creation of the TBK1CC2 point mutants, co-IP studies were performed to identify the specific binding properties between the adaptors and TBK1. Co-IPs were performed in HEK293T cells after co-transfection of Myc-tagged TBK1CC2 point mutants and V5-tagged adaptor proteins.

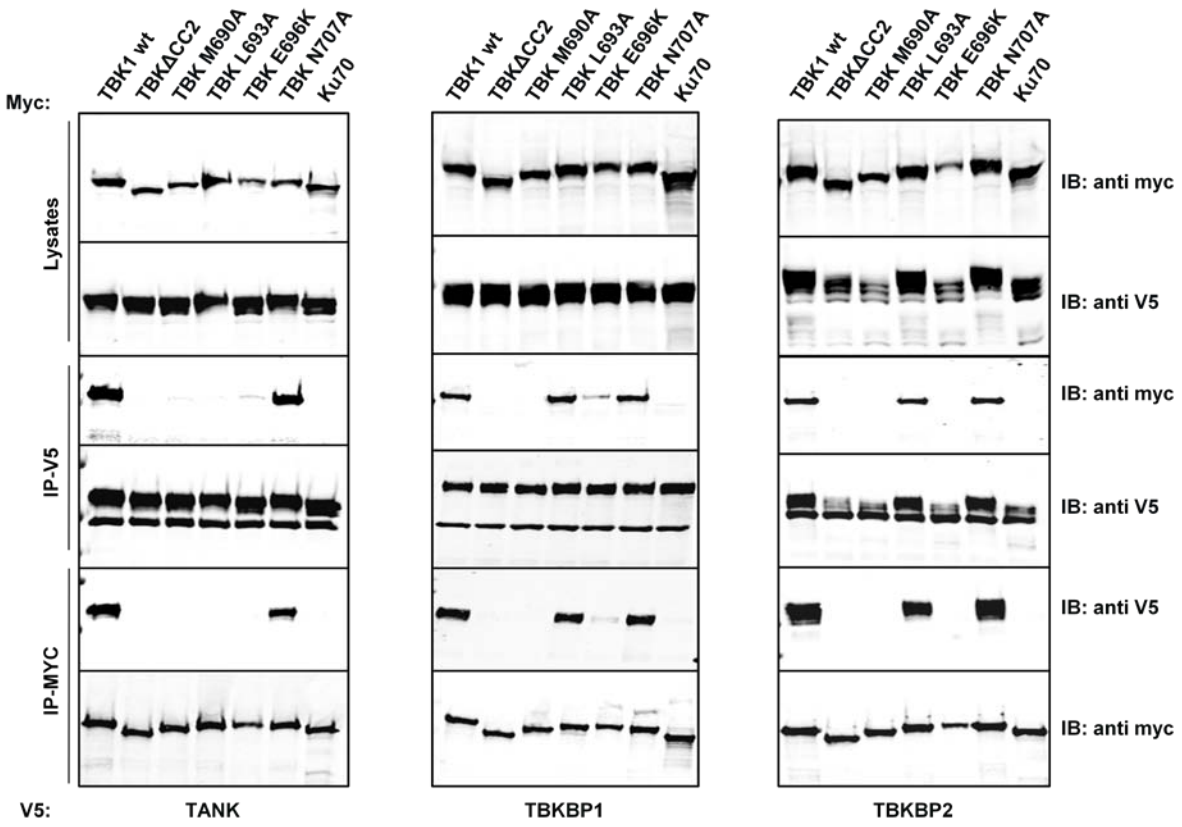

Figure 15: HEK293T cells were co-transfected with Myc-tagged TBK1wt or TBK1mutants and the V5-tagged adaptors. After co-IP the anti-Myc Western Blot of the V5-IP and vice versa show the binding results. The mutants M690A and E696K abolish the binding to the three adaptors, whereas the mutant L693A specifically abolishes TANK binding.

The Western Blot results show that TBK1wt and TBK1 mutants as well as the adaptors are all well expressed. The co-immunoprecipitation result shows that two of the point mutants (M690A, E696K) specifically abolish the binding to TBKBP1, TBKBP2 and TANK. In addition to those two mutations a third mutation (L693A) inhibits the binding to TANK only. The point mutant N707A binds as strongly as TBK1wt to all the adaptors.

In summary we created one TBK1CC2 point mutant (TBKL693A) that specifically abolishes the binding to TANK but still binds to TBKBP1 and TBKBP2.

A model of the specific binding sites of the adaptors to TBK1/IKKi can be found below.

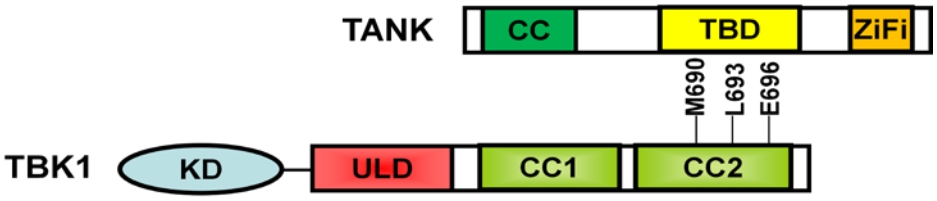

Figure 16: Binding model of TANK and TBK1

TANK binds with its TBK1/IKKi binding domain (TBD) to the coiled coil 2 region of TBK1. For the interaction the 3 amino acids M690, L693 and E696 are crucial.

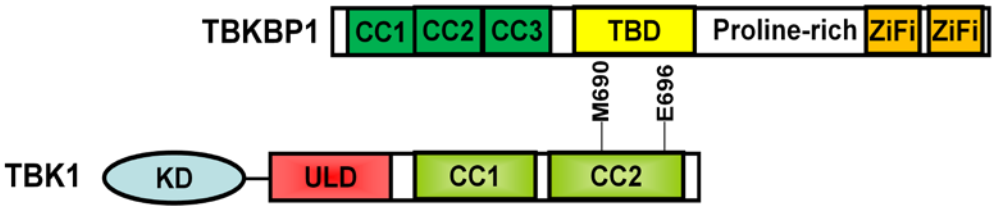

Figure 17: Binding model of TBKBP1 and TBK1

TBKBP1 also binds with its TBD to the coiled coil 2 region of TBK1 whereas for the interaction only 2 amino acids, M690 and E696 are necessary.

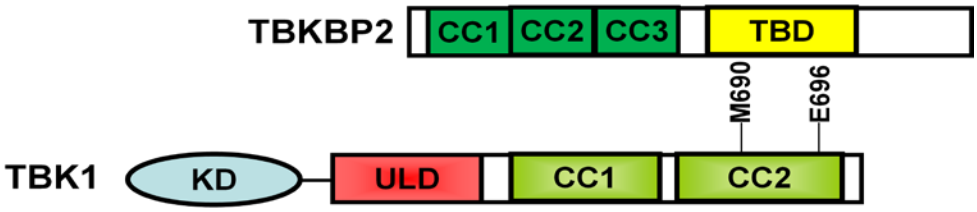

Figure 18: Binding model of TBKBP2 and TBK1

TBKBP2 binds with its TBD to the coiled coil 2 region of TBK1. Like in TBKBP1, the 2 amino acids M690 and E696 are crucial for the interaction.

The TBK1 adaptors all bind to the coiled coil 2 domain in TBK1 but their binding mode is likely to differ significantly as certain amino acids can distinguish between TANK on one side and TBKBP1/TBKBP2 on the other side.

3.3 TBK1/IKKi Activity

After identifying the sites in TBK1/IKKi that bind to the adaptors, we wanted to examine whether the deletion of certain domains also has an impact on TBK1 activity. Another interesting question we wanted to address was whether the binding of the adaptors effects the activity of TBK1.

Several methods can be used as read-outs to investigate TBK1/IKKi activity. One of the most prominent is autophosphorylation of TBK1 at serine 172, which is located in the activation loop of TBK1. We used a phospho-specific antibody that has been created in the lab before and is sensitive for recognizing only the active form of TBK1 (Soulat, Burckstummer et al. 2008). Another assay measures kinase activity by the use of radioactive labeled [γ32P] ATP. A third assay is more indirect and measures TBK1 activity using a reporter gene assays. Here the activation of either IFN-β or NF-κB reporter genes was used to investigate TBK1 activity.

3.3.1 TBK1 Phosphorylation at Ser172

We first investigated the phosphorylation of TBK1 at Ser172 for all the deletion mutants and TBK1wt. HEK293T cells were transfected with the Myc-tagged TBK1wt or the different Myc-tagged TBK1 deletion mutants. The phosphorylation status was then examined by Western blotting using the phosphor-specific antibody

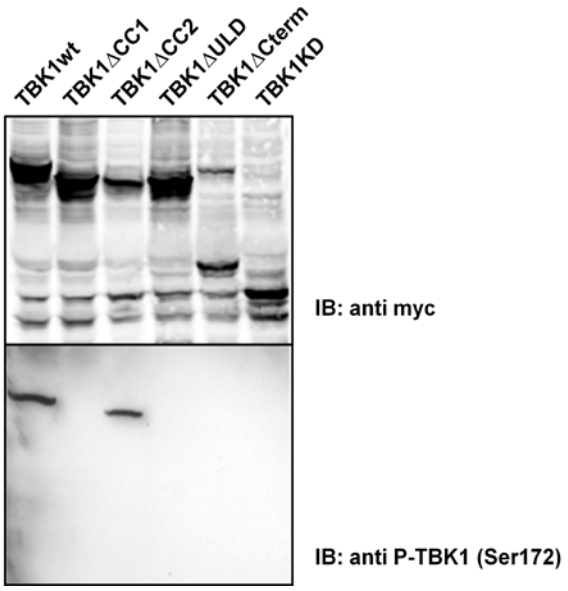

Figure 19: HEK293T cells were transfected with Myc-tagged TBK1wt or TBK1 mutants. Autophosphorylation at serine 172 is shown by Western Blot analysis with the use of a phospho-specific TBK1 antibody. The only mutant that shows activity is TBK1ΔCC2, all the other mutants are inactive.

The anti-Myc Western Blot shows that TBK1wt and all the TBK1 deletion mutants are expressed. As already shown before, we confirmed with the phospho-specific anti-TBK1 antibody that if you delete the ULD, TBK1 is not phosphorylated. Also the kinase domain alone is not active and TBK1ΔCC1 and TBK1ΔC-terminus seem to be inactive but we don't know if this is physiological. But, surprisingly, we see that deletion of the TBK1 coiled coil 2 region, which is the region crucial for binding the adaptor proteins, does not affect TBK1 activity at the level of autophosphorylation.

TBK1 point mutants were also tested for Ser172 phosphorylation using the same method.

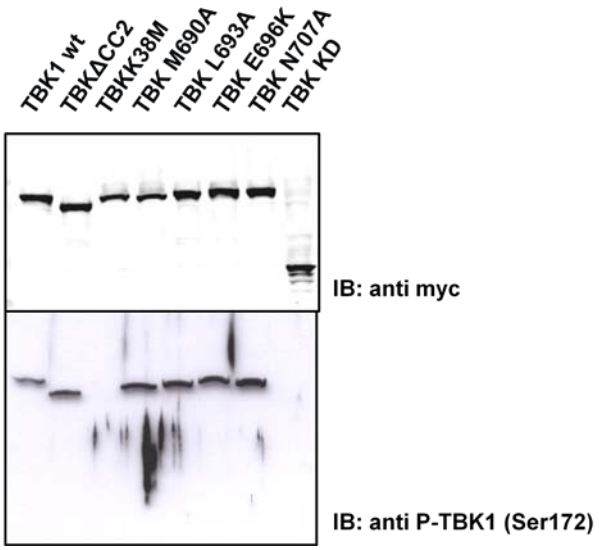

Figure 20: HEK293T cells were transfected with Myc-tagged TBK1wt or TBK1 point mutants. Western Blot analysis detects the autophosphorylation at serine 172 with the use of a phospho-specific TBK1 antibody. All of the TBK1CC2 point mutants show activity similar to TBK1wt and TBK1ΔCC2.

The anti-Myc Western Blot shows that TBK1wt and all the point mutants are expressed. The result of phospho-specific anti-TBK1 Western Blot shows that all the TBK1CC2 point mutants (independent of adaptor binding) are active at the level of serine 172 phosphorylation.

The findings of the activity of TBK1 deletion mutants and point mutants support each other and indicate that TBK1 activity upon overexpression of TBK1 is independent of its ability to bind to the TBK1 adaptors. Nevertheless, it does not rule out that the TBK1 adaptors contribute to the activation process of endogenous TBK1, which is elicited by upstream receptor ligation.

3.3.2 Kinase assay

Next, we performed a kinase assay in order to identify whether the findings we observed at the level of autophosphorylation hold true for IRF3 phosphorylation. Here we used radioactive labeled ATP as a source to examine the ability of TBK1wt and each TBK1 mutant to phosphorylate an IRF3 peptide. The respective Myc-tagged plasmids were transfected into HEK293 cells and the cells were lysed

after 24h. After using immunoprecipitation with anti-Myc-agarose, the final eluates were used in a kinase assay.

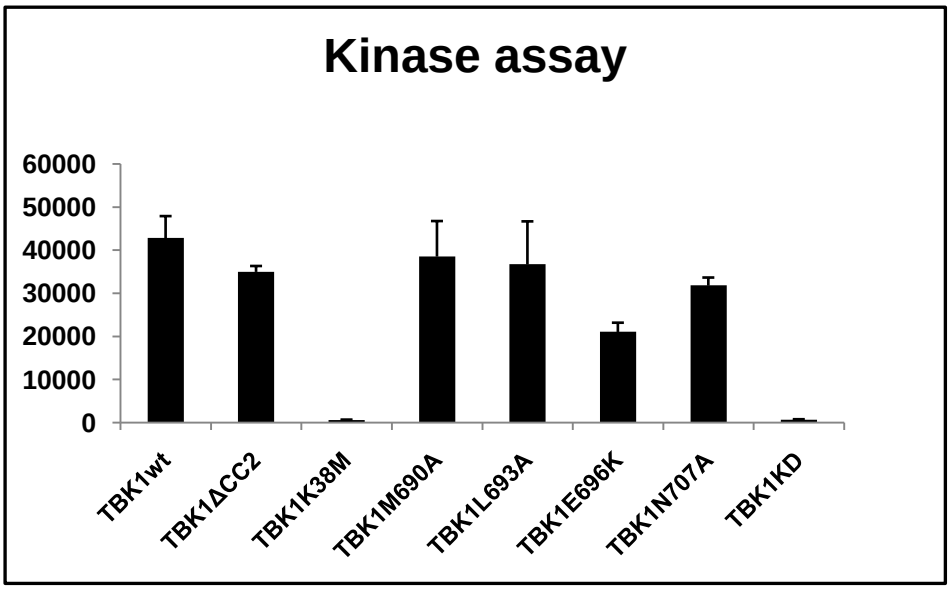

Figure 21: HEK293 cells were transfected with Myc-tagged TBK1wt or TBK1 mutants. After immunoprecipitation by the use of anti-Myc-agarose, Kinase assay was performed. An IRF3 peptide was used to detect phosphorylation, performed by any active TBK1 version. The kinase dead mutant TBK1K38M and the kinase domain only show impaired kinase activity. TBK1wt and the respective TBK1CC2 mutants are all active.

The kinase assay shows no kinase activity in the kinase dead TBKK38M mutant as expected and also no kinase activity in the kinase domain only. TBK1ΔCC2 and the TBK1CC2 point mutants, no matter whether they bind the adaptors or not, show almost as much kinase activity as the TBK1wt. This supports the results of the autophosphorylation study.

3.3.3 NF-κB reporter gene assay

To further examine the activity of TBK1wt and the different TBK1 mutants, reporter gene assays were performed. HEK293 cells were transfected with 0.1, 0.2 or 0.4 µg of TBK1wt or the respective TBK1 mutants and with the reporter constructs. After 24h of incubation the cells were lysed and the lysates were examined by a reporter gene assay. The NF-κB reporter assay covers the TBK1 mediated NF-κB activation.

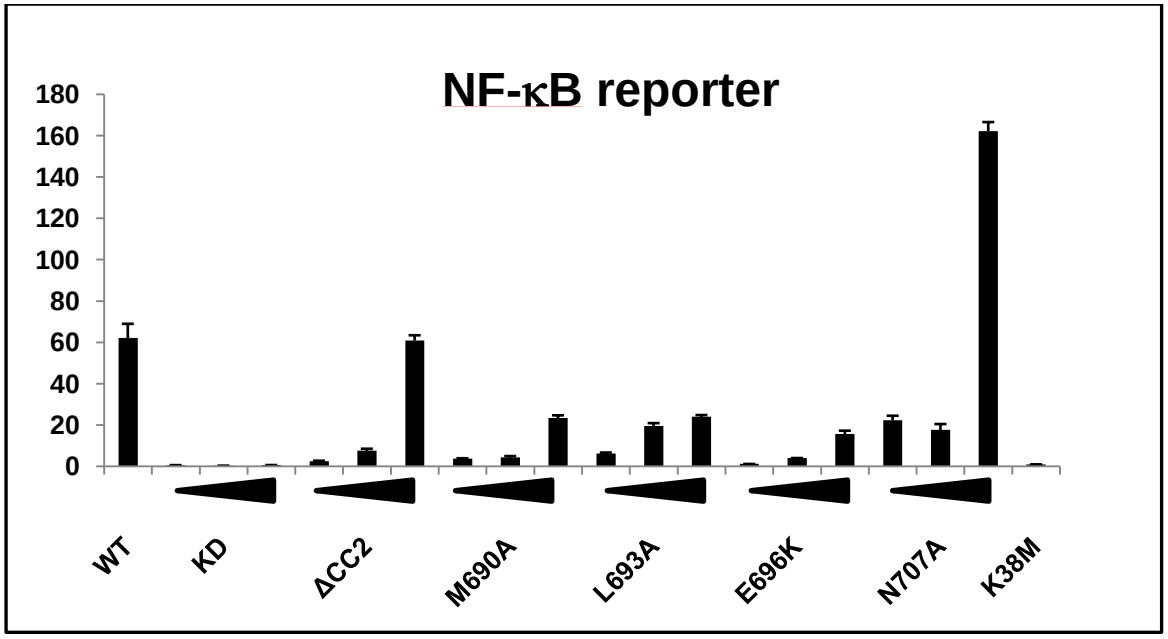

Figure 22: HEK293 cells were transfected with 0.1, 0.2 or 0.4 µg of TBK1wt or the respective TBK1 mutants and with the reporter constructs. After 24h of incubation the cells were lysed and NF-κB reporter gene assay was performed. TBK1wt, TBK1ΔCC2, and TBK1 point mutants show reporter gene expression, whereas TBK1KD and TBK1K38M do not.

Supportive to the kinase assay and the phosphorylation at Ser172 the NF-κB reporter gene assay shows no activity for the kinase domain only and the kinase dead mutant TBKK38M, whereas all the point mutants and TBK1ΔCC2 are active, with only minor differences. TBK1N707A shows higher NF-κB reporter gene activation but has also higher expression levels (Figure 23).

The expression levels of the lysates were depicted in an anti-Myc blot.

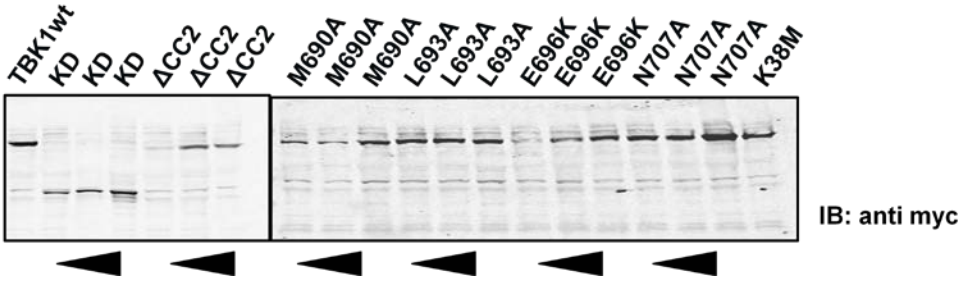

Figure 23: Expression levels of NF-κB and IFN-β reporter gene assay examined by an anti-Myc Western Blot

All of the titrated TBK1 mutants are expressed with only minor differences within expression levels.

3.3.4 IFN-β reporter gene assay

Additionally an IFN-β reporter gene assay was conducted which covers the IRF3 and IRF7 activation via TBK1/IKKi. Again HEK293 cells were transfected with 0.1, 0.2 or 0.4 µg of TBK1wt or the respective TBK1 mutant and with the reporter constructs. After 24h of incubation the cells were lysed and the lysates were taken for reporter gene assay.

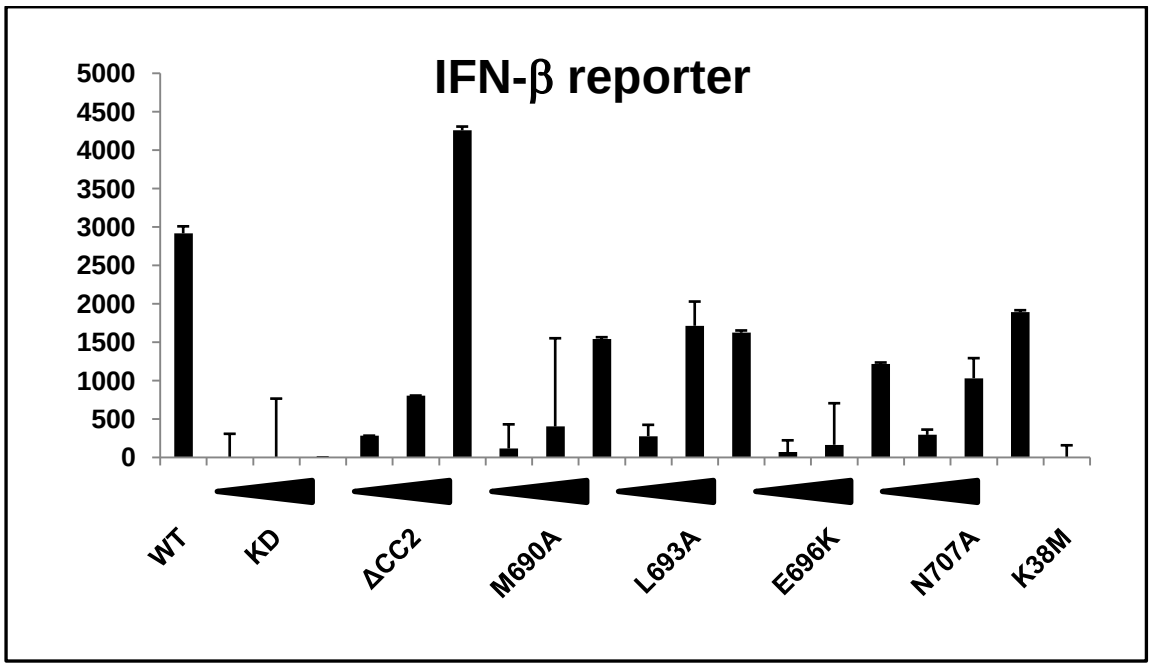

Figure 24: HEK293 cells were transfected with 0.1, 0.2 or 0.4 µg of TBK1wt or the respective TBK1 mutants and with the reporter constructs. After 24h of incubation the cells were lysed and IFN-β reporter gene assay was performed. TBK1wt, TBK1ΔCC2, and TBK1 point mutants show reporter gene expression, whereas TBK1KD and TBK1K38M do not.

In support of the NF-κB reporter gene assay, TBK1wt as well as TBK1ΔCC2 and all the point mutants induce IFN-β reporter gene expression. This confirms that binding of the adaptors is not correlated with IFN activity in over expression studies. The expression levels can be seen in Figure 23.

3.3.5 Impact of the adaptors on NF-κB and IFN-β reporter gene assay

We next investigated the effect of the adaptor proteins on either TBK1wt, or non-binding TBK1ΔCC2 in reporter gene assays. HEK293 cells were transfected with either 0.4µg of plasmid for the single transfections or 0.3µg of TBK1wt or TBK1ΔCC2 and 0.6µg of the respective adaptor for the double transfections.

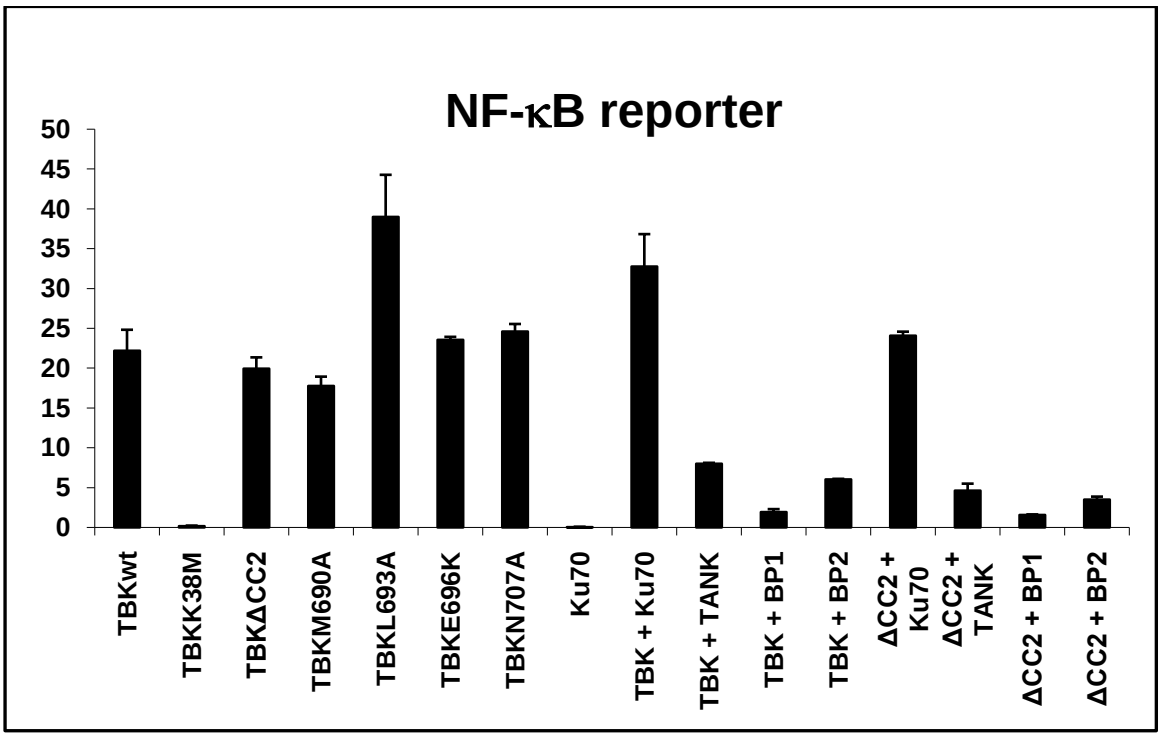

Figure 25: HEK293 cells were transfected with TBK1wt or the respective TBK1 mutants or co-transfected with TBK1wt or TBK1ΔCC2 together with the adaptors and always with the reporter constructs. After 24h of incubation the cells were lysed and NF-κB reporter gene assay was performed. Co-transfection of TBK1wt or TBK1ΔCC2 with the adaptors but not with the control shows reduced reporter gene expression.

As already shown in the previous experiment TBK1CC2 point mutants are as active as the TBK1wt when transfected alone. TBK1wt and TBK1ΔCC2 show almost the same reporter induction whereas when transfected together with the adaptors, in both cases less NF-κB reporter gene activity was detected. The co-transfected negative control Ku70 doesn't show any decrease at all. The minor differences in activity might result from differences in transfection efficiencies (Figure 27).

This shows that coexpression of the adaptor proteins has a negative impact on NF-κB activation as elicited by TBK1. Furthermore, surprisingly, this negative impact of the adaptors is still observed for the TBK1ΔCC2 mutant, suggesting that it is independent of the binding of the TBK1 adaptors to TBK1.

The same experiment was then conducted for IFN-β induction.

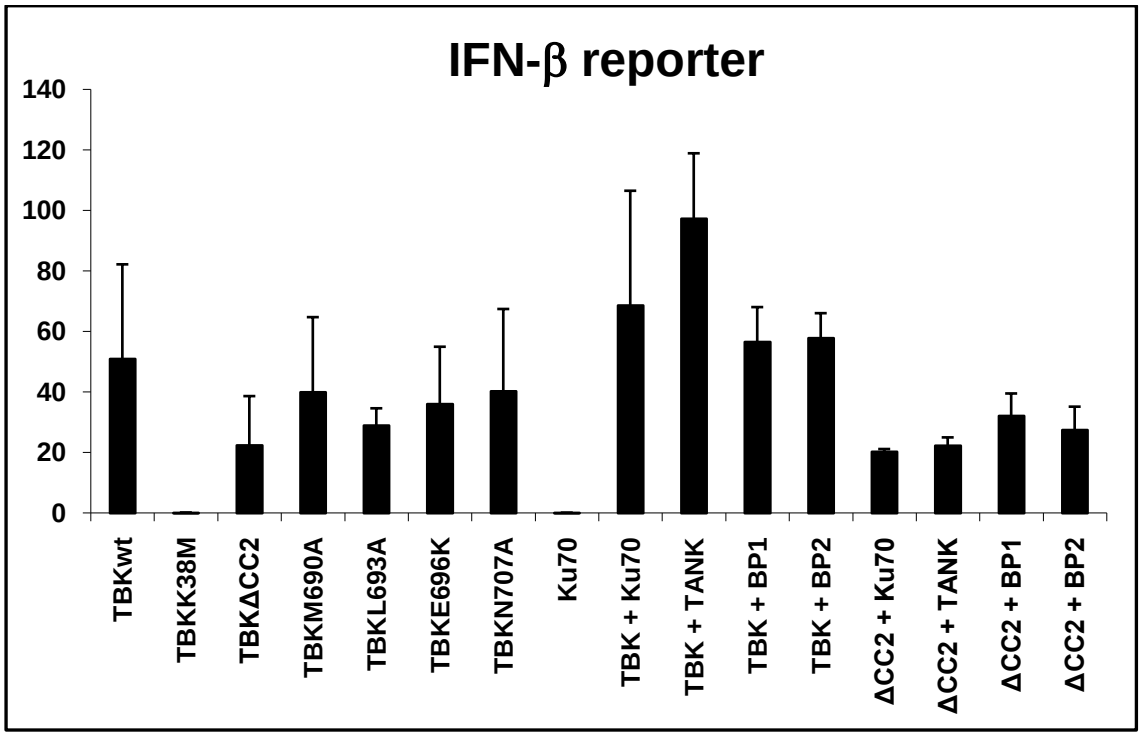

Figure 26: HEK293 cells were transfected with TBK1wt or the respective TBK1 mutants or co-transfected with TBK1wt or TBK1ΔCC2 together with the adaptors and always with the reporter constructs. After 24h of incubation the cells were lysed and IFN-β reporter gene assay was performed. There is no change in reporter gene expression visible when co-transfecting TBK1wt or TBK1ΔCC2 with the adaptors.

As shown before TBK1CC2 point mutants are as active as the TBK1wt, depending on their expression levels (Figure27) when transfected alone. Regarding the IFN-β reporter gene expression, co-transfection of the adaptor proteins with either TBK1wt or TBK1ΔCC2 does not make much of a difference. The minor differences in activity might result from differences in transfection efficiencies (Figure 27).

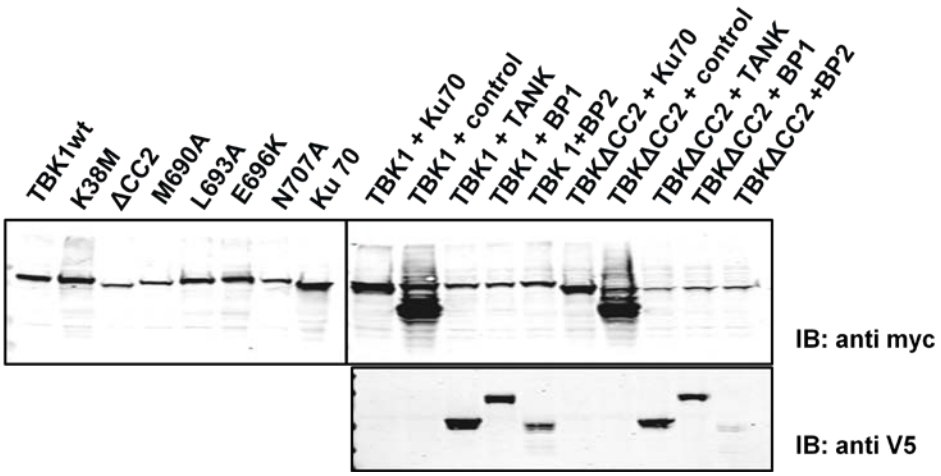

Figure 27: Expression levels of the samples used for NF-κB and IFN-β reporter gene assay detected by an anti-Myc Western Blot.

TBK1ΔCC2 is expressed to a lesser extent than TBK1wt in the co-transfection experiment. Therefore, and independent of adaptor binding, all TBK1ΔCC2 samples are less active on IFN-β and NF-κB reporter gene assay (Figure 26, 25).

3.4 Localization of TBK1 and Binding proteins

It looks like TBK1 adaptor binding is not directly associated with the activity of overexpressed TBK1. In order to investigate whether the adaptor proteins may play a role in the localization of TBK1 we looked at the subcellular localization of these proteins. We transfected HeLa cells on a cover slide with Myc-tagged TBK1 (and respective mutants) or V5-tagged adaptor proteins. After fixing them on the slide and staining them with respective antibodies we took pictures at the fluorescent microscope.

First we stained TBK1wt, TBK1ΔCC2, kinase dead TBKK38M and the different non-binding TBK1CC2 point mutants in order to see whether the binding to the adaptors or activity has any impact on TBK1 localization.

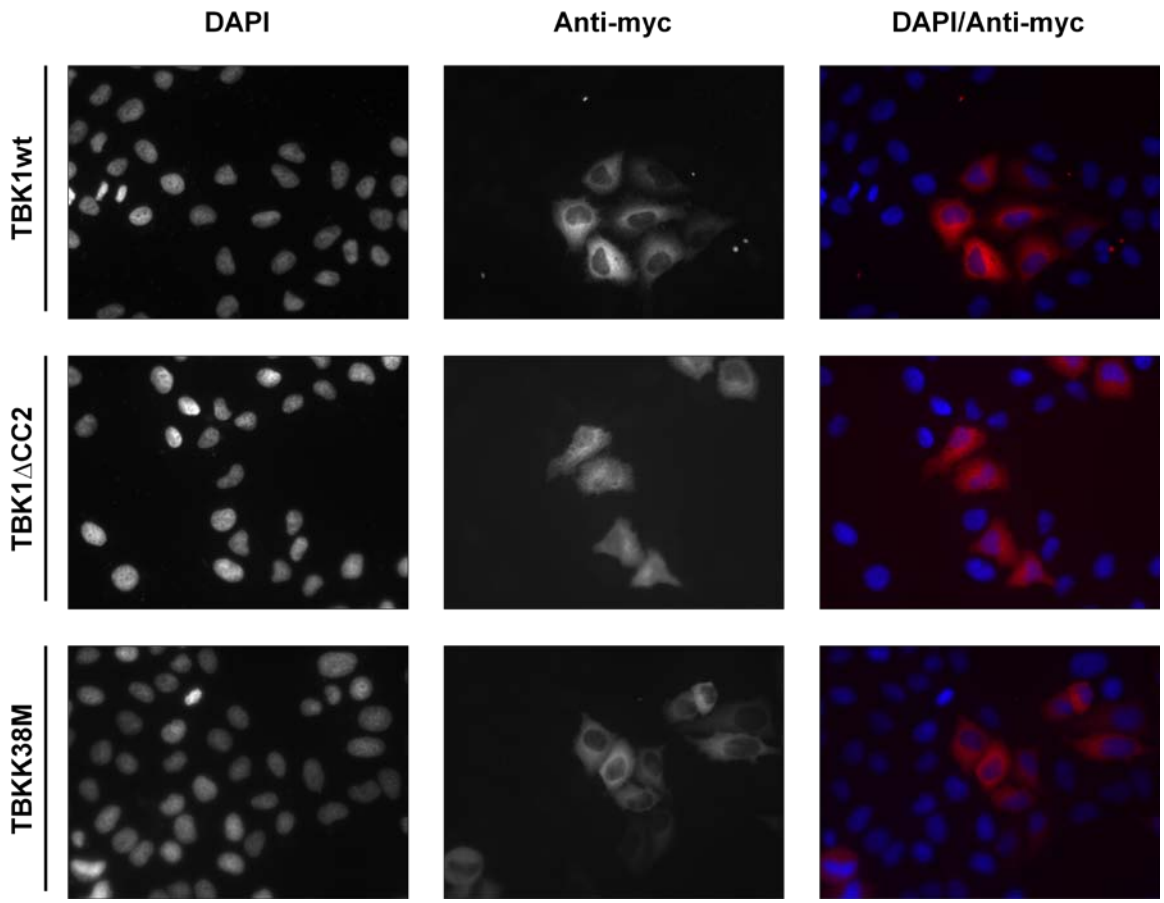

Figure 28: HeLa cells were transfected with Myc-tagged TBK1wt or TBK1 mutants. After fixing them, they were stained with an anti-Myc antibody and analyzed at the fluorescent microscope. TBK1wt, TBK1ΔCC2 and TBKK38M are all found to occur in the cytosol.

TBK1 was evenly distributed throughout the whole cytosol. Similar results were obtained for TBK1ΔCC2 mutant, which is unable to bind any of the adaptors, and TBKK38M which is not active.

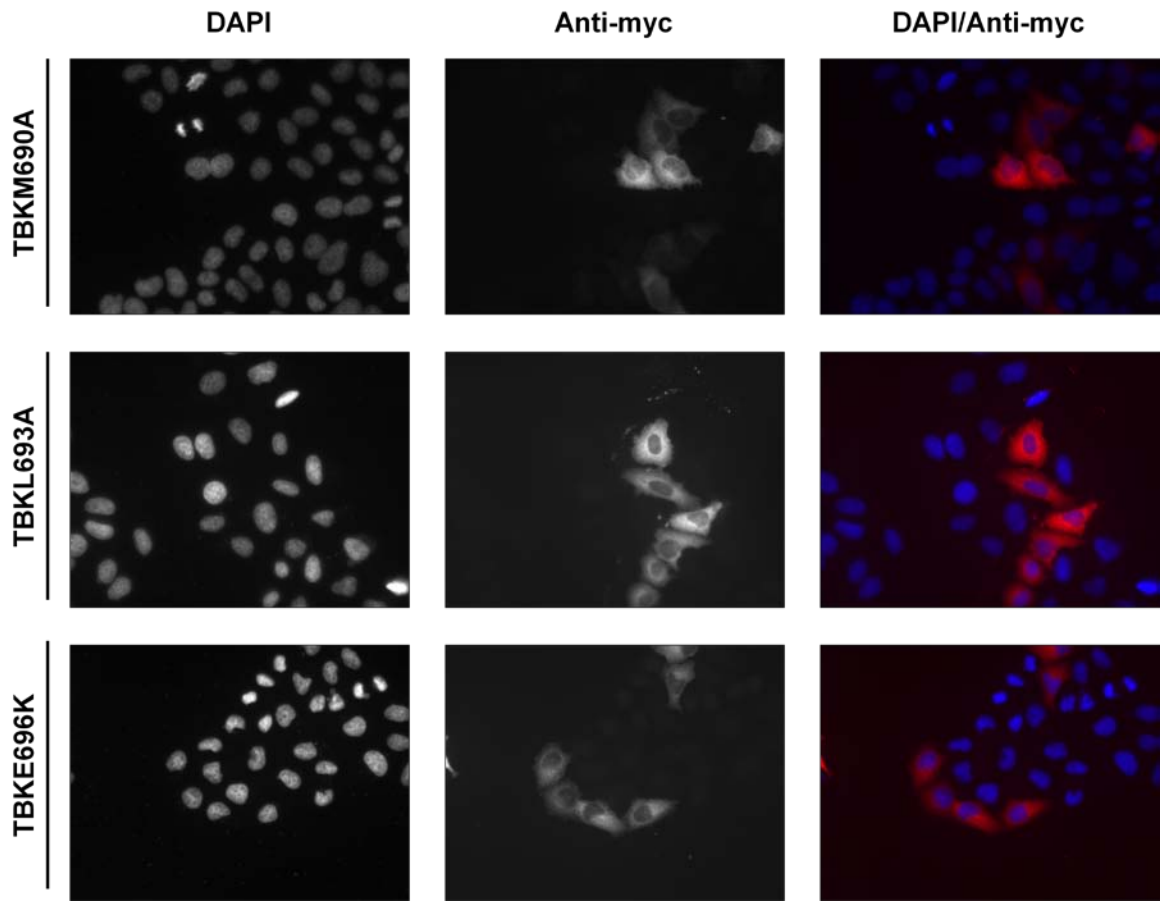

Figure 29: HeLa cells were transfected with Myc-tagged TBK1wt or TBK1 mutants. After fixing them, they were stained with an anti-Myc antibody and analyzed at the fluorescent microscope. TBK1CC2 point mutants are all found to occur in the cytosol.

In summary, the immunoflourescence experiment shows that the different TBK1 point mutants and the TBK1wt show no difference in localization within cells when they are expressed. Nevertheless the situation might be different in physiological conditions.

Additionally we stained the adaptor proteins TBKBP1, TBKBP2 and TANK upon over expression.

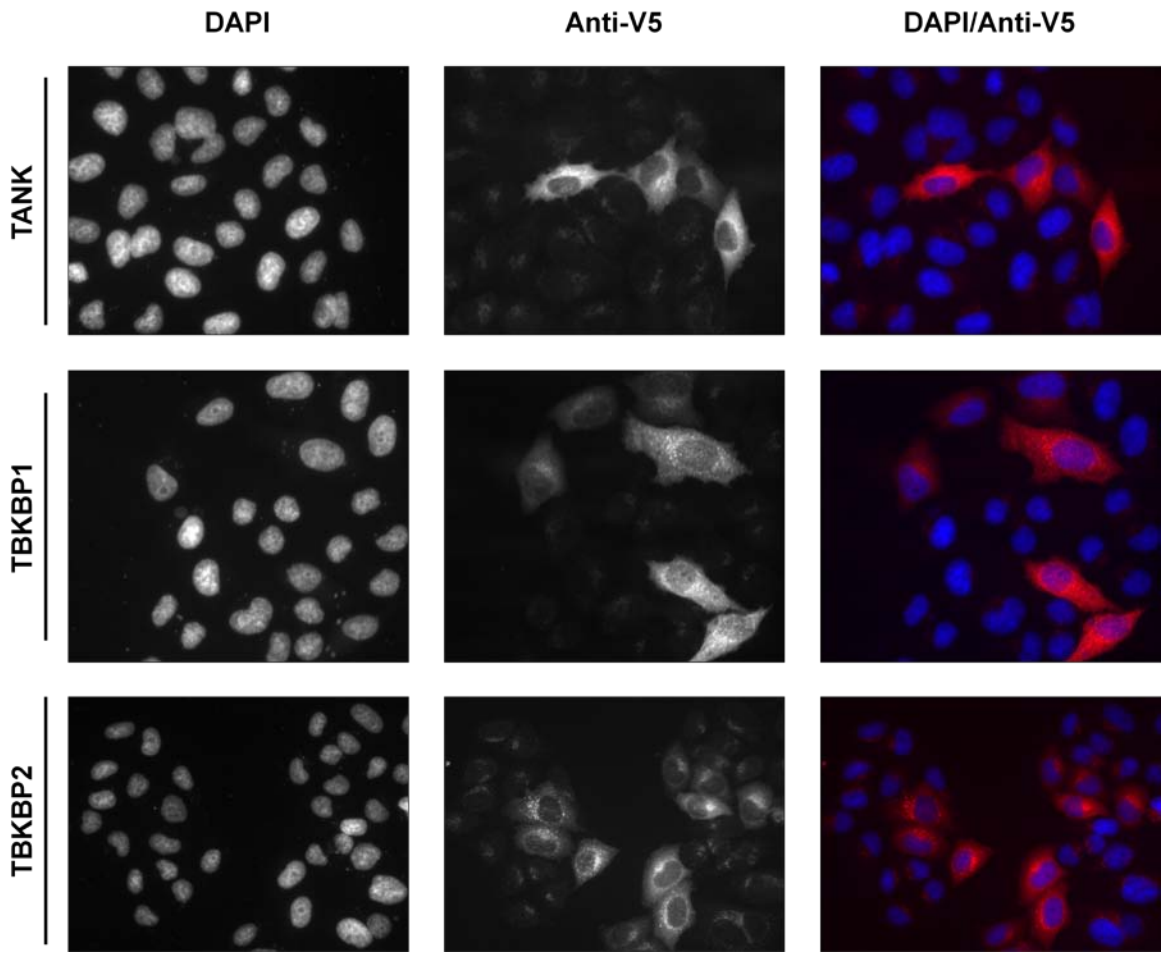

Figure 30: HeLa cells were transfected with V5-tagged TBK1 adaptors. After fixing them, they were stained with an anti-V5 antibody and analyzed at the fluorescent microscope. TANK can be found in the cytosol, but TBKBP1 and TBKBP2 show interesting speckles around the cytosol.

TANK, like TBK1 shows an even distribution around the cytoplasm. Interestingly TBKBP1 and TBKBP2 show a cytoplasmic staining but with many speckles in the cell very close to the nucleus. In comparison with MAVS, a mitochondrial protein, TBKBP1 and TBKBP2 look rather like they were located in smaller vesicles in the cell, like lysosomes or endosomes (data not shown). This suggests that the different adaptors might locate TBK1 to different compartments of the cell.

4 Discussion

Even though TBK1 and IKKi have an important role in innate immunity signaling upon bacterial or viral infection, little is known about their exact mode of action. The aim of the study was to obtain a better understanding of the core complex and the details of how TBK1 and IKKi interact with the respective binding proteins TBKBP1, TBKBP2 and TANK.

4.1 Tandem affinity purification

To investigate the interaction pattern of TBK1 and IKKi with their binding proteins we used the method of TAP purification as it reveals complex composition under close-to-physiological conditions. For this approach we used RAW 264.7 macrophages, which are competent for innate immunity signaling and therefore appropriate for our demands. Under non-stimulated conditions we used either the kinases TBK1 and IKKi or the adaptor proteins TBKBP1, TBKBP2 or TANK as bait protein to pull down specific binding proteins.

We wanted to perform side-by-side TAP analysis of TBK1/IKKi and the adaptor proteins TBKBP1, TBKBP2 and TANK to get an idea of the molecular architecture of the complex. As expected, when we analyzed the pulldowns with TBK1 and IKKi as bait proteins, the adaptor proteins were present in high abundance in TBK1 pulldowns, but fewer peptide counts appeared for IKKi. ProtFollow analysis showed that IKKi didn't pull down TBKBP1 in the previous experiments at CeMM, and also in these pulldowns TBKBP1 was only found in one of the two IKKi pulldowns. This may indicate that either TBKBP1 binds to IKKi with lower affinity or that TBKBP1 was not detected because a more abundant protein was "covering" TBKBP1.

The pulldown results of the adaptor proteins gave additional insight into the composition of the core complex. Against our expectations, the adaptor proteins did not show any binding to each other (Figure 9). Since we always found the three adaptor proteins in a quite high abundance with TBK1 in TBK1 pulldowns, we expected them to occur in a core complex. However our data seem to suggest that TBK1 is forming different sub-complexes with each of the binding proteins.

4.2 Interaction of TBK1 and the adaptor proteins

The TAP results directed our attention towards the interaction mechanism between TBK1 and its adaptors. The only finding that has been shown so far in this field was that only the C-terminal part (last 43 amino acids) of TBK1 was necessary to bind TANK (Pomerantz and Baltimore 1999). For TBKBP1 and TBKBP2 nothing was reported so far. So which domains of TBK1 are required for binding to the different binding proteins? To answer this question we created several deletion mutants of TBK1 (Figure 11) and investigated the binding properties with the adaptors.

Surprisingly TBKBP1, TBKBP2 and TANK all appeared to bind to the coiled coil 2 domain in the C-terminal part of TBK1 (Figure 12). The ULD domain or the coiled coil 1 domain of TBK1 can be deleted without affecting the binding of the TBK1 adaptors. To narrow down the exact region for TBK1 adaptor binding, we introduced several point mutants in the coiled coil 2 domain of TBK1. We again performed co-IPs and found 3 mutants which abolish the binding to the adaptors. One of the mutants also specifically binds TBKBP1 and TBKBP2 but not TANK (Figure 15). These mutants provide important insights by themselves. Although the TBK1 adaptors bind to similar regions in TBK1, their binding mode is likely to differ significantly as certain amino acids can distinguish between TANK on one side and TBKBP1/TBKBP2 on the other side. At the same time, these mutants represent valuable tools to study the function of the TBK1 adaptor proteins in cells, possibly by reintroducing the TBK1 mutants into a TBK1-/- background.

In general these findings support our TAP data, in that the formation of a core complex is unlikely since the adaptors don't bind to each other but bind the same domain in TBK1. Therefore the question was raised whether they bind in a pathway specific manner. This would imply that TBK1 induced activation of NF-κB or IRF3/7 is mediated by different stimuli, which in turn require different adaptors (Figure 31).

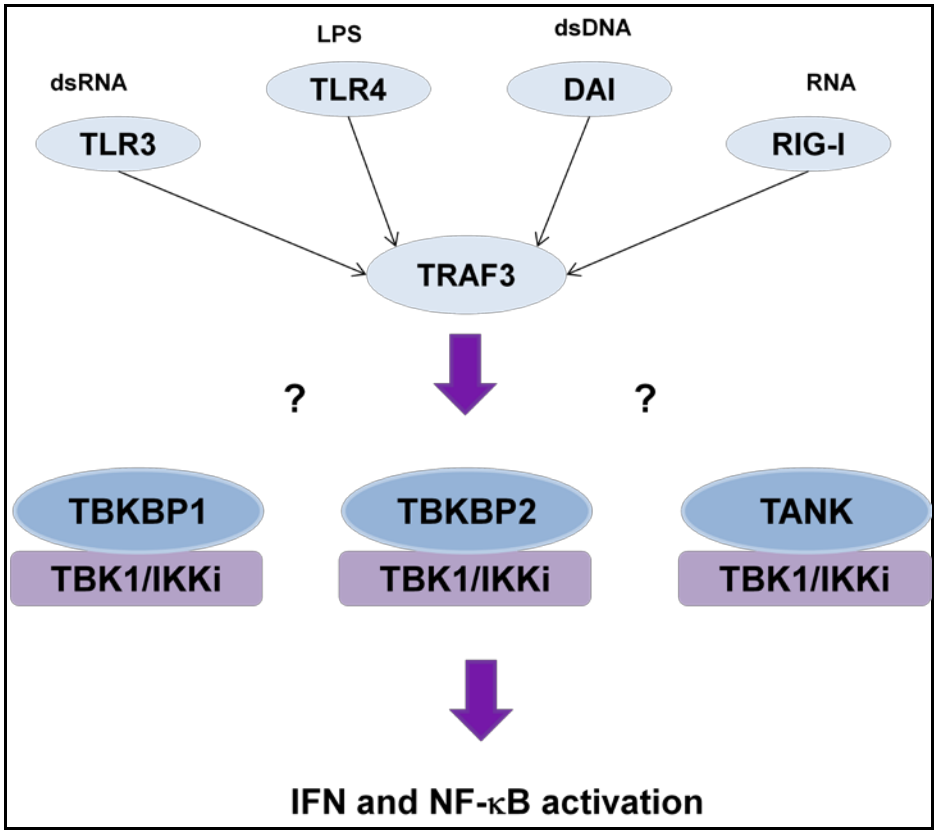

Figure 31: Schematic model of the stimulus dependent requirement of the different adaptors in IFN and NF-κB activation mediated via TBK1/IKKi

The exact mechanism of how, upon which stimuli and with which interaction partner(s) TBK1/IKKi performs its action to induce which transcription factor is not yet fully understood. But the fact that the three adaptors show pair wise sequence homology concerning their domains and bind the same region of TBK1 suggests that there might be functional significance for that. They might act in a non-redundant way during TBK1 signaling upon different stimuli. It would be worth testing whether knock down of each one of the adaptors gives a different signaling pattern upon different stimulation.

4.3 TBK1 Activity

There are several methods used as a read-out to measure the activity of TBK1. In this study we used autophosphorylation on serine 172, kinase assays, IFN-β and NF-κB reporter gene assays.

It was initially believed that the binding of the adaptor proteins to TBK1 has an impact on its activity. However, when we created the deletion mutants of TBK1 and investigated their binding abilities to the adaptor proteins, and afterwards the activity of those mutants we found that binding and activity are not correlated in an overexpression setting (Figure 19). Also the respective non-binding TBK1CC2 point mutants don't have a negative impact on TBK1 phosphorylation (Figure 20) or kinase activity (Figure 21). It additionally seems within IFN-β and NF-κB reporter gene assays, that the non-binding TBK1ΔCC2 and TBK1CC2 point mutants show the same activity as TBK1wt (Figure 22, Figure 24). Slight differences, however, occur due to different expression levels (Figure 23).

The reason for this might be that upon overexpression experiments TBK1 is active by itself. Therefore the process of activation, within which the adaptors still might play an important role, cannot be monitored by these means.

When we went one step further and co-transfected the adaptors with either TBK1wt or non-binding TBK1ΔCC2 to monitor again reporter gene activation, we expected that the effects of the adaptors are dependent on their binding to TBK1. Therefore less reporter gene activity was expected for the non-binding TBK1ΔCC2 mutant compared to the TBK1wt. Nevertheless we found that IFN-β induction is at about the same level whenever TBK1wt or TBK1ΔCC2 (non-binding mutant) is co-transfected with either the adaptors or the unrelated control protein (Figure 26). Minor changes only result from slightly different expression levels (Figure 27).

Interestingly NF-κB induction is decreased whenever the adaptors are co-transfected, no matter if with TBK1wt or the non-binding mutant TBK1ΔCC2. This is not the case for the unrelated control protein and therefore we suggest that the adaptors might have a negative function on the NF-κB pathway, independent of binding to TBK1 (Figure 25). Our results show that all 3 adaptors TBKBP1, TBKBP2 and TANK reduce the activation of NF-κB reporter genes upon co-transfection with

TBK1, indicating that the TBK1 adaptors mediate different upstream signals towards TBK1.

This result also enables the possibility of the adaptors acting as negative regulators on TBK1/IKKi activation. This idea is also supported by recent findings proposing that TANK is a negative regulator of TLR signaling (Kawagoe, Takeuchi et al. 2009).

As explained we have not been able to monitor the process of TBK1 activation since over expressed TBK1 is active already. But taken together our findings and the data from the literature indicate that the adaptors have different functions in the pathways TBK1 is activating.

Unfortunately there are no commercial antibodies available which detect endogenous TBKBP1, TBKBP2 and TANK. Although we made a great effort to purify the adaptors to generate antibodies it was not possible since the proteins are hardly soluble. This is why we had problems to conduct experiments on the endogenous levels of TBK1 and its adaptors.

4.4 Localization

In the beginning of this study we expected the adaptors of TBK1, TBKBP1, TBKBP2 and TANK to be required for the activity of the kinase. But after creating the mutants, which were still active, even though the binding to TBK1 was abolished, the possibility that they have a different role in innate immunity signaling arose. The idea was that the adaptors could play a role in the localization of TBK1. Therefore we used HeLa cells to perform immunofluorescence staining of TBK1, the TBK1 mutants and the 3 adaptor proteins.

When we first investigated TBK1 it was evenly distributed across the cytoplasm of the cell but there was no difference visible between the wild type, the non-binding TBK1ΔCC2 or the inactive TBKK38M mutant (Figure 28). This was also confirmed by the staining of the non-binding TBK1 point mutants (Figure 29). Activity or binding to the adaptors seems not to be necessary for proper location of TBK1 upon overexpression.

We next stained the adaptors and they showed an interesting staining pattern. TANK was distributed across the cytoplasm comparable to TBK1, but TBKBP1 and TBKBP2 appeared in interesting speckles (Figure 30). TBKBP2 seemed to appear even in bigger speckles than TBKBP1. These different location patterns also highly support our hypothesis of the occurrence of distinct signaling pathways. For the future it would be interesting to investigate localization of the endogenous proteins as well as upon cell stimulation.

5 Conclusion

This study provides evidence that the binding site of the adaptor proteins TANK TBKBP1 and TBKBP2 to TBK1 lies within the coiled coil 2 region. We also were able to map the binding site of the adaptors to TBK1 at the level of single amino acids. This enables us to distinguish precisely the binding site of TANK to TBK1 and the binding of TBKBP1 and TBKBP2 to TBK1, which differentiate by only a single amino acid. This is a crucial finding for identifying the role of the adaptor proteins, since the occurrence of one single core complex during TBK1 activation is almost excluded. We tend to believe that there are several non-redundant sub-complexes required for TBK1 activation.

There are still many questions that arose from this study. Are the adaptors needed for TBK1 activation? Could there also be some negative regulation mechanism? What does the localization pattern of the adaptors, especially TBKBP1 and TBKBP2 mean? Is there any stimulus dependent function for the different adaptor proteins and are the different sub-complexes needed for that? To answer these questions, antibodies that recognize the endogenous proteins are important. This tool will be needed for studying the endogenous activation process of TBK1 and the role of the adaptors.

TBK1 and IKKi are important kinases involved in innate immunity signaling via different receptors. Understanding the activation mechanism is intrinsic to immunity research. This study unravaled the binding mechanism of TBK1 and its adaptors and is the initial step of a publication that may arise.

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