TECHNISCHE UNIVERSITÄT MÜNCHEN

LEHRSTUHL FÜR GENETIK

Functions of the germinal center kinase TNIK in signal transduction of the viral oncoprotein LMP1 and the related CD40 receptor

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Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktor der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. M. Hrabé de Angelis
Prüfer:	1. Priv.-Doz. Dr. A. Kieser
	2. Univ.-Prof. Dr. D. Langosch

Die Dissertation wurde am 01.06.2012 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 9.10.2012 angenommen.

Parts of this thesis have been submitted in a revised version of the manuscript for publication in PLoS Biology:

The germinal center kinase TNIK is required for JNK and canonical NF-κB signalling by the viral LMP1 oncoprotein and CD40.

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The research for this thesis was performed at the

HelmholtzZentrum münchen
German Research Center for Environmental Health

Research Unit Gene Vectors

Signal Transduction Group

For Gerda Town
and in loving memory of
Harold Town

“Let the future tell the truth, and evaluate each one according to his work and accomplishments. The present is theirs; the future, for which I have really worked, is mine”

— Nikola Tesla

1. Introduction

1.1. The germinal center kinase TNIK

1.1.1. Overview of the germinal center kinase family

Epstein-Barr virus (EBV) is associated with various human malignancies and has been classified as a type I carcinogen. EBV transforms cells by means of a highly efficient viral pseudoreceptor called latent membrane protein 1 (LMP1) that exploits a wide range of cellular signal transduction pathways (see 1.2). Our research group is dedicated to the study of LMP1-induced signal transduction in order to understand how EBV usurps normal cellular processes for its means and ultimately aims to find new therapies for EBV associated malignancies. The first step towards this ultimate goal is to gain a closer insight into the composition and function of the signalling complex that is induced by LMP1. To this end a functional proteomics approach was engaged in order to find new interaction partners of LMP1. This screen revealed the germinal center kinase (GCK) family member TRAF2-and Nck-interacting kinase (TNIK) as a new interaction partner of LMP1.

TNIK was first discovered in 1999 in a screen for tumour necrosis factor receptor (TNFR) associated factor 2 (TRAF2) and Nck-interacting proteins and has afterwards found little mention in the literature for 10 years. Now TNIK has emerged as an important factor for several biological functions in health and disease. TNIK plays a critical role for the outgrowth of colorectal cancer and chronic myoelogenous leukemia (Satow et al, 2010; Schurch et al, 2012). In addition, TNIK is essential for mammalian nerve cell development and is implicated as a risk factor for psychiatric diseases, such as schizophrenia and bipolar disorder (Kawabe et al, 2010; Wang et al, 2010a). Moreover, TNIK is involved in embryonic development and mediates axis formation in Xenopus embryos (Satow et al, 2010).

TNIK is a member of the sterile 20 (Ste20) kinase family that comprises 30 protein serine/threonine kinases in mammals in addition to homologues in *Drosophila melanogaster* and *Caenorhabditis elegans*. The catalytic domains of Ste20 kinases are homologous to the budding yeast Ste20p (Ste20 protein) (Dan et al, 2001). The Ste20 kinase family can be subdivided into the p21-activated protein kinases (PAKs) and germinal center kinases (GCKs) that mediate various cellular functions including regulation of apoptosis, rearrangement of the cytoskeleton and development. The GCK family is further classified into eight subfamilies based on the extent of conservation of their kinase domain (Dan et al, 2001). TNIK belongs to the GCK IV subfamily together with Nck-interacting kinase (NIK), Misshapen-NIKs-related kinase (MINK)/hMINKβ, HPK/GCK-like kinase (HGK), and Nck-interacting kinase (NIK)-related kinase (NRK)/NIK-like embryo-specific kinase (NESK)

as well as *Drosophila melanogaster* Misshapen (Msn) and *Caenorhabditis elegans* Mig-15 (Dan et al, 2001). Members of the GCK IV subfamily have an N-terminal kinase domain, a highly variable intermediate domain with several proline-rich motifs and a C-terminal germinal center kinase homology (GCKH) domain, also referred to as citron homology (CNH) domain (Dan et al, 2001).

Like many members of the GCK family the GCK IV kinases have the ability to activate the c-Jun N-terminal kinase (JNK) pathway. The JNK signalling pathway is a mitogen-activated protein kinase (MAPK) pathway that is activated in response to inflammatory cytokines and environmental stresses (Johnson & Nakamura, 2007). The GCK IV members activate MAPK kinase kinases (MAP3K) and thus act as MAP4K. However, the activation of the downstream MAP3K and induction of JNK signalling does not depend on the kinase domain. Members of the GCK IV family activate downstream signalling events either solely through their GCKH domain or require both their kinase and GCKH domains for full activation (Dan et al, 2001; Diener et al, 1997; Fu et al, 1999; Hu et al, 2004; Pombo et al, 1995; Su et al, 1997). GCK IV kinases are thought to activate their associated MAP3K not through direct phosphorylation, but rather by binding through their GCKH domains which then causes a conformational change and auto-phosphorylation of the MAP3K (Dan et al, 2001; Kyriakis, 1999).

1.1.2. Structure and function of TNIK

TNIK consists of 1360 amino acids and has an apparent molecular weight of 155 kDa. It has an N-terminal kinase domain (amino acids 1 - 306), an intermediate domain (amino acids 307 – 1017) and a germinal center kinase homology domain (amino acids 1018 – 1360) (Fu et al, 1999) (figure 1.1). The kinase and GCKH domains of TNIK share high sequence homology with NIK, MINK and HGK (about 90 %) as well as *Drosophila* Msn and *C.elegans* Mig-15 (about 80 %). The intermediate domain is less conserved with only about 53 % sequence homology. NRK/NESK is more divergent from the other GCK IV family members and shares only about 59 % sequence homology in its kinase and GCKH domains and about 37 % sequence homology in its intermediate domain (Dan et al, 2001; Fu et al, 1999; Wright et al, 2003).

Three regions within the intermediate domain of TNIK can be alternatively spliced resulting in eight different isoforms. The function of theses isoforms is still unknown (Fu et al, 1999). TNIK is ubiquitously expressed and has a number of important physiological functions in different cell types (figure 1.1). Northern blot analysis of TNIK expression revealed high levels of TNIK messenger RNA in heart, brain and skeletal muscle (Fu et al, 1999).

Additionally, immunoblot analysis of TNIK expression in mouse tissues showed high levels of TNIK protein in brain, lung, lymph nodes and thymus. Intermediate levels of TNIK were detected in heart and skeletal muscle (Katharina Vogel and Dr. Vigo Heissmeyer, personal communication).

TNIK plays a role in the reorganization of the cytoskeleton and exerts a negative effect on cell spreading by inducing the disassembly of F-actin in a kinase dependent manner (Fu et al, 1999). It is unclear, how the kinase activity of TNIK is involved in actin fibre disassembly. TNIK phosphorylates the F-actin fragmenting and capping enzyme Gelsolin *in vitro* and it is thus possible that TNIK regulates F-actin assembly through this enzyme (Fu et al. 1999). Autophosphorylation of TNIK might also play a role in TNIK-mediated regulation of the cytoskeleton. Autophosphorylation induces the translocation of TNIK to the cytoskeletal fraction and is induced by Rap2, a member of the Ras family of small GTP-binding proteins. Rap2 interacts with the GCKH domain of TNIK and enhances the inhibitory function of TNIK against cell spreading (Taira et al, 2004).

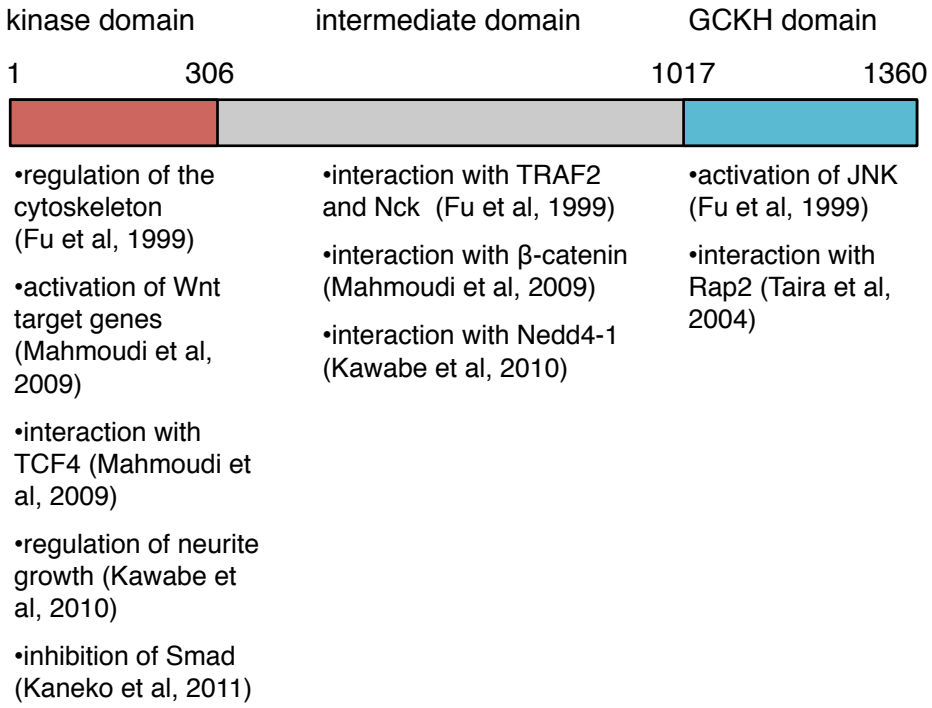


Figure 1.1. Schematic diagram of TNIK
The numbers represent amino acids of TNIK. The functions mediated by each domain are listed.

Effects on the cytoskeleton have also been reported for other members of the GCK IV family. NESK, which is expressed exclusively during the early stages of embryogenesis in skeletal muscle, is involved in the regulation of actin cytoskeletal organization through

phosphorylation of the actin-depolymerizing factor cofilin (Nakano et al, 2003). HGK and hMINKβ both exert a negative effect on cell spreading. Kinase-inactive forms of HGK and hMINKβ enhance cell-cell adhesion and block cell migration and invasion thus linking these kinases to tumour progression (Hu et al, 2004; Wright et al, 2003). Consequently, HGK is found expressed in many human tumour cells and potentiates cell transformation of tumour cells (Wright et al, 2003).

In addition to its effects on the cytoskeleton the kinase activity of TNIK is also involved in the activation of the Wnt pathway. Activation of Wnt receptors leads to a stabilization of β-catenin, which translocates into the nucleus, where it forms a complex with TCF/LEF transcription factors and serves as a transcriptional co-activator. Deregulated Wnt signalling is associated with several human malignancies (Polakis, 2000). TNIK is an essential mediator of the Wnt signalling pathway. Activation of Wnt signalling induces autophosphorylation of TNIK at serine 764. Phosphorylated TNIK translocates into the nucleus where it interacts with the TCF4-β-catenin transcriptional complex and phosphorylates and thus activates TCF4 (Mahmoudi et al, 2009; Shitashige et al, 2010). Since a different study reported translocation of phosphorylated TNIK to the cytoskeletal fraction (Taira et al, 2004), phosphorylation of TNIK seems to have differential effects depending on the cell type and type of inducer. The kinase activity of TNIK is essential for the outgrowth of colorectal cancer thus making TNIK a promising target for the treatment of this type of cancer (Mahmoudi et al, 2009; Shitashige et al, 2010).

The role of TNIK as an activator of Wnt signalling is also involved in embryonic development. A recent study shows that the Xenopus otholog XTNIK is essential for Xenopus development and the knock down of XTNIK or expression of a kinase-inactive XTNIK results in severe malformations with a complete loss of head and axis structures (Satow et al, 2010).

Other members of the GCK IV family also play important roles in developmental processes. The kinase activity of TNIK, MINK and the *Drosophila* Msn has been associated with phosphorylation and thus inhibition of Smad, a regulator of transforming growth factor β (TGFβ)/bone morphogenic protein (BMP) signalling, which is critical for proper embryonic patterning and homeostasis (Kaneko et al, 2011). Msn additionally acts downstream of the Frizzled receptor to mediate epithelial planar cell polarity and mediates dorsal closure during embryogenesis by activating basket, the *Drosophila* homologue of JNK (Paricio et al, 1999; Su et al, 1998). NIK-deficient mice die in postgastrulation between embryonic day (E) 9.5 and E10.5 and exhibit mesodermal patterning defects (Xue et al, 2001). The *C.elegans* ortholog Mig-15 mediates several developmental processes in *C. elegans* including regulation of

Q-neuroblast polarity and migration as well as axon pathfinding (Chapman et al, 2008; Poinat et al, 2002).

TNIK is highly expressed in the brain and has important functions in neurite development. TNIK acts as an adapter protein between the E3 ubiquitin ligase Nedd4-1 and Rap2 that is essential for the ubiquitination and inactivation of Rap2. Ubiquitination inhibits Rap2 function, which reduces the activity of its effector kinases TNIK and MINK and promotes dendrite growth (Kawabe et al, 2010). TNIK has also received attention for its implication in mental diseases, such as schizophrenia and bipolar disorder (Wang et al, 2010a). TNIK interacts with DISC1 (disrupted-in-schizoprenia1) and regulates synaptic activity and composition by stabilizing levels of post-synaptic proteins (Bradshaw & Porteous, 2012; MacLaren et al, 2011; Wang et al, 2010a).

The intermediate domain of TNIK is the interaction site for the adapter proteins TRAF2 and Nck. TRAF2 is a member of the TRAF family, which are important signalling mediators of the TNFR superfamily, the interleukin-1 receptor (IL-1R)/Tolllike receptor(TLR) superfamily as well as of the viral oncoprotein LMP1 (Chung et al, 2002) (see 1.7). Nck belongs to a family of adapter proteins with Src homology (SH2/SH3) domains and has been implicated in the regulation of many growth receptor mediated signal transduction pathways (Buday et al, 2002). Nck interacts with proline rich motifs within the intermediate domain of TNIK, which are potential SH3 binding sites (Fu et al, 1999). A biological function for the interaction of TNIK with Nck has not been described so far. A recent study suggested a role of the TNIK-TRAF2 interaction for the activation of the Wnt pathway by the TNFR family member CD27 in leukaemia stem cells. TRAF2 interacts with the cytosolic domain of CD27 and may link TNIK to CD27-mediated Wnt signalling (Schurch et al, 2012).

NIK and MINK also interact with Nck via proline-rich regions within their intermediate domains (Hu et al, 2004; Su et al, 1997). NIK is linked to receptor tyrosine kinases of the Eph family via Nck and mediates activation of integrin and the JNK pathway (Becker et al, 2000). Msn functions downstream of dreadlocks (dock), the *Drosophila* homologue of Nck, to mediate photoreceptor axon pathfinding (Ruan et al, 1999; Su et al, 2000).

As mentioned before, activation of the JNK pathway is a common feature of GCK family members (see 1.1.1). While TNIK and hMINKβ activate JNK signalling solely via their GCKH domains independently of their kinase domain (Fu et al, 1999; Hu et al, 2004), NIK and Msn require both their kinase and intermediate domains for full activation of the JNK pathway (Su et al, 1997; Su et al, 2000). Both NIK and NESK activate the JNK pathway via the MAP3K MAPK/ERK kinase 1 (MEKK1) (Nakano et al, 2000; Su et al, 1997), whereas HGK employs TAK1 as a downstream MAP3K (Yao et al, 1999). Msn binds *Drosophila* DTRAF1 and

coexpression of Msn and DTRAF1 leads to a synergistic activation of JNK (Liu et al, 1999). It is not known to date which downstream kinase TNIK employs for activation of JNK. Neither has a receptor engaging TNIK for JNK signalling nor the biological function of JNK activation by TNIK been described to date.

A functional proteomics screen conducted in our laboratory has revealed TNIK as a novel interaction partner of the viral oncoprotein LMP1 from the B-cell specific EBV. Further experiments have designated an important role of TNIK in the signal transduction of LMP1 and the cellular counterpart of LMP1, the CD40 receptor.

1.2. The viral oncoprotein latent membrane protein 1 (LMP1)

1.2.1. LMP1 as the major oncoprotein of EBV

LMP1 is a viral receptor-like oncoprotein that is essential for cellular transformation by EBV. EBV is a member of the herpesvirus family. Herpesviruses are a family of large double-stranded DNA viruses with eight human herpesviruses that are subdivided according to their genomic structure into the three subfamilies: α-, β- and γ-herpesviruses. EBV belongs together with Kaposi's sarcoma-associated virus to the γ-herpesviruses (Sedy et al, 2008). EBV latently infects more than 90 % of the human population worldwide. Primary infection with EBV usually occurs during childhood and is mostly asymptomatic, while delayed primary infection in adolescence or adulthood is often accompanied by infectious mononucleosis. Naive B-cells are the main targets of EBV infection but in some circumstances infection of T-cells, epithelial cells, monocytes or dendritic cells occurs (Kuppers, 2003). After infection the linear viral DNA forms a circle and persists as an episome in infected cells thereby establishing latent infection and from then on persists in memory B-cells throughout life. Activation of memory B-cells can lead to a switch to the lytic cycle, which results in the production of new virus particles (Babcock et al, 1998; Kuppers, 2003). Due to constant immune surveillance infection with EBV is usually innocuous in healthy individuals. However, in immuno-compromised persons EBV infection can cause lymphoproliferative diseases such as post-transplant lymphoproliferative disorders. In addition, EBV is associated with malignancies such as Hodgkin's lymphoma, Burkitt's lymphoma, gastric carcinoma and nasopharyngeal carcinoma and has therefore been classified as a type I carcinogen by the World Health Organization (WHO) (Niedobitek, 1999; Young & Rickinson, 2004).

LMP1 is considered the primary oncogene of EBV that efficiently exploits cellular signal transduction pathways for viral cell transformation and continuous *in vitro* proliferation of

latently infected B-cells called lymphoblatoid cell lines (LCLs) (Kieser, 2007; Kieser, 2008; Young & Rickinson, 2004). LMP1 is essential for LCL outgrowth and EBV-mediated transformation of B-cells (Dirmeier et al, 2003; Kaye et al, 1993). It is sufficient to induce transformation of rodent fibroblasts in vitro (Moorthy & Thorley-Lawson, 1993; Wang et al, 1985) and tissue specific expression of LMP1 in B-cells of transgenic mice results in the development of B-cell lymphomas (Kulwichit et al, 1998).

LMP1 acts as a highly efficient viral pseudo-receptor that is independent of a ligand and induces activation of a wide range of signalling pathways that ensure cell survival, proliferation and evasion of apoptosis which are essential for malignant outgrowth (Kieser, 2007).

1.2.2. The structure and function of LMP1

LMP1 consists of 386 amino acids with an apparent molecular weight of 63 kDa. LMP1 is a transmembrane protein with a short cytoplasmic amino-terminal domain, 6 transmembrane helices and a cytoplasmic carboxy-terminal domain (figure 1.2). The amino-terminal domain (amino acids 1-24) is responsible for the correct insertion and orientation of LMP1 in the membrane (Coffin et al, 2001). Additionally, this domain mediates protein turnover of LMP1, which has a short half-life ranging from 1.5 – 5 hours. LMP1 is degraded by the ubiquitin proteasome system upon ubiquitination of its amino-terminal domain (Aviel et al, 2000; Baichwal & Sugden, 1987; Mann & Thorley-Lawson, 1987; Martin & Sugden, 1991). Amino acids 9 – 20 contain putative SH3 binding motifs which affect patching of LMP1 in the membrane and contribute towards the transformation potential of LMP1 (Bloss et al, 1999).

The transmembrane domain (amino acids 25 – 186) mediates clustering of LMP1 molecules in the membrane, which is essential and sufficient for the activation of LMP1 and induction of signal transduction (Gires et al, 1997). LMP1 is distributed between plasma membranes and intracellular domains (Lam & Sugden, 2003). A FWLY motif (amino acids 38 - 41) located in the transmembrane helix 1 mediates oligomerization and is also essential for localization of LMP1 in lipid rafts. About 30 % of LMP1 reside in lipid rafts at steady state. LMP1 recruits signalling adapters, such as TRAFs and TRADD, into the lipid raft fraction and induction of signal transduction takes place in this compartment (Kaykas et al, 2001; Lam & Sugden, 2003).

The carboxy-terminal domain (amino acids 187 – 386) of LMP1 constitutes the signalling active domain. It incorporates the two C-terminal activating regions, CTAR1 (amino acids 194 – 232) and CTAR2 (amino acids 351 – 386) which are essential for B-cell transformation by

EBV. The CTARs recruit signalling molecules and induce a wide range of signal transduction pathways (Huen et al, 1995; Kaye et al, 1995; Kieser, 2007; Mitchell & Sugden, 1995). CTAR1 recruits the TRAF family members 1, 2, 3 and 5 through its P_{204}QQAT motif (see 1.7.2) and mediates activation of the non-canonical nuclear factor-κB (NF-κB), phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signal-regulated kinase (ERK)-MAPK pathways and contributes towards p38 MAPK activation (Devergne et al, 1996; Kaye et al, 1996; Kieser, 2007; Sandberg et al, 1997). In some cell lines the CTAR1 domain mediates activation of the JNK pathway (Eliopoulos et al, 2003). CTAR2 recruits the adapter proteins TRAF6 and tumour necrosis factor receptor type1-associated death domain protein (TRADD) and induces canonical NF-κB signalling as well as activating the JNK and p38 MAPK pathways (Izumi & Kieff, 1997; Kieser, 2007; Kieser et al, 1997; Luftig et al, 2003; Schneider et al, 2008; Schultheiss et al, 2001). A stretch of six amino acids (P_{379}VQLSY$_{384}$) at the far C-terminus of LMP1 was determined as the NF-κB and the JNK activating region (Floettmann & Rowe, 1997; Kieser et al, 1999) and incorporates the TRADD binding motif (amino acids 384 – 386) (Izumi & Kieff, 1997) (figure 1.2).

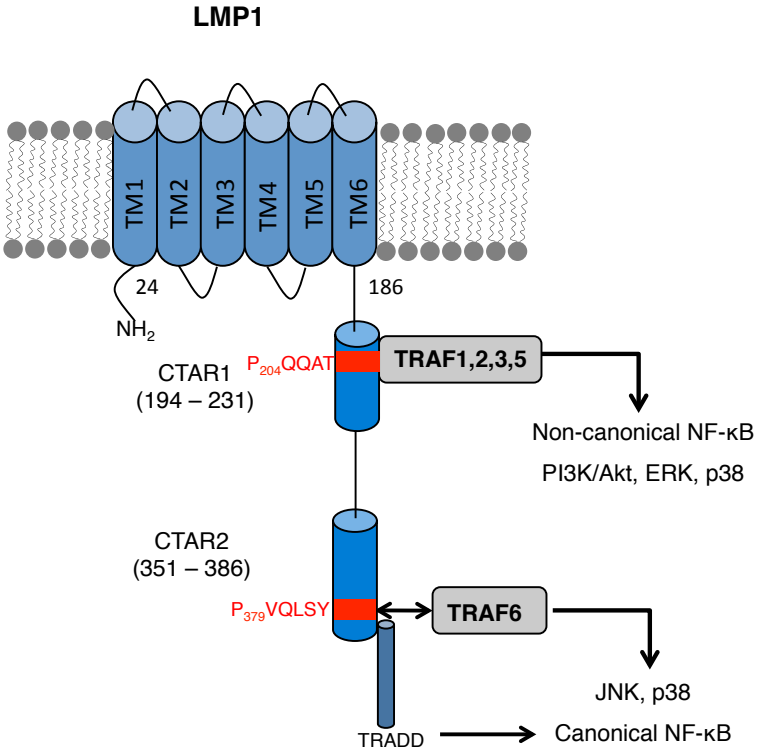


Figure 1.2. Schematic diagram of LMP1
The numbers respresent amino acids of LMP1. LMP1 consists of a short amino-terminus, 6 transmembrane helices (TM1-6) and the C-terminal domain incorporating CTAR1 and CTAR2. Functional sites within the C-terminus are indicated. LMP1 recruits TRAF family members and TRADD through its PQQAT and PVQLSY sites respectively and activates the signal transduction pathways listed on the right.

Both the NF-κB and the JNK pathways are essential for B-cell transformation by EBV. NF-κB signalling up-regulates a panel of anti-apoptotic genes, cytokines and B-cell activation markers, while JNK signalling mediates cell cycle progression and continuous proliferation of LCLs (Cahir-McFarland et al, 1999; Kutz et al, 2008). The PI3K/Akt pathway promotes cell survival, proliferation and contributes towards morphological transformation of rat fibroblasts (Dawson et al, 2003; Mainou et al, 2005). The p38 MAPK pathway leads to cytokine induction, while the ERK-MAPK pathway plays a role in LMP1-induced epithelial cell motility and transformation (Dawson et al, 2008; Eliopoulos et al, 1999b; Mainou et al, 2007; Vockerodt et al, 2001).

LMP1 additionally mediates activation of the JAK/STAT pathway that might be triggered by binding of JAK3 to consensus motifs located between CTAR1 and CTAR2 (Gires et al, 1999). However, the involvement of the putative JAK3 binding motifs has been contradicted by another study (Higuchi et al, 2002).

1.3. The CD40 receptor

1.3.1. Structure and function of CD40

CD40 is a member of the TNFR superfamily and a co-stimulatory receptor on B-cells that is important for the adaptive immune response. Additionally, CD40 is the cellular counterpart of the EBV pseudoreceptor LMP1 which mimics functions of CD40 in an amplified and sustained manner. Initiation of an adaptive immune response involves binding of the T-cell antigen receptor (TCR) to polypeptides presented by the major histocompatability complex II (MHCII) on the surface of antigen-presenting cells (APCs) and binding of a native antigen by the B-cell receptor complex of B-cells. Subsequent secondary signals are involved in the fine-tuning of the immune response and are mediated by costimulatory molecules expressed as receptor ligand pairs on T-cells and APCs. Due to its important role in immunity, CD40/CD40 ligand (CD40L) is the best characterized costimulatory receptor/ligand pair (Kawabe et al, 2011). CD40 mediates antibody production, immunoglobulin isotype switching, generation of memory B-cells, T-cell co-stimulation, antigen presentation and the production of cytokines which underlines an essential role of CD40 in the humoral immune response. In addition to B-cells, CD40 is also found expressed on dendritic cells, macrophages and under specific conditions on epithelial cells, basophils, eosinophils, monocytes, T-cells and neuronal cells (Elgueta et al, 2009; Graham et al, 2010). CD40 is a 40 – 45 kDa type I transmembrane protein, which contains a 193 amino acid extracellular domain with 22 conserved cystein residues, a linker sequence of 21 amino acids, a transmembrane domain

of 22 amino acids and an intracellular domain of 62 amino acids. CD40 is activated by binding of its ligand CD40L, also known as CD154 or gp39. CD40L is a member of the TNF superfamily with a molecular weight between 32 and 39 kDa. It is a type II transmembrane protein, which is expressed predominantly on activated T cells.

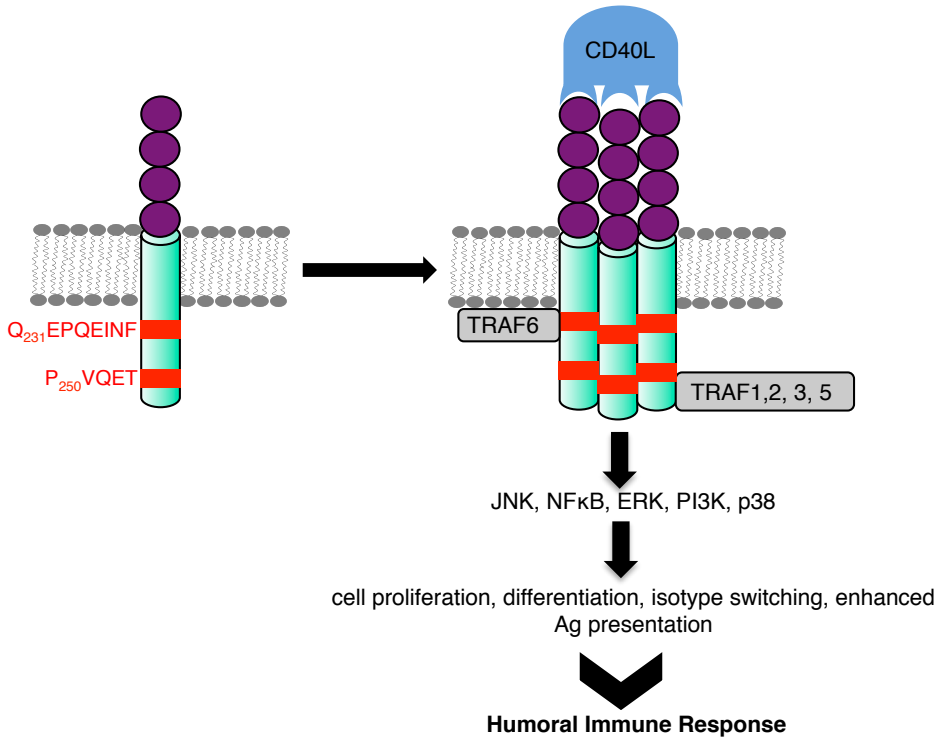


Figure 1.3. Schematic diagram of the CD40 receptor
CD40 is shown in its inactive state on the left hand side. Activation of CD40 by binding of CD40L leads to a clustering of CD40 in the membrane. Activated CD40 recruits TRAF family members and activates the indicated signal transduction pathways (right hand side).

Binding of CD40L by CD40 leads to clustering of CD40 and subsequent activation of the receptor (Elgueta et al, 2009). CD40 lacks intrinsic kinase activity in its cytoplasmic tail and signals are transduced through ligand-dependent recruitment of adapter proteins of the TRAF family. CD40 recruits the TRAF family members TRAF 1,2,3,5 and 6 and activates the ERK, JNK and p38 MAPK pathways, the canonical and non-canonical NF-κB pathways, as well as the PI3K and JAK/STAT pathways (figure 1.3). These pathways act in concert to mediate the diverse functions of CD40 (Kawabe et al, 2011).

1.3.2. The role of CD40 in cancer and autoimmune diseases

While CD40 is critical for the functional immune response, it is also involved in cancer and auto-immune diseases. CD40 plays an important role in the pathogenesis of a number of autoimmune diseases, such as type I diabetes, multiple sclerosis, psoriasis, inflammatory

bowel disease, rheumatoid arthritis and systemic lupus erythematosus (Peters et al, 2009). Deregulated CD40 activity is also involved in the development and progression of several haematopoietic and non-haematopoietic human malignancies. CD40 engagement promotes proliferation and survival of B lymphoma cells (Choi et al, 1995; Greiner et al, 1997; Lee et al, 1999a; Planken et al, 1996; Renard et al, 1996; Sarma et al, 1995). Additionally, a study conducted with transgenic mice expressing a constitutively active LMP1-CD40 fusion protein consisting of the transmembrane domain of LMP1 and the intracellular signalling domain of CD40 showed that constitutive CD40 signalling promotes the development of B-cell lymphomas (Homig-Holzel et al, 2008). CD40 is also found expressed in melanoma and carcinomas of the lung, breast, colon and prostrate (Vonderheide, 2007).

CD40 can favour tumour progression in several different ways. In some cases, tumour cells aberrantly express both CD40 and CD40L and are thus able to self-stimulate (van den Oord et al, 1996). CD40 can also bind and down-regulate CD40L on T-cells, thus decreasing T-cell-mediated inhibition of tumour growth (Batrla et al, 2002). However, the role of CD40 in cancer is a double-edged sword as CD40 signalling can also defend against malignancies. Activation of CD40 can be directly cytotoxic to some tumours or can lead to the up-regulation of the expression of pro-apoptotic factors, such as TNF-related apoptosis-inducing ligand (TRAIL), TNF and CD95/FAS thus inducing apoptosis (Dicker et al, 2005; Eliopoulos et al, 2000; Funakoshi et al, 1994). Additionally, CD40 activates APCs to induce T-cell activation that directly targets tumour cells (Graham et al, 2010; Vonderheide, 2007).

1.3.3. LMP1 as a viral mimic of the CD40 receptor

As described above, deregulated CD40 activity has many adverse effects and leads to the development of cancer or autoimmune disease. The viral oncoprotein LMP1 makes use of the transforming potential inherent in deregulated CD40 signals by mimicking CD40 in an amplified and sustained manner. LMP1 exploits cellular signalling pathways that normally contribute towards the immune response and thus help to fight against disease to ultimately achieve cellular transformation.

Both LMP1 and CD40 engage TRAFs for signalling and induce an overlapping set of signal transduction pathways including the canonical and non-canonical NF-κB pathways and the JNK, p38 and ERK MAPK pathways (Graham et al, 2010; Lam & Sugden, 2002). Studies conducted with a transgenic mouse strain expressing a fusion protein of the extracellular signalling domain of CD40 and the cytoplasmic C-terminus of LMP1 in B-cells showed that LMP1 can substitute for CD40 signalling *in vivo* (Rastelli et al, 2008). However, the

expression of the CD40-LMP1 fusion protein can lead to hyperactivation of B-cells and disruption of lymphoid architecture which indicates that LMP1 signals in an amplified manner compared to CD40 (Stunz et al, 2004). The amplified nature of LMP1 signalling is also underlined by a study showing that while both receptors are translocated to lipid rafts upon activation, LMP1 induces a more efficient signalling complex compared to CD40 (Kaykas et al, 2001). While the activation of CD40 is tightly controlled and relies on the binding of CD40L, LMP1 is constitutively active and signals independently of a ligand (Gires et al, 1997).

1.4. General overview of the NF-κB pathway

Nuclear factor-κB (NF-κB) is a collective term for a family of dimeric transcription factors that are critical regulators of immunity, inflammation, stress responses, apoptosis and differentiation. Deregulated NF-κB activity is linked to cancer, metabolic and auto-immune diseases as well as inflammatory disorders (Oeckinghaus et al, 2011). NF-κB has received considerable attention for being the key transcription factor involved in cancer and is constitutively active in most cancers (Chaturvedi et al, 2011). Activation of the NF-κB pathway is also critical for LMP1-induced transformation (Cahir-McFarland et al, 1999; Devergne et al, 1996; He et al, 2000).

The NF-κB family comprises five members in mammals: p65 (RelA), RelB, c-Rel and the precursors p105 (NF-κB1) and p100 (NF-κB2) that are processed into p50 and p52 respectively. All members of the NF-κB family are characterized by the presence of an N-terminal Rel-homology domain (RHD), which mediates homo-and heterodimerization as well as sequence-specific DNA binding. While p65, c-Rel and Rel-B contain a C-terminal transactivation domain to activate gene transcription, p52 and p50 depend on interactions with other NF-κB subunits. p65 and c-Rel mainly heterodimerize with p50, whereas RelB forms heterodimers with p100. In its inactive state NF-κB dimers are retained in the cytoplasm by NF-κB inhibitor (I-κB) proteins or the precursor proteins p100 and p105 (Hayden & Ghosh, 2008). NF-κB is activated mainly via two pathways: the canonical and non-canonical pathway. The canonical NF-κB pathway is induced by stimuli emanating from receptors such as the TNFR, IL-1R and TLR families as well as LMP1 and leads to the activation of the IκB kinase (IKK) complex which consists of the two catalytically-active kinases IKKα and IKKβ and the regulatory subunit IKKγ (NEMO) (Oeckinghaus et al, 2011). The kinase subunits are activated by phosphorylation at two serine residues within an activation loop. To date it is unclear, whether phosphorylation occurs through trans-autophosphorylation or through phosphorylation by an upstream kinase (Israel, 2010;

Mercurio et al, 1997). The canonical pathway depends on IKKβ and NEMO and leads to the phosphorylation of I-κBα by IKKβ. I-κBα is subsequently ubiquitinated and degraded via the ubiquitin-proteasome system. This leads to the liberation of NF-κB transcription factors, mainly p65/p50 dimers, which travel into the nucleus and activate transcription of target genes (Hayden & Ghosh, 2008).

The non-canonical pathway is initiated in response to CD40L, B-cell activating factor (BAFF) and lymphotoxin-β as well as LMP1 activity and depends on activation of IKKα by NIK. Activated IKKα phosphorylates p100, which leads to partial processing of p100 to p52 and travels in complex with RelB into the nucleus to initiate transcription (Hayden & Ghosh, 2008).

1.5. General overview of the JNK pathway

The c-Jun N-terminal kinases are critical regulators of various physiological processes in mammals including cell survival, cell death, proliferation, inflammation and the immune response. JNK together with p38 belongs to a family of serine/threonine kinases, the MAP kinases (MAPK). Since JNK and p38 can be activated by cellular stresses, they are also known as stress-activated kinases (SAPKs). Three members of the JNK family are known (JNK1-3), of which only JNK1 and 2 are expressed in the haematopoietic system (Huang et al, 2009; Rincon & Davis, 2009). JNKs are activated through MAPK signalling cascades, which involve the consecutive phosphorylation and activation of MAPKs. Induction of a receptor such as the CD40 receptor leads to the activation of a MAP3K, which in turn activates a MAP2K that then phosphorylates the MAPK JNK. A major target of activated JNK is the c-Jun subunit of the activator protein-1 (AP-1) transcription factor, which leads to the subsequent induction of AP1 target genes. The main MAP2Ks mediating activation of JNK are MKK4/SEK1 and MKK7. A much larger number of MAP3Ks are involved in activation of JNK, among which TAK1 is of particular importance for the immune response (Huang et al, 2009; Sato et al, 2005; Shim et al, 2005). Both LMP1 and CD40 induce JNK signalling via TAK1 (Sato et al, 2005; Uemura et al, 2006; Wan et al, 2004). While in non-transformed B-cells, the JNK pathway is a critical regulator of survival and proliferation and can mediate apoptosis and inhibit proliferation, aberrant JNK signalling in tumour cells can provide critical survival signals (Rincon & Davis, 2009). Induction of the JNK pathway by LMP1 is essential for the outgrowth and proliferation of LCLs (Kutz et al, 2008).

1.6. The role of post-translational protein modifications in signal transduction pathways

1.6.1. The role of ubiquitination

In order to understand the mechanisms by which receptors like CD40 and the viral pseudoreceptor LMP1 induce signal transduction, it is necessary to understand the function of post-translational protein modifications in cellular signalling cascades. Post-translational modifications are a highly efficient way to increase the versatility of protein function using only a limited number of genes. Reversible covalent modification by phosphorylation, ubiquitination or SUMOylation changes the activation state of a target protein, effects sub-cellular localization or creates new protein-protein interaction sites (Hunter, 2007).

Ubiquitin is a small evolutionary highly conserved and ubiquitously expressed protein consisting of 76 amino acids and a molecular weight of approximately 8.5 kDa. A three-step enzymatic reaction mediates the attachment of ubiquitin via its C-terminal glycine residue to the ε-amino group of a lysine residue within a substrate protein. Firstly, an E1 enzyme activates ubiquitin in an ATP-dependent manner, which results in the formation of a thioester linkage between the C-terminal carboxy group of ubiquitin and a cysteine residue within E1. Ubiquitin is subsequently transferred to the active site cysteine of an E2 enzyme by a trans-esterification reaction. In the final step an E3 ubiquitin ligase transfers the activated ubiquitin to the lysine residue of a substrate protein (Hershko et al, 1983; Pickart, 2001). The human genome encodes two E1 enzymes, approximately 38 E2 enzymes and more than 600 E3 enzymes. A vast number of combinations of E2 and E3 ubiquitin ligases are thus available to specify the target protein and type of ubiquitin chains to be attached. E2 enzymes play an important role in determining the length and type of ubiquitin chain, whereas E3 ubiquitin ligases confer substrate specificity to the ubiquitin reaction by acting as adapters that bind both the E2 and the target protein (Malynn & Ma, 2010). E3 ligases are classified according to the protein domain that mediates substrate recognition. There are RING (really interesting new gene), HECT (homologous to E6-associated protein carboxyl terminus) and U box ubiquitin ligases where RING ligases make up the vast majority of E3s (Deshaies & Joazeiro, 2009; Rotin & Kumar, 2009).

Ubiquitination of proteins was originally related to protein turn-over and degradation of the target protein. However, the discovery of diverse ubiquitin chains characterized by chain length and type or the lysine residue used for chain growth has greatly extended the knowledge about the function of ubiquitination. Mono-ubiquitination, the attachment of only a single ubiquitin residue, mediates receptor internalization, vesicle sorting and is involved in

the DNA damage response (Sigismund et al, 2004). Polyubiquitin chains can be formed by using any of the seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) or the N-terminal amino-group to connect the individual ubiquitin proteins within a chain. K48-linked ubiquitin chains target proteins for proteolytic degradation by the proteasome system, whereas K63-linked ubiquitin chains are more flexible and involved in a wider range of functions, including kinase activation, DNA repair and protein trafficking. Ubiquitination processes are also essential for the activation of NF-κB and JNK signal transduction pathways (Komander, 2009; Malynn & Ma, 2010) (see 1.8).

1.6.2. The role of SUMOylation

Post-translational modification of proteins by members of the small ubiquitin-related modifier (SUMO) family has emerged as a highly dynamic process with diverse effects on the target protein ranging from altered activity, intracellular localization and modified protein-protein interactions. SUMOylation is involved in multiple cellular processes including progression of the cell cycle, genome stability and sub-cellular transport (Geiss-Friedlander & Melchior, 2007). SUMO is a 10 kDa protein that is essential for the normal function of all eukaryotic cells. The human genome encodes four SUMO proteins (SUMO1 – 4) of which SUMO1 – 3 are ubiquitously expressed (Geiss-Friedlander & Melchior, 2007).

The mechanism of conjugation of SUMO to target proteins is related to ubiquitination and also involves a three-step enzymatic cascade. SUMO proteins are first activated in an ATP-dependent manner by an E1-activating enzyme. This step involves the formation of a thioester bond between the active site cysteine of E1 and the C-terminal glycine residue of SUMO. SUMO is then transferred to an E2 conjugating enzyme generating an E2-SUMO thioester. Ubc9, the only known SUMO-conjugating enzyme can directly interact with target proteins and transfer SUMO to an acceptor lysine residue within a SUMOylation consensus motif comprising ψKxD/E, where ψ is a large hydrophobic amino acid. This process is usually facilitated by E3 enzymes. In contrast, there are no defined consensus sites at which ubiquitination takes place. Another difference between ubiquitination and SUMOylation is that SUMOylation usually results in the addition of a single SUMO residue as compared to ubiquitin chains (Geiss-Friedlander & Melchior, 2007).

SUMOylation is involved in JNK and NF-κB signalling. In the case of JNK signalling SUMOylation of the c-fos/c-jun AP1 dimer down-regulates its transcriptional activity (Bossis et al, 2005). The regulation of IκBα in the context of canonical NF-κB signalling is a good example that proteins can be multiply modified by different types of post-translational

modification which can act in combination. Modification of IκBα with SUMO-1 inhibits its ubiquitination as both modifications occur at the same target lysine residue (Desterro et al., 1998). Ubiquitination of IκBα usually leads to its degradation and subsequent liberation of NF-κB transcription factors. SUMOylation thus exerts a negative effect on NF-κB signalling. Phosphorylation of IκBα inhibits its SUMOylation and is thus a prerequisite for its degradation (Desterro et al, 1998). While a role of SUMOylation has not been demonstrated for CD40, LMP1 interacts with the SUMO-conjugating enzyme Ubc9 and mediates SUMOylation of cellular proteins (Bentz et al, 2011).

1.6.3. Regulation of the NF-κB subunit p65 by phosphorylation

Post-translational modifications of NF-κB subunits constitute alternative mechanisms of NF-κB regulation that can be independent of the IKK-complex and IκBα. NF-κB subunits are subject to a number of post-translational modifications including phosphorylation, ubiquitination and acetylation (Perkins, 2006).

Phosphorylation of p65 is the best-studied modification and several putative phosphorylation sites have been described which are located both within the N-terminal RHD as well as within the C-terminal transactivation domain. The effects of p65 phosphorylation are stimulus and cell type-specific and range from repression of genes to transcriptional activation and an increase in nuclear retention time of p65 (Neumann & Naumann, 2007; Perkins, 2006). The following section intends to give a short overview of the effects of p65 phosphorylation at different sites, but in order to keep this introduction concise not each of the p65 phosphorylation sites is discussed.

Phosphorylation of p65 at threonine 505 is induced by the tumour suppressor ARF and has a suppressive effect on the transcriptional activity of p65 (Campbell et al, 2006; Rocha et al, 2005). Phosphorylation of p65 at serine 276 by protein kinase A enhances its transcriptional activity by increasing its association with the transcriptional coactivator cAMP response element-binding-binding protein/p300 (CBP/p300) (Zhong et al, 2002; Zhong et al, 1998). Transcriptionally inactive NF-κB consists of p50 and p65 homodimers complexed with inhibitory histone deacetylase-1 (HDAC-1) in the nucleus. Only the p50 complexes bind to DNA and suppress transcription. In stimulated cells phosphorylated p65 enters the nucleus, interacts with CBP/p300 and displaces the p50 subunits. Phosphorylation of p65 determines whether it can interact with HDAC-1 or CBP-p300 (Perkins, 2006; Zhong et al, 2002). Phosphorylation of p65 at threonine 254 occurs in response to TNF stimulation by an unknown kinase (Ryo et al, 2003). Phospho-p65(T254) interacts with the peptidyl-prolyl

isomerase Pin-1 and results in prolyl isomerization thus disrupting the interaction of p65 with IκBα and inducing nuclear translocation and stabilization of p65 (Ryo et al, 2003). Induction of the tumour suppressor p53 results in ribosomal S6 kinase 1 (RSK1) kinase activation, which can then phosphorylate p65 at serine 536. This leads to a nuclear accumulation of p65 through a disruption of the shuttling of NF-κB/IκBα complexes (Bohuslav et al, 2004). Serine 536 of p65 is also phosphorylated by IKKs in response to cytokine stimulation and is essential for p65 transcriptional activity (Sakurai et al, 1999). A pool of phospho-p65(S536) exists in T-cells which are translocated into the nucleus upon stimulation. Interestingly, phospho-p65(S536) is not associated with p50 or IκBα and nuclear translocation occurs independently of IκBα (Sasaki et al, 2005). Phosphorylation and thus activation of p65 at serine 536 is also involved in LMP1-induced NF-κB-signalling and depends on interleukin 1-receptor associated kinase (IRAK1) (Song et al, 2006).

1.7. The role of TRAFs in signalling by LMP1 and CD40

1.7.1. Overview of the TRAF family

As neither LMP1 nor CD40 possess any enzymatic activity, both receptors rely on adapter proteins, such as TRAF family members to induce signal transduction (Graham et al, 2010; Kieser, 2007).

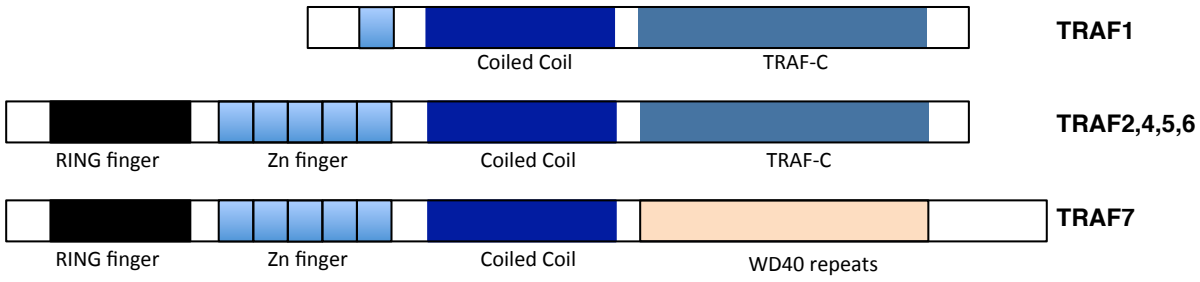


Figure 1.4. Schematic domain organization of mammalian TRAF proteins
TRAF2,4,5 and 6 each consist of a RING finger, Zn finger, coiled coil and TRAF-C domain. TRAF1 has a single Zn finger in its N-terminus and TRAF7 contains WD40 repeats instead of the TRAF-C domain.

The tumour necrosis factor receptor associated factor (TRAF) family constitutes a conserved family of adapter proteins that are involved in a wide range of biological functions including innate and adaptive immunity, stress response, embryonic development and bone

metabolism. Seven TRAF family members (TRAF1 - 7) have been identified in mammals (Arron et al, 2002; Xu et al, 2004). The N-terminal domains of TRAF family members except for TRAF1 consist of a RING finger domain, which confers ubiquitin ligase activity followed by several Zinc (Zn) fingers. The C-terminal TRAF domain consisting of a coiled coil domain and the TRAF-C domain mediates oligomerization of TRAFs and facilitates binding to receptors or other signalling molecules (Arron et al, 2002). TRAF7 the most recently identified member of the TRAF family, shares the N-terminal RING and Zn finger motifs, but differs in its TRAF domain which contains seven WD40 repeats (Xu et al, 2004) (figure 1.4). A characteristic feature of TRAF proteins is their ability to couple receptors to intracellular signalling pathways. TRAFs participate in the signal transduction of a large number of receptors including members of the TNFR and IL-1R/TLR families (Wang et al, 2010b). Clustering of TRAF proteins at the intracellular domains of activated receptors facilitates recruitment and activation of downstream signalling molecules and initiates signal transduction (Arron et al, 2002).

1.7.2. The role of TRAFs in LMP1-mediated signalling

Both LMP1 and CD40 recruit TRAF1, 2, 3, 5 and 6, but engage them in distinct and in some cases contrasting manners. LMP1 directly binds TRAF1, 2, 3 and 5 through a consensus TRAF binding motif (PxQxT/S) (Franken et al, 1996) within the sequence P_{204}QQAT in the CTAR1 domain (Devergne et al, 1996). TRAF6 is recruited to LMP1, but no direct interaction between TRAF6 and LMP1 could be shown to date (Schultheiss et al, 2001). BS69 has been suggested as a potential mediator of the interaction between TRAF6 and the CTAR2 domain of LMP1 (Wan et al, 2006), whereas a study conducted with TRAF6-deficient B-cells showed binding of TRAF6 to the consensus TRAF binding motif within the CTAR1 domain (Arcipowski et al, 2011).

LMP1 interacts with TRAF proteins with different affinities. TRAF3 exhibits the highest *in vitro* binding affinity, followed by TRAF1 and TRAF2 (Sandberg et al, 1997). Consistent with this finding the majority of TRAF1 and TRAF3 is associated with LMP1 in LCLs whereas only 5 % of TRAF2 binds to LMP1 (Devergne et al, 1996). Interestingly, an LMP1-mutant lacking the CTAR2 domain recruits TRAF1 and 2 more efficiently than full length LMP1 while binding affinities of TRAF3 and TRAF5 remain unchanged (Devergne et al, 1996; Sandberg et al, 1997; Xie & Bishop, 2004).

The contribution of individual TRAF family members to LMP1- and CD40-induced signalling has been studied in detail and has in some cases produced conflicting results. TRAFs can

form different hetero- and homodimers. Thus, results obtained by TRAF overexpression studies may have limited significance due to unspecific competitive effects. In addition, certain TRAF family members may have distinct roles in different cell types (Kieser, 2007).

The role of TRAF1 in signalling by LMP1 is cell type-specific and depends on the levels of TRAF1 in individual cell types. Epithelial cells express very low levels of TRAF1, but TRAF1 expression was shown to be up-regulated by LMP1 in nasopharyngeal carcinoma cells (Siegler et al, 2004; Wang et al, 2003). In contrast, TRAF1 expression is readily detected in cells from B-cell lymphoma cell lines (Eliopoulos et al, 2003). The CTAR1 domain of LMP1 did not induce the JNK pathway in the TRAF1 deficient epithelial cell lines but activated JNK signalling in TRAF1-expressing B-cell lines. Additionally, overexpression of TRAF1 in epithelial HEK293 cells augments CTAR1-mediated activation of JNK and NF-κB signalling (Devergne et al, 1996; Eliopoulos et al, 2003).

An initial study on the role of TRAF2 in LMP1-induced signalling showed that a dominant-negative TRAF2 lacking its amino-terminal RING finger domain inhibited NF-κB signalling by the CTAR1 domain by 75 % and NF-κB signalling by the CTAR2 domain by 40 % suggesting that TRAF2 is involved in LMP1-induced NF-κB signalling (Kaye et al, 1996). Expression of dominant-negative TRAF2 was additionally shown to reduce LMP1-mediated activation of the JNK pathway (Eliopoulos et al, 1999a). These findings were partially contradicted by studies analysing the effect of overexpression of TRAF2 wildtype on activation of the non-canonical pathway by the CTAR1 domain of LMP1. While one study showed that the overexpression of TRAF2 had almost no effect on NF-κB signalling by CTAR1-domain of LMP1 (Devergne et al,1996), another study demonstrated a negative effect of overexpressed TRAF2 on LMP1-induced activation of the non-canonical NF-κB pathway (Song & Kang, 2010) (see 1.8).

Studies conducted with TRAF2-deficient cell lines showed no contribution of TRAF2 towards JNK and NF-κB signalling by LMP1. LMP1-induced canonical NF-κB or JNK signalling was normal in TRAF2-deficient mouse B-cells (Xie et al, 2004). Additionally, LMP1-induced activation of the JNK pathway was not affected in TRAF2-deficient MEF cells (Wan et al, 2004). Activation of the NF-κB pathway by LMP1 was also normal in TRAF2/5-deficient MEF cells with regard to the nuclear translocation of p65 and NF-κB-dependent reporter activity suggesting that neither TRAF2 nor TRAF5 are essential for LMP1 induced NF-κB signalling (Luftig et al, 2003). Thus these studies argue against a role of TRAF2 for LMP1-induced JNK and NF-κB signalling.

However, a recent study employing a genome wide siRNA screen to identify novel components of the canonical NF-κB pathway in HEK293 cells with inducible expression of a LMP1 CTAR1 null mutant showed a role of TRAF2 in CTAR2-mediated canonical NF-κB

signalling (Gewurz et al, 2012). The knockdown of either TRAF6 or TRAF2 significantly reduced CTAR2-induced degradation of IκBα in this study (Gewurz et al, 2012). However, as in contrast to TRAF6, TRAF2 is not recruited to the CTAR2 domain of LMP1 (Kieser, 2007), TRAF2 might function in a cytosolic complex to mediate canonical NF-κB signalling by LMP1.

TRAF6 is an important positive regulator of LMP1-induced signalling and several studies have shown a role of TRAF6 as an essential mediator of the JNK and p38 MAPK pathways as well as the canonical NF-κB pathway by the CTAR2 domain of LMP1 (Luftig et al, 2003; Schneider et al, 2008; Schultheiss et al, 2001; Wan et al, 2004). LMP-induced JNK and NF-κB signalling is highly defective in TRAF6-deficient MEF cells with LMP1 being unable to induce activation of JNK, IKKβ and nuclear translocation of p65/RelA in the absence of TRAF6 (Luftig et al, 2003; Wan et al, 2004; Wu et al, 2006). Additionally, experiments conducted with TRAF6-deficient mouse B-cells expressing the inducible CD40-LMP1 fusion protein that consists of the extracellular and transmembrane domains of CD40 and the intracellular signalling domain of LMP1 showed that in the absence of TRAF6, LMP1 is unable to mediate activation JNK, canonical NF-κB or p38 signalling (Arcipowski et al, 2011).

Overexpression of TRAF3 in epithelial cells exerted a negative effect on CTAR1-mediated non-canonical NF-κB signalling (Devergne et al, 1996; Song & Kang, 2010). A different study using TRAF3-deficient mouse B-cells reported a critical role of TRAF3 as a positive regulator for canonical NF-κB, JNK and p38 signalling as well as in the up-regulation of CD23 and CD80 surface molecules and antibody secretion (Xie et al, 2004a,b).

The role of TRAF5 in signalling by LMP1 is under debate and might be cell-type-specific. One study using TRAF5-deficient mouse B-cells expressing a CD40-LMP1 fusion protein consisting of the extra cellular and transmembrane domains of CD40 and the cytoplasmic C-terminal signalling domain of LMP1 demonstrated an essential role of TRAF5 in LMP1-induced JNK and Akt signalling as well as the production of IL-6, TNFα and IL-17 (Kraus et al, 2009). However, studies conducted with epithelial cells and TRAF5-deficient MEF cells showed no involvement of TRAF5 in JNK and NF-κB signalling by LMP1 (Eliopoulos et al, 2003; Luftig et al, 2003; Wan et al, 2004).

1.7.3. The role of TRAFs in CD40-mediated signalling

CD40 directly interacts with TRAF1, 2 and 3 through the P_{250}VQET motif (TRAF1,2,3-binding site), whereas TRAF5 does not bind directly to CD40, but is recruited by TRAF3 (Ishida et al, 1996b; Pullen et al, 1998). TRAF6 interacts with CD40 at a distinct binding site (TRAF6-binding site) proximal to the membrane via the Q_{231}EPQEINF motif (Ishida et al, 1996a;

Pullen et al, 1998) (see figure 1.2). As described for LMP1, binding affinities of individual TRAF family members also differ in the case of CD40 with TRAF2 and TRAF3 showing a stronger interaction with CD40 than TRAF1 and TRAF6 (Lee et al, 1999b; Pullen et al, 1998).

Studies analysing the effects of TRAF1 overexpression in TRAF1-negative epithelial cells and overexpression of dominant-negative TRAF1 in B-cells showed no role of TRAF1 for CD40-induced JNK signalling (Eliopoulos et al, 2003). However, a study conducted with TRAF1-and TRAF2-deficient mouse B-cell lines suggested that while TRAF1 alone does not contribute towards CD40-mediated activation of the JNK and canonical NF-κB pathways, TRAF1 may act in concert with TRAF2 to enhance activation of these pathways (Xie et al., 2006). CD40-mediated activation of the canonical NF-κB and JNK pathways was normal in the absence of TRAF1 and defective in the absence of TRAF2. However, a more profound defect was observed in mouse B-cells deficient in both TRAF1 and TRAF2, which indicates a cooperative effect between TRAF1 and TRAF2 in the context of CD40-mediated signalling (Xie et al, 2006).

Activation of the JNK, p38 and canonical NF-κB pathway by exogenously expressed CD40 is diminished in epithelial cells depleted of TRAF2 by RNA interference and in fibroblasts isolated from TRAF2-deficient mice (Davies et al, 2005). Additionally, studies using B-cell lines in which the *TRAF2* gene was disrupted by targeted homologous recombination show that while TRAF2 is essential for CD40-mediated JNK signalling, it has no or only a minor contribution towards activation of the canonical NF-κB pathway (Hostager et al, 2003; Rowland et al, 2007; Xie et al, 2006). In contrast, studies conducted with TRAF2-deficient primary murine B-cells showed that TRAF2 is essential for CD40-mediated proliferation and activation of the canonical NF-κB pathway (Grech et al, 2004; Nguyen et al, 1999). The contrasting results regarding the role of TRAF2 in CD40-induced activation of the canonical NF-κB pathway probably reflect differences in the signal transduction of primary B-cells used in the studies by Grech et al. and Nguyen et al. (Grech et al, 2004; Nguyen et al, 1999) and the B-cell lines used in the former studies (Hostager et al, 2003; Rowland et al, 2007; Xie et al, 2006) that might be attributed to different expression levels of individual TRAF family members.

Several studies suggested a partial overlap of TRAF2 and TRAF6 functions in CD40-induced canonical NF-κB signalling. Only a partial defect in activation of the canonical NF-κB pathway was observed in TRAF6-deficient mouse B-cells, while activation of the canonical NF-κB pathway was completely abolished in B-cells deficient in both TRAF2 and TRAF6 (Rowland et al, 2007). Additionally, a study conducted with dominant-negative TRAFs lacking the N-terminal RING and Zn-finger domains showed that expression of dominant-negative

TRAF6 but not dominant-negative TRAF3 reduced residual NF-κB activity in CD40L stimulated TRAF2-deficient fibroblasts cells stably transfected with CD40 (Davies et al., 2005).

The importance of TRAF6 for CD40-induced signalling has been shown by studies conducted with splenocytes, dendritic cells and fibroblasts isolated form TRAF6-deficient mice that showed an essential role of TRAF6 for the activation of NF-κB, JNK and p38 by CD40 (Davies et al, 2005; Kobayashi et al, 2003; Lomaga et al, 1999; Rowland et al, 2007). However, experiments conducted with CD40 mutants showed that a mutation of the TRAF6-binding site (Q_{231}EPQEINF) within the cytoplasmic domain of CD40 does not affect JNK or canonical NF-κB signalling as long as the second TRAF1,2,3-binding site (P_{250}VQET) remains intact (Ahonen et al, 2002; Davies et al, 2005; Hostager et al, 2003; Jabara et al, 2002). Mutation of both TRAF binding sites, however, markedly decreases induction of JNK, NF-κB and p38 (Ahonen et al, 2002; Davies et al, 2005; Jabara et al, 2002). These findings indicate that while TRAF6 is essential for CD40-mediated JNK and canonical NF-κB signalling, the binding of TRAF6 to the TRAF6-binding site is dispensable for CD40-induced signal transduction. However, a functional TRAF1,2,3-binding site is essential to transduce signals from activated CD40. To explain these findings, it has been proposed that TRAF2 may participate in the indirect recruitment of TRAF6 to CD40. Such a model is supported by findings that the interaction between TRAF2 and TRAF6 is induced in HeLa cells following stimulation of a CD40 mutant deficient in TRAF6 binding, while this interaction is not induced by a CD40 mutant deficient in TRAF2 binding (Davies et al, 2005). Additionally, in the absence of TRAF2 mutation of the TRAF6 binding site within CD40, which normally has no effect on CD40-induced signalling, leads to the abrogation of canonical NF-κB signalling in response to CD40 stimulation (Davies et al, 2005; Hostager, 2007; Hostager et al, 2003).

However, this model has been challenged by findings of a study showing that TRAF6 mutants that are unable to interact with CD40 are still able to rescue CD80 up-regulation as well as the activation of the JNK and canonical NF-κB pathway in B-cells deficient in both TRAF2 and TRAF6 (Rowland et al, 2007). This suggests that either TRAF6 can be recruited to the CD40 signalling complex independently of TRAF2 by a different molecule that is recruited to or activated by the TRAF1,2,3-binding site of CD40 or that TRAF6 mediates signalling within a cytoplasmic complex.

Studies with B-cells from TRAF5-deficient mice have revealed only a minor contribution of TRAF5 towards CD40-induced signalling and B-cell activity (Nakano et al, 1999). TRAF5-deficient B-cells showed defects in the up-reglation of surface molecules, immunoglobulin production and proliferation in response to CD40 stimulation. However, CD40-mediated

activation of the JNK and NF-κB pathway was not affected in the absence of TRAF5 (Nakano et al, 1999).

TRAF3 plays a negative role in signalling by CD40 and activation of JNK and p38 is enhanced in TRAF3-deficient B-cells (Matsuzawa et al, 2008; Xie et al, 2004a). Activation of JNK and p38 signalling requires translocation of CD40 from the membrane to the cytosol. This translocation is inhibited by TRAF3, which is degraded in a TRAF2-dependent and cellular inhibitor of apoptosis 1/2 (cIAP1/2) dependent manner upon activation of CD40 (Hostager et al, 2003; Matsuzawa et al, 2008). The non-canonical NF-κB pathway is hyperactive in TRAF2-and TRAF3-deficient B-cells. TRAF2 and TRAF3 exert a regulatory function on the non-canonical NF-κB pathway and lead to a stabilization of NIK upon activation of CD40 (Grech et al, 2004; He et al, 2006; Hostager et al, 2003; Karin & Gallagher, 2009; Vallabhapurapu et al, 2008; Xie et al, 2006) (see 1.9).

1.8. Induction of NF-κB and JNK signalling by LMP1

LMP1 activates the non-canonical NF-κB pathway via the CTAR1 domain (Atkinson et al, 2003). Overexpression studies suggested that TRAF2 and TRAF3 negatively regulate activation of the non-canonical NF-κB pathway by inducing the degradation of NIK (Song & Kang, 2010). NIK is constitutively ubiquitinated with K48-linked ubiquitin chains by TRAF3 and thus constitutively degraded in non-stimulated cells. TRAF2 functions as an adapter in this complex that links NIK to TRAF3 (Vallabhapurapu et al, 2008). LMP1 sequesters TRAF2 and TRAF3 away from NIK, which leads to a stabilization of NIK and subsequent activation of IKKα (Song & Kang, 2010). Activated IKKα phosphorylates p100, which is then partially degraded to yield p52 that travels in a complex with RelB into the nucleus to activate transcription. p100 exists in a complex with RelB and retains RelB in the cytoplasm in non-stimulated cells (see 1.4 and figure 1.6). There is a crosstalk between the CTAR1 and CTAR2 domains of LMP1. While the CTAR1 domain mediates processing of p100 to p52 and induces nuclear translocation of p52-RelB complexes, the CTAR2 domain of LMP1 mediates up-regulation of p100 and RelB (Atkinson et al, 2003). In epithelial cells the CTAR1 domain induces nuclear translocation of alternative p50-p50, p50-p52 and p65-p52 NF-κB dimers in addition to p52-RelB complexes (Paine et al, 1995; Song & Kang, 2010; Thornburg et al, 2003; Thornburg & Raab-Traub, 2007b).

The CTAR2 domain of LMP1 mediates activation of the JNK and canonical NF-κB pathways. Activation of both signalling pathways depends on TRAF6, TAK1 and the TAK1-binding

proteins TAB1 and TAB2 (Luftig et al, 2003; Schultheiss et al, 2001; Uemura et al, 2006; Wan et al, 2004; Wu et al, 2006). Activation of the JNK pathway by LMP1 leads to the consecutive phosphorylation of MKK6/SEK1 and JNK1. Activated JNK travels into the nucleus and phosphorylates the c-jun subunit of the c-jun/c-fos dimer and thus enhances transcriptional activity (figure 1.5).

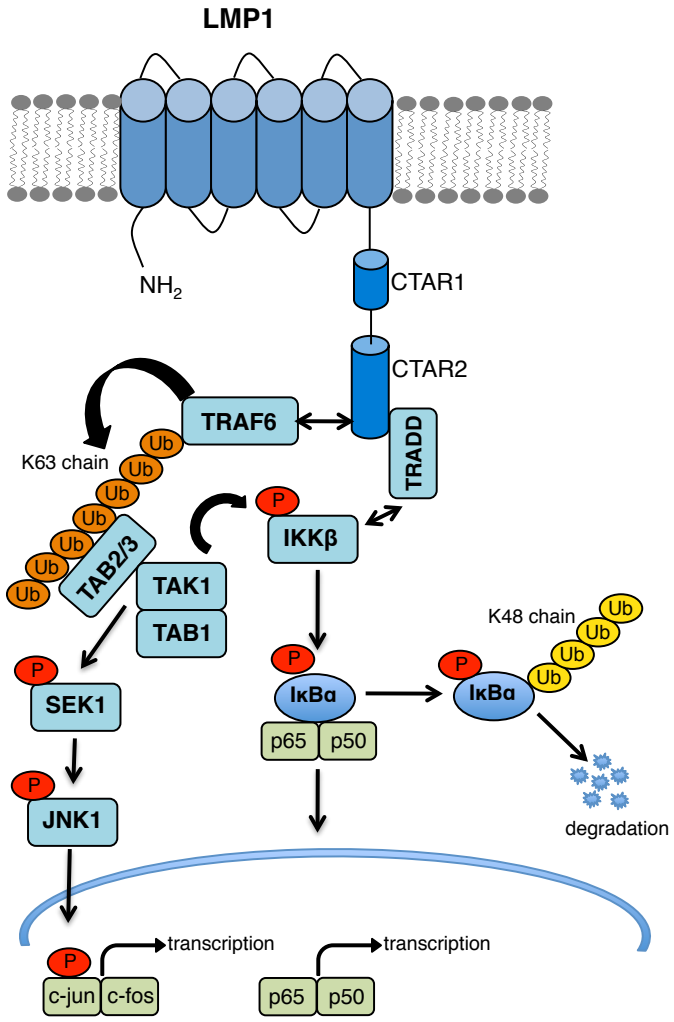


Figure 1.5. Activation of the JNK and canonical NF-κB signal transduction pathways by the CTAR2 domain of LMP1
Activation of both pathways depends on TRAF6 and TAK1. TRAF6 is recruited to the CTAR2 domain of LMP1 and ubiquitination of TRAF6 with K63-linked ubiquitin chains is induced. This facilitates binding of the TAK1-TAB complex. In the case of JNK signalling SEK1 and JNK1 are consecutively phosphorylated and thus activated. Activated JNK1 travels into the nucleus and phosphorylates the c-jun subunit of AP1 transcription factors thus inducing transcriptional activity.
Activation of the canonical NF-κB pathway requires the recruitment of IKKβ through TRADD. IKKβ is activated by TRAF6 and the TAK1-TAB complex and phosphorylates I-κBα, which leads to ubiquitination of I-κBα with K48-linked ubiquitin chains and subsequent degradation. p65-p50 NF-κB transcription factors are liberated and induce transcription of target genes in the nucleus.

Canonical NF-κB signalling additionally depends on TRADD which interacts with the CTAR2 domain of LMP1 and is important for the efficient recruitment of IKKβ (Schneider et al, 2008). IKKβ is activated in a TRAF6-dependent manner and subsequently phosphorylates IκBα, which is ubiquitinated with K48-linked ubiquitin chains and then degraded. p50-p65 NF-κB transcription factors are thus liberated and travel into the nucleus to initiate transcription of target genes (Herrero et al, 1995; Schultheiss et al, 2001) (figure 1.5).

The role of TAK1 in the activation of the canonical NF-κB pathway by LMP1, as well as the contribution of the TAK1-binding protein TAB2 to LMP1-induced JNK and canonical NF-κB signalling is controversially discussed. Since activation of the canonical NF-κB and JNK pathways by LMP1 was not affected in TAB2-deficient MEF cells it was suggested that TAB2 is not involved in signalling by LMP1 (Luftig et al, 2003; Wan et al, 2004). However, TAB2 and TAB3 are homologous and play redundant roles in the activation of TAK1 (Besse et al, 2007; Cheung et al, 2004; Ishitani et al, 2003) suggesting that in the case of the studies conducted with TAB2-deficient MEF cells, TAB3 might have rescued LMP1-induced signalling.

The role of TAK1 in the activation of the LMP1-induced NF-κB pathway is also under debate. One study analysing the effect of dominant-negative TAK1 protein with a deletion of the ATP binding site and down-regulation of TAK1 by siRNA on LMP1 induced NF-κB reporter activity reported no contribution of TAK1 to LMP1 induced NF-κB signalling (Uemura et al, 2006). In contrast a different study showed that the down-regulation of TAK1 by siRNA abolished LMP1-mediated activation of IKKβ thus demonstrating an essential role of TAK1 in LMP1-induced activation of the canonical NF-κB pathway (Wu et al., 2006).

Ubiquitination of TRAF6 is important for cellular signal transduction pathways and is also induced by LMP1 (Song et al, 2006). In the context of signalling by IL1/TLR, TNFR and LMP1, TRAF6 functions as an E3 ubiquitin ligase that acts in concert with the E2 complex Ubc13/Uev1A to mediate the formation of K63-linked ubiquitin chains (Deng et al, 2000; Luftig et al, 2003; Song et al, 2006). Auto-ubiquitination of TRAF6 mediates the recruitment and activation of TAK1 through binding of the regulatory subunits TAB2 and TAB3 to the K63-ubiquitin chains (Kanayama et al, 2004; Wang et al, 2001). The TAB1 subunit is also present in TAK1-TAB2/3 complexes after receptor activation and is recruited to the LMP1-induced signalling complex (Wan et al., 2004). TAB1 seems to contribute to the activation of TAK1 (Ono et al, 2001; Sakurai et al, 2000; Sakurai et al, 2002). TAK1 is activated through auto-phosphorylation and K63-polyubiquitination by TRAF6 and then mediates activation of NF-κB and JNK signalling (Fan et al, 2010; Hamidi et al, 2012; Singhirunnusorn et al, 2005; Yamazaki et al, 2009). Additionally, TRAF6-derived unanchored ubiquitin chains bind to

TAB2 and help to activate TAK1 (Xia et al, 2009). The role of auto-ubiquitination of TRAF6 in the recruitment of the TAK1-TAB complex and activation of the NF-κB and JNK pathways is currently under debate and a necessary but insufficient role of TRAF6 auto-ubiquitination in the activation of NF-κB has been suggested (Lamothe et al, 2007; Megas et al, 2011; Walsh et al, 2008).

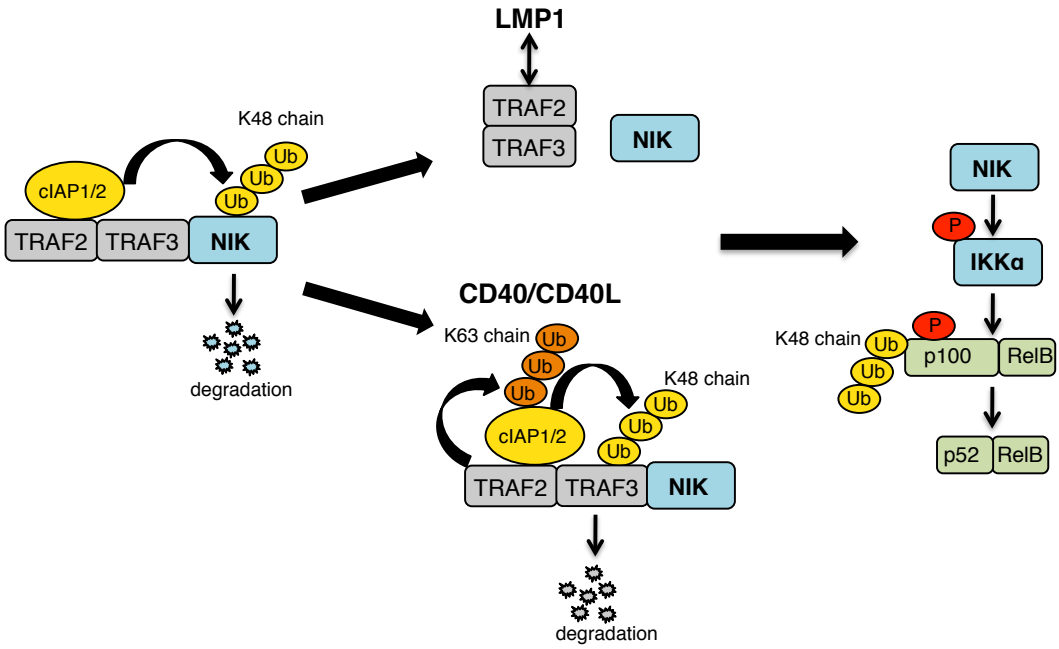


Figure 1.6. Activation of the non canonical NF-κB pathway by LMP1 and CD40
In non-stimulated cells NIK is ubiquitinated with K48-linked ubiquitin chains by the cIAP1/2 complex and constitutively degraded. TRAF2 and TRAF3 function as adapters between NIK and cIAP1/2. LMP1 stabilizes NIK by sequestering TRAF2 and TRAF3 away from NIK. Induction of CD40 leads to ubiquitination of cIAP1/2 with K63-linked chains by TRAF2. cIAP1/2 then ubiquitinates TRAF3 with K48 linked ubiquitin chains and thus induces its degradation, which prevents association of NIK with the cIAP1/2-TRAF2 complex and leads to a stabilization and accumulation of NIK. NIK phosphorylates IKKα, which phosphorylates p100. Phosphorylated p100 is subsequently ubiquitinated and partially degraded to p52, which travels in a complex with RelB into the nucleus to activate transcription of target genes.

To date the exact composition of the signalling complex at the CTAR2 domain of LMP1 is still elusive and it has not been clarified how the individual components of the complex are recruited and activated. The existence of unidentified molecular players that organize the signalling complex and facilitate the activation of signalling pathways thus seems probable.

1.9. Induction of NF-κB and JNK signalling by CD40

Activation of the non-canonical NF-κB pathway by CD40 is regulated by TRAF2 and TRAF3. In non-stimulated cells cIAP1/2 acts as a K48-specific ubiquitin ligase towards NIK. TRAF2 functions as an adapter in this complex that links cIAP1/2 to TRAF3 which then interacts with NIK. Activation of CD40 leads to the ubiquitination with K48-linked ubiquitin chains and subsequent degradation of TRAF3 by the ubiquitin proteasome system. This depends on TRAF2 which ubiquitinates cIAP1/2 with K63-linked ubiquitin chains and enhances the catalytic activity of cIAP1/2 towards TRAF3 leading to its degradation. Degradation of TRAF3 prevents association of NIK with the cIAP1-cIAP1-TRAF2 ubiquitin ligase complex and results in a stabilization and accumulation of NIK and subsequent activation of the non-canonical NF-κB pathway (Karin & Gallagher, 2009; Vallabhapurapu et al, 2008) (figure 1.6).

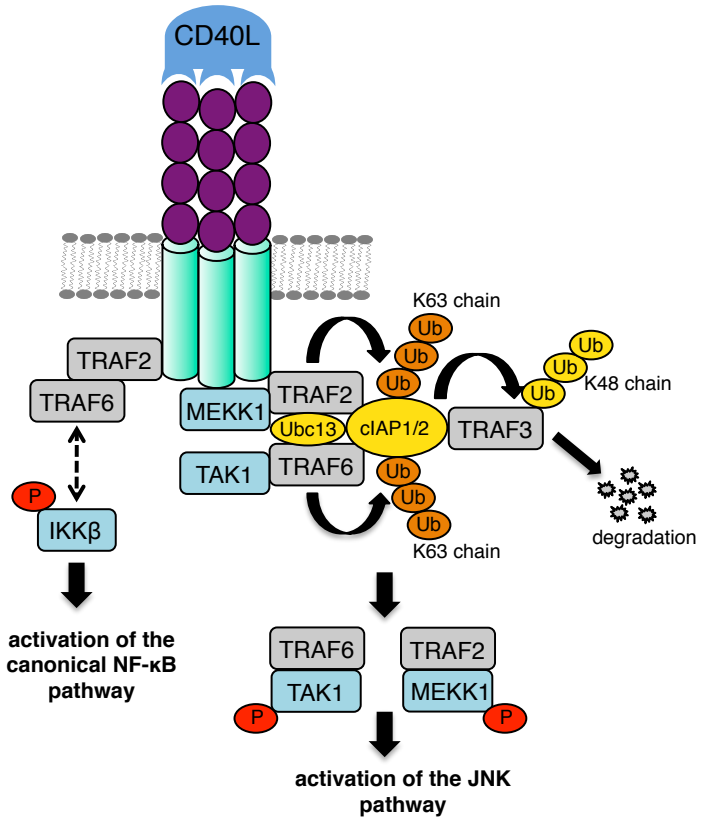


Figure 1.7. Activation of the canonical NF-κB and JNK signal transduction pathways by CD40
Activation of canonical NF-κB and JNK signalling depends on TRAF2 and TRAF6. In the case of the canonical NF-κB signalling IKKβ is recruited to the cytoplasmic part of the CD40 receptor and subsequently activated. In the case of JNK signalling a receptor-associated signalling complex forms with TRAF2,3 and 6, the E2 enzyme Ubc13, the E3 enzyme cIAP1/2 and the MAP3K MEKK1 and TAK1. TRAF2 and TRAF6 induce ubiquitination of cIAP1/2 with K63-linked chains. Activated cIAP1/2 then ubiquitinates TRAF3 with K48-linked chains, which leads to the degradation of TRAF3 and facilitates the release of the TRAF6-TAK1 and TRAF2-MEKK1 signalling complexes into the cytosol. TAK1 and MEKK1 are subsequently activated by an unknown mechanism and induce JNK signalling.

A recent study suggests that activation of the canonical NF-κB and JNK pathways by CD40 is spatially and temporally separated (Matsuzawa et al, 2008). Upon induction of CD40 by binding of CD40L IKKβ is recruited rapidly to CD40 and initiates activation of the canonical NF-κB pathway in a mechanism that depends on TRAF2 and TRAF6 (Hostager, 2007; Hostager et al, 2003; Pullen et al, 1999; Rowland et al, 2007) (figure 1.7). Induction of the JNK pathway has slower kinetics and is mediated by two signalling complexes, a TRAF2-MEKK1 and a TRAF6-TAK1 signalling complex that are recruited to CD40 along with TRAF3, the E2 conjugating enzyme Ubc13 and the E3 activating enzyme cIAP1/2. TRAF2 and TRAF6 ubiquitinate and thus activate the cIAP1/2 complex with K63-linked ubiquitin chains. Activated cIAP1/2 then ubiquitinates TRAF3 with K48-linked ubiquitin chains which leads to the degradation of the latter. Degradation of TRAF3 is followed by the release of the TRAF2-MEKK1 and TRAF6-TAK1 signalling complexes into the cytoplasm (figure 1.7). Complex translocation into the cytoplasm is the prerequisite for the activation of TAK1 and MEKK1, which in turn activate JNK and p38 MAPK signalling (Karin & Gallagher, 2009; Matsuzawa et al, 2008).

The mechanism by which MAP3K TAK1 and MEKK1 are activated in the cytoplasm, however, remains elusive. It is also poorly understood how the activation of JNK and NF-κB signalling is orchestrated. As in the context of LMP1 signal transduction, the existence of still unidentified molecular players that organize the signalling complexes and mediate activation of the two pathways is thus hypothesized.

1.10. Aims of this thesis

LMP1 is the primary oncoprotein of EBV and a functional mimic of the B-cellular CD40 receptor. However, despite intense research it has remained unclear how the signalling complex at the C-terminus of LMP1 forms and induction of the wide range of signalling pathways is orchestrated. The existence of still unidentified proteins that help to organize the signalling complex and facilitate signalling has thus been hypothesized. In a quest to identify new interaction partners of LMP1 our group performed a functional proteomics screen that has revealed the germinal center kinase family member TNIK as a new interaction partner of LMP1.

It was the aim of this thesis to characterize the role of TNIK in signalling by LMP1 and the related cellular CD40 receptor. Results obtained in our laboratory designating TNIK as an essential signalling mediator of the JNK and canonical NF-κB pathways initiated by the CTAR2 domain of LMP1 were verified and further defined. The composition of the signalling complex at the CTAR2 domain was analysed by co-immunoprecipitation experiments in HEK293D cells and a number of new interaction partners of TNIK were identified. TNIK is a novel inducer of the NF-κB pathway and the mechanisms of TNIK-induced canonical NF-κB signalling were analysed using a set of HA-tagged TNIK mutants.

As CD40 is the cellular counterpart of the viral pseudoreceptor LMP1, a role of TNIK in signalling by CD40 was investigated and TNIK was revealed as an essential mediator of JNK and canonical NF-κB signalling by CD40. The signalling complex at CD40 was analysed by co-immunoprecipitation studies in HEK293D cells. Additionally the time course of complex formation was investigated in the B-cell line BL41 after activation of CD40 with CD40 ligand.

Post-translational modifications of proteins are involved in many cellular signal transduction pathways. To further understand, how TNIK mediates signalling by LMP1 and CD40, it was investigated in which way TNIK itself is modified and how TNIK affects post-translational modifications within the signalling complex.

2. Materials

2.1. Plasmids

pRK5: Mammalian expression vector with a cytomegalovirus (CMV) promoter (Schall et al, 1990)

pSV-LMP1: LMP1 wildtype in pHEBO (Sugden et al, 1985) vector under the control of an SV40 promoter/enhancer (Kieser et al, 1997)

pCMV-HA-LMP1-TNFR1ΔDD: LMP1-TNFR1 fusion protein with a deleted death domain in a pHEBO expression vector with CMV promotor (Schneider et al, 2008)

pCMV-HA-LMP1-TNFR1ΔDD-CTAR2: LMP1-TNFR1 fusion protein carrying amino acids 371-386 of LMP1 instead of the death domain of TNFR1 in a pHEBO expression vector (Schneider et al, 2008)

pCMV-HA-LMP1wt: LMP1wildtype (amino acids 6-386) from EBV strain B95.8 with N-terminal HA-tag in a pHEBO expression vector under the control of a CMV promoter (Kieser et al, 1997)

pCMV-HA-LMP1(PQT): LMP1 mutant with an N-terminal HA-tag containing a mutated PxQxT motif within CTAR1 in a pHEBO expression vector (Schneider et al, 2008)

pCMV-HA-LMP1(Y384G): LMP1 mutant with an N-terminal HA-tag harbouring a point mutation within the CTAR2 domain which abolishes JNK and IKKβ activation in a pHEBO expression vector (Schneider et al, 2008)

pCMV-HA-LMP1(Δ371–386): LMP1 deletion mutant with an N-terminal HA-tag lacking the 16 C-terminal amino acids of the CTAR2 domain in a pHEBO expression vector (Schneider et al, 2008)

pCMV-HA-LMP1(Δ194-386): LMP1 with an N-terminal HA-tag lacking the C-terminal signalling domain (amino acids 194 -386) in a pHEBO expression vector (Schneider et al, 2008)

pCMV-HA-LMP1(PQT/Δ371–386): LMP1 mutant with an N-terminal HA-tag and mutated PxQxT motif within CTAR1, in which the 16 C-terminal amino acids of CTAR2 are deleted, in a pHEBO expression vector (Schneider et al, 2008)

pCMV-HA-LMP1(Δ194-386): LMP1 with an N-terminal HA-tag lacking the C-terminal signalling domain (amino acids 194 -386) in a pHEBO expression vector (Schneider et al, 2008)

pRK5-Flag-TNIK: Wildtype TNIK with an N-terminal Flag-tag in a pRK5 expression vector (Kutz H. and Kieser A., unpublished)

pRK5-HA-TNIKwt: TNIKwt with an N-terminal HA-tag in a pRK5 expression vector (J.Town, cloned in Master thesis)

pRK5-HA-TNIK-KM: Full length TNIK (amino acids 1 – 1360) with an N-terminal HA-tag in a pRK5 expression vector harbouring a K54R mutation within the TNIK kinase domain (Kutz H., unpublished) was subcloned from pYCI-TNIK(KM) (Fu et al, 1999)

pRK5-HA-TNIK(K780R): Full length TNIK with an N-terminal HA-tag, harbouring a K780R mutation within the intermediate domain in a pRK5 expression vector (A. Winter, unpublished)

pRK5-HA-TNIK-KD: Kinase domain of TNIK (amino acids 1 – 289) with an N-terminal HA-tag in a pRK5 expression vector (J. Town, cloned in Master thesis)

pRK5-HA-TNIK-IMD: Intermediate domain of TNIK (amino acids 290 – 1046) with an N-terminal HA-tag in a pRK5 expression vector (J. Town, cloned in Master thesis)

pRK5-HA-TNIK-GCKH: GCKH domain of TNIK (amino acids 1047 - 1360) with an N-terminal HA-tag in a pRK5 expression vector (J. Town, cloned in Master thesis)

pRK5-HA-TNIK-ΔKD: TNIK lacking the kinase domain (amino acids 290 - 1360) with an N-terminal HA-tag in a pRK5 expression vector (J. Town, cloned in Master thesis)

pRK5-HA-TNIK-ΔGCKH: TNIK lacking the GCKH domain (amino acids 1 - 1046) with an N-terminal HA-tag in a pRK5 expression vector (J. Town, cloned in Master thesis)

pRK5-HA-TNIK-KDwob: TNIK kinase domain with an N-terminal HA-tag that contains silent wobble mutations at the nucleotide level to eliminate targeting by the human Dharmacon TNIK ON TARGETplus SMARTpool siRNA (J-004542-10) (Kutz H., unpublished)

pRK5-Flag-TRAF6: Wildtype TRAF6 with an N-terminal Flag-tag in a pRK5 expression vector (see 3.4.8)

pRK5-Flag-TRAF6(274-522): TRAF6 deletion mutant (amino acids 274 – 522) lacking the N-terminal RING and Zn-finger domains with an N-terminal Flag-tag in a pRK5 expression vector (see 3.4.8)

pRK5-Flag-TAK1: Wildtype TAK1 with an N-terminal Flag-tag in a pRK5 expression vector (see 3.4.8)

pRK5-HA-JNK1: c-Jun-N-terminal kinase with an N-terminal HA-tag in a pRK5 (Schneider et al. 2008)

pGST-c-Jun: Fusion of the transactivation domain of c-Jun with glutathione-S-transferase (GST) in a bacterial expression vector (Kieser et al, 1999)

pcDNA3-Flag-IKKβ: I-κBα kinase β (IKKβ) with an N-terminal Flag-tag in a pcDNA3 expression vector (Jun-Hsiang Lin, Philadelphia)

pGST-I-κBα: Fusion of GST with I-κBα in a bacterial expression vector (Schneider et al, 2008)

pRK5-TRAF2wt: Wildtype TRAF2 in a pRK5 expression vector (Dennis J. Templeton)

pCMV-HA-TAB2: Wildtype TAB2 with an N-terminal HA-tag in a pHEBO expression vector with a CMV promotor (Uemura et al, 2006)

pESBOS-CD40: Full length CD40 in a mammalian expression vector pESBOS (Kieser et al, 1997)

pRK5-HA-Ubiquitin: Wildtype ubiquitin with an N-terminal HA-tag in a pRK5 expression vector (was obtained from Ted Dawson through Adgene.com)

pRK5-Myc-SUMO: Wildtype SUMO1 with an N-terminal Myc-tag in a pRK5 expression vector (provided by Daniel Krappmann)

2.2. Antibodies

2.2.1. Primary antibodies

TNIK (cl. 53): mouse, monoclonal (BD Biosciences)

LMP1 (1G6-3): rat, monoclonal, provided by E. Kremmer (Nicholls et al, 2004)

LMP1 (CS1-4): mouse, mixture of 4 monoclonal antibodies (Dako)

His (2F12): mouse, monoclonal, provided by E.Kremmer

EBNA1 (1H4): rat, monoclonal, provided by E.Kremmer

Roche:
HA (12CA5): mouse, monoclonal

HA (3F10): rat, monoclonal

Santa Cruz Biotechnology:
JNK1 (C17): rabbit, polyclonal

IKKβ (H470): rabbit, polyclonal

TRAF6 (H-274): rabbit, polyclonal

TRAF2 (C20): rabbit, polyclonal

TAK1 (M-579): rabbit, polyclonal

CD40 (C20): rabbit, polyclonal

p50(C-19): goat, polyclonal

RelB (C-19): rabbit, polyclonal

SAM68 (C-20): rabbit, polyclonal

Tubulin (B-5-1-2): mouse, monoclonal

Sigma:
Flag (M2): mouse, monoclonal

Flag (6F7): rat, monoclonal

New England Biolabs:
NF-κB p65 (C22B4): rabbit, monoclonal

NF-κB p52/p100 (18D10): rabbit, monoclonal; human specific

TAB2 (3744S): rabbit, polyclonal

2.2.2. Secondary antibodies

Secondary antibodies for immunoblots:

Anti-mouse IgG: horseradish peroxidase (HRP)-linked antibody (Cell Signalling Technology)

Anti-rabbit IgG: HRP-linked antibody (Cell Signalling Technology)

Anti-rat IgG: HRP-coupled antibody (Dianova)

Secondary antibodies for immunofluorescence studies:

Goat Anti-Mouse IgG (H+L): CY3-conjugated, minimal cross reactivity to rat, human, bovine, horse, rabbit serum proteins (Dianova)

Goat Anti-Rat IgG (H+L): FITC-conjugated, minimal cross reactivity to rat, human, bovine, horse, rabbit serum proteins (Dianova)

2.3. Cell lines

HEK293D: Human epithelial kidney cells, transformed by adenovirus type 5 derived DNA (Graham et al, 1977)

BL41: EBV-negative Burkitt's lymphoma cell line BL41 (Marchini et al, 1992)

LCL 721: EBV-positive lymphoblastoid cell line LCL721 (Kavathas et al, 1980)

MEFwt: Wildtype mouse embryonic fibroblast cells (Lomaga et al, 1999)

TRAF6 -/- MEF: Mouse embryonic fibroblast cells with a homozygous knock-out of the *traf6* gene (Lomaga et al, 1999)

2.4. Equipment and chemicals

Agfa Healthcare: Medical X-ray film, CEA Blue sensitive

Beckman, Palo Alto, USA: J25 centrifuge, Coulter Counter® Z1TM Series (cell and particle counter)

Becton Dickinson GmbH, Heidelberg: Disposable polypropylene tubes (Falcon) 15 ml and 50 ml, 6-well plates, cell scraper

Biochrom AG: Fetal bovine serum (FCS)

BioRad, München: Genepulser II, electroporation cuvettes

Eppendorf Gerätebau, Hamburg: 1.5 ml and 2 ml reaction tubes, BioPhotometer, centrifuge 5415R, thermomixer compact

Fermentas Life Sciences: PageRuler Plus Prestained Protein Ladder (protein marker for SDS-PAGE), GeneRuler DNA ladder mix (marker for agarose gel electrophoresis)

GE Healthcare: Protein G Sepharose, Glutathione Sepharose 4B, Amersham Hyperfilm for autoradiography, Fuji FLA-5100 Phosphoimager

Genomed GmbH: Jet Star Maxi Plasmid Purification Kit

Gibco®, Invitrogen: RPMI 1640 and DMEM cell culture medium, penicillin/streptomycin, L-glutamine, Trypsin-EDTA

Hoefer Scientific Instruments: Mighty Small II gel electrophoresis unit, Immunoblot Chamber

Kendro Laboratory Products GmBH: Hareus centrifuge 3 L-R

Leica Microsystems: TCS SP2 Confocal Microscope

Macherey-Nagel: NucleoSpin Extract II (nucleic acid purification kit)

New England Biolabs: Restriction enzymes, VENT® DNA polymerase, T4 DNA ligase

Qiagen: PolyFect® transfection reagent, Ni^{2+}-NTA agarose beads

Roche Diagnostics GmbH: dNTP mix

Scientific Volume Imaging: Huygens Essential Suite 3.2, Image processing software

Source BioScience: Recombinant human soluble CD40 Ligand

Stratagene: Robocycler Gradient 96

Thermo Fisher Scientific: Dharmafect, cryotubes 3 ml, cell culture dishes, cell culture flasks, 6-well plates

Whatman: Protran BA79 Blotting Membrane Roll (0.1 μm nitrocellulose membrane for immunoblotting)

Zeiss: Axiovert 200 Inverted Fluorescence Microscope

2.5. siRNA oligonucleotides

siRNA oligonucleotides were obtained from Thermo Fisher Scientific.

Human ON TARGETplus SMARTpool TNIK siRNA (pool of four siRNAs J-004542-10 to 13):

siRNA	sequence	target sequence
J-004542-10	GAACAUACGGGCAAGUUUA	100 – 119 of TNIK splice variant 1
J-004542-11	UAAGCGAGCUCAAAGGUUA	3954 – 3972 of TNIK splice variant 1
J-004542-12	CGACAUACCCAGACUGAUA	2574 – 2592 of TNIK splice variant 1
J-004542-13	GACCGAAGCUCUUGGUUAC	1969 – 1987 of TNIK splice variant 1

ON TARGET plus non-targeting control siRNA: Pool of siRNAs that has no targets in human cells.

2.6. shRNA

shRNA vectors were obtained from Open Biosystems: pSM2c-shTNIK: shRNA (short hairpin RNA) vector targeting human TNIK (RHS1764-949310), pSM2c-shCONTROL: non-silencing shRNA control vector (RHS1707-OB)

pSM2c-shTNIK:

UGCUGUUGACAGUGAGCGAGGCUCCUAAACCGUAUCAUAAUAGUGAAGCCACAGAUG

UAUUAUGAUACGGUUUAGGAGCCCUGCCUACUGCCUCGGA

3.Methods

3.1. Methods in cell biology

3.1.1. Cell culture

All cell lines were cultured at 37°C, 5 % CO_2. HEK293D cells, the EBV-negative Burkitt's lymphoma cell line BL41and the EBV-positive lymphoblastoid cell line LCL721 were kept in RPMI full medium supplemented with 10 % fetal calf serum, 1mM glutamine and 100 IU/ml penicillin/streptomycin. Wildtype and TRAF6-/- mouse embryonic fibroblasts were grown in DMEM containing 10 % fetal calf serum, 1 mM glutamine and 100 IU/ml penicillin/streptomycin.

3.1.2. Transient transfection of HEK293D cells

Transfection is a method by which DNA can be delivered into eukaryotic cells to achieve expression of the desired gene. Transient transfection of HEK293D cells was carried out using the PolyFect reagent from Quiagen. Cells were transfected at 70 - 80 % confluence with the expression vectors indicated in the figure. Total DNA was adjusted to 15 μg for transfection in 15 cm cell culture dishes, 8 μg in 10 cm cell culture dishes and 3 μg in 6-wells using the respective empty vector. 15 minutes prior to transfection, the medium was changed to RPMI minimal medium without FCS or antibiotics. DNA was mixed with 2 μl of PolyFect reagent per μg of DNA in 200 μl RPMI minimal medium and incubated for 10 minutes at room temperature to allow complex formation and subsequently diluted in 10 ml, 7 ml or 1 ml RPMI minimal medium for transfection in 15 cm cell culture dishes, 10 cm dishes or 6-well plates respectively. Cells were kept at 37°C and the medium was changed to RPMI full medium after 4-5 hours.

3.1.3. Electroporation of MEF cells

MEF cells were transiently transfected by electroporation which is a transfection method used for cell lines that are difficult to transfect and is based on the permeabilization of the cell membrane by an electrical field applied externally. Cells were seeded in 15 cm cell culture dishes. For electroporation cells were detached with trypsin-EDTA, spun down by centrifugation for 5 minutes at 900 rpm and resuspended in DMEM without FCS or antibiotics (DMEM minimal medium). 1.5 x 10^6 cells in 250 μl minimal medium per reaction were mixed with 20 μg DNA and incubated at room temperature for 10 minutes. Cells were subsequently electroporated using a Biorad Gene Pulser II at 240 V and 975 μF. 500 μl pre-warmed FCS

was added immediately after the pulse, cells were subsequently transferred to 6-well plates, 2.5 ml full medium was added and the cells were cultured at 37°C.

3.1.4. Protein knockdown by siRNA

HEK293 cells were seeded in 6-well plates the day before transfection and transfected twice within 24 hours at 50 – 60 % confluence with 100 nM human ON TARGETplus SMARTpool TNIK siRNA (pool of four siRNAs J-004542-10 to 13, Dharmacon) or corresponding ON TARGETplus non-targeting control siRNA. 10 µl siRNA from 20 µM stock solution was resuspended in 200 µl RPMI without FCS or antibiotics (RPMImin) per 6-well and mixed with 4 µl Dharmafect transfection reagent in 200 µl RPMImin. The reaction mixtures were incubated at room temperature for 20 minutes. The medium was aspirated from the cells and 1.6 ml antibiotics-free RPMI supplemented with 10 % FCS and 1 mM glutamine and the transfection mix was added to the cells. 24 hours after the second siRNA transfection, cells were transfected with the indicated expression vectors using Polyfect transfection as described in 3.1.2.

3.1.5. Protein knockdown by shRNA

To achieve protein knock down with shRNA, HEK293D cells were seeded at 60 % confluence in 10 cm or 15 cm cell culture dishes, as indicated in the figure and transfected once using the Polyfect reagent (see 3.1.2.) with pSM2c-shTNIK or pSM2c-shCONTROL (Open Biosystems) and the indicated expression vectors. 4 µg shRNA vectors were used for transfection in 10 cm cell culture dishes and 7 µg shRNA vectors in the case of 15 cm cell culture dishes. Cells were lysed and used for experiments 48 hours post transfection.

3.2. Protein methods

3.2.1. Determination of protein concentration by the Bradford Assay

Protein concentrations were determined using the Bradford Assay (Bradford, 1976). The assay is based on a differential absorbance shift in the dye Coomassie Brilliant Blue G-250 in the presence of various protein concentrations. The absorbance maximum shifts from 465 nm in the brown-red region of the visual spectrum to 595 nm in the blue region of the visual spectrum upon binding of Coomassie to proteins. For the assay 800 µl water was

mixed with 200 µl Bradford solution and 1 - 3 µl protein sample. A reaction without protein served as the blank value for the measurement. After an incubation period of 2 minutes at room temperature the absorption at 595 nm was determined and protein concentrations were calculated with the aid of the BioPhotometer software.

3.2.2. SDS-PAGE and immunoblotting

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins according to their apparent molecular weight. The proteins are denatured by heating in Laemmli sample buffer, which contains dithiothreitol (DTT) to reduce disulfide bridges and SDS as an anionic detergent. On the gel the proteins first travel through the stacking gel and are focussed at the border with the more finely meshed separation gel which functions as a molecular sieve allowing smaller proteins to move faster towards the positive pole upon application of an electrical field. 5 % stacking gels and 12.5 % separation gels were used. Protein solutions were mixed with ¼ volume of 4x Laemmli sample buffer or ½ volume of 2 x Laemmli sample buffer and heated to 95°C for 5 minutes before applying the samples to the gel. Proteins were separated at 35 mA and 250 V per gel chamber.

Stacking gel (400 ml): 307 ml H_2O; 60 ml 30 % acrylamide, 25 ml 2 M Tris-HCl (pH 6,8), 4 ml 10 % (w/v) SDS; 4 ml 0.5 M EDTA. 2 ml was used per gel to which 30 µl APS and 8 µl TEMED were added.

Separation gel (100 ml): 41 ml H_2O; 41.6 ml 30 % polyacrylamide; 16.6 ml 2 M Tris-HCl (pH 8.9); 700 µl 0.5 M EDTA; 1 ml APS; 100 µl TEMED. 8 ml was used per gel.

Laemmli sample buffer (4x): 100 mM Tris/HCl (pH 6.8), 4 % SDS, 20 % glycerol, 0.1 M DTT, 0.2 % bromophenol blue

10 x SDS running buffer: 0.25 M Tris, 2 M glycine, 1 % SDS

After separation of protein samples by SDS-PAGE, proteins were transferred onto nitrocellulose membranes by tank-style immunoblotting for 45 minutes at 100 V. Nitrocellulose membranes were subsequently blocked for one hour with 5 % non-fat dry milk in TBS-T (5 % milk) at room temperature and incubated with primary antibody overnight at 4°C. Membranes were subsequently washed three times with TBS-T buffer and incubated in 5 % milk with horseradish peroxidase (HRP)-coupled secondary antibody for 90 minutes at room temperature. The membranes were washed three times with TBS-T buffer and

developed with 4 ml ECL (enhanced chemiluminescence) reagent per membrane for which 1 ml ECL solution A was mixed with 3 µl ECL solution B.

TBS-T (Tris-buffered saline-Tween 20) buffer: 150 mM NaCl, 10 mM Tris pH 7.5 , 0.1 % Tween 20

Blotting buffer: 25 mM Tris, 120 mM glycine

ECL solution A: 100 mM Tris-HCl pH 8.8, 200 mM p-coumaric acid, 1.25 mM luminol

ECL solution B: 3 % H_2O_2 (v/v)

3.2.3. Covalent coupling of antibodies to protein G Sepharose beads

Antibodies covalently coupled to protein G Sepharose beads were used for immunoprecipitation experiments. Antibodies bind to protein G derived from *Streptococcus* but can be detached together with the bound proteins under denaturing conditions. This is prevented by covalently coupling antibodies to beads with the aid of dimethyl pimelimidate (DMP) as a cross-linking reagent and has the advantage of avoiding interference in immunoblot analysis by antibody-derived heavy chains.

0.1 mg antibody was used per 1 ml of protein G Sepharose (GE Healthcare) bead slurry. Beads and antibodies were mixed and incubated in 10 volumes of PBS buffer for 1.5 hours at room temperature under agitation. The beads were subsequently washed twice with 10 volumes of 0.2 M sodium borate (pH 9.0) by centrifugation at 15000 g for 0.5 minutes. The beads were resuspended in 10 volumes 0.2 M sodium borate using 20 mM DMP and incubated at room temperature for 30 minutes under gentle agitation. The reaction was stopped by washing the beads once in 0.2 M ethanolamine (pH 8.0). The beads were then incubated with 10 volumes of 0.2 M ethanolamine for 2 hours at room temperature with gentle mixing. After washing 3 times with PBS buffer the beads were resuspended in PBS and stored at 4°C. For longer storage times 0.1 % sodium azide was added.

PBS (phosphate-buffered saline) buffer: 4.3 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4, pH 7.4

3.2.4. Immunoprecipitation and cell lysis

3.2.4.1. General procedures for immunoprecipitation

Antibody-coupled protein G Sepharose beads were added to cellular lysates as a 50 % slurry in PBS for immunoprecipitation in 15 ml Falcon tubes. Subsequently beads were precipitated by centrifuging the tubes for 5 minutes at 1000 g, 4°C. The beads were resuspended in 1 ml cold IP-lysis buffer or PBS and transferred to 1.5 ml Eppendorf tubes. Washing steps were then carried out by centrifuging for 30 seconds at 15000 g, 4°C. The supernatant was aspirated and the beads were resuspended in 1 ml cold IP-lysis buffer or PBS per washing step.

3.2.4.2. Immunoprecipitation experiments in HEK293D cells

Twenty-four hours post transfection, HEK293D cells were washed once with PBS and then lysed with IP-lysis buffer. 2 ml IP-lysis buffer was used for 15 cm cell culture dishes and 1ml for 10 cm cell culture dishes. Cells were detached by scraping with a cell scraper and lysates were subsequently incubated on ice for 5 minutes. To determine protein expression in cellular lysates, 60 µl cell lysate per sample was mixed with 25 µl 4 x Laemmli sample buffer and heated to 95°C for 5 minutes. The remaining lysates were centrifuged at 16000 g for 10 minutes at 4°C and protein concentrations in the supernatants were determined by the Bradford assay. Protein concentrations were adjusted to 1 mg/ml in 15 ml tubes using 4 – 6 mg total protein in the case of 10 cm cell culture dishes and to 2 mg/ml using 8 – 10 mg total protein in the case of 15 cm cell culture dishes. 35 µl protein G Sepharose beads covalently coupled to an antibody directed against the protein of interest were added per sample. Samples were subsequently rotated for 1.5 – 3 hours at 4 °C. The beads were washed 2 - 3 times with cold IP-lysis buffer and finally resuspended in 45 µl 2 x Laemmli sample buffer, heated to 95°C for 5 minutes and analysed by SDS-PAGE and immunoblotting.

IP lysis buffer: 150 mM NaCl, 50 mM HEPES pH 7.5, 5 mM EDTA, 0.1 % NP-40, 0.5 mM sodium orthovanadate, 0.5 mM NaF, 0.5 mM sodium molybdate, 0.5 mM β-glycerophosphate, 0.5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF)

Laemmli sample buffer: 25 mM Tris-HCl pH 6.8, 1 % SDS, 5 % glycerol, 25 mM DTT

3.2.4.3. Immunoprecipitation experiments in BL41 cells

BL41 cells were stimulated with 500 - 600 ng/ml human recombinant soluble CD40 ligand (Source BioScience) for the indicated times using 1.0 - 1.5 x 10^8 cells in 1 ml serum-free RPMI medium per time point. Stimulation was stopped by centrifuging cells at 1500 g for 1.5 minutes at 4°C. The supernatants were discarded and cells were resuspended in 1 ml cold PBS. A 90 µl sample of each cell suspension was transferred to a separate tube for control of protein expression in total cell lysates and the samples were again centrifuged at 1500 g for 1.5 minutes at 4°C. Samples of total cell lysates were lysed with 200 µl 2 x Laemmli sample buffer and heated to 95°C for 5 minutes. Immunoprecipitation samples were lysed with 2 ml IP-lysis buffer per sample and incubated for 5 minutes on ice, followed by centrifugation at 16000 g for 10 minutes at 4°C. Protein concentrations in the supernatants were determined by the Bradford assay and adjusted to 4 – 7 mg/ml as indicated in the figure. 35 µl protein G Sepharose beads covalently coupled to an antibody directed against the protein of interest was added per sample and samples were rotated at 4 °C for 1.5 – 2 hours. In the case of immunoprecipitation of TNIK and TRAF6, beads were washed 3 times with IP-lysis buffer. In the case of immunoprecipation of TRAF2, beads were washed 3 times with PBS. Beads were subsequently resuspended in 40 µl 2 x Laemmli sample buffer and heated to 95°C for 5 minutes.

3.2.4.4. Immunoprecipitation experiments in LCL721 cells

Two 650 ml cell culture flasks containing 150 ml densely grown cell culture were used per immunoprecipitation sample. Cells were pelletted by centrifugation at 300 g for 10 minutes, washed once with PBS and subsequently lysed with 2 ml IP-lysis buffer. Lysates were incubated for 5 minutes on ice and centrifuged at 16000 g for 10 minutes at 4°C. The supernatants were transferred to a fresh tube and the protein concentration was determined by the Bradford assay. 3 mg total protein in 5 ml IP-lysis buffer was used per sample, 35 µl LMP1(1G6-3) antibody covalently coupled to protein G Sepharose beads or the unrelated rat isotype IgG (IsoG), EBNA(1H4) covalently coupled to protein G Sepharose beads, as a negative control was added and immunoprecipitation was carried out by incubating samples under agitation for 2 hours at 4°C. Beads were subsequently washed three times with PBS, resuspended in 45 µl 2 x Laemmli sample buffer and heated for 5 minutes to 95°C.

3.2.5. Subcellular fractionation and nuclear shift analysis

Subcellular fractionation was carried out in cooperation with Dr. Michael Romio from our laboratory. HEK293D cells were seeded in 10 cm cell culture dishes and transiently transfected with the indicated constructs. Cells were lysed 24 hours after transfection in the case of overexpression studies and 48 hours after transfection in the case of shRNA experiments. The medium was aspirated and cells were scraped from the dishes in the presence of 5 ml cold PBS. Cells were subsequently washed twice with cold PBS by centrifugation for 10 minutes at 300 g, 4°C. Cells were then lysed with 100 µl swelling buffer and incubated for 10 minutes on ice. 7 µl of 10 % NP40 (v/v) was subsequently added, samples were incubated for 1 minute on ice and then centrifuged for 1 minute at 16000 g, 4°C. The supernatant representing the cytosolic fraction was transferred to a different tube. The pellet was washed once with 200 µl swelling buffer and was then lysed with 50 µl nuclear extraction buffer. The samples were incubated under agitation for 30 minutes at 4°C and then centrifuged for 10 minutes at 16000 g, 4°C. The supernatant represents the nuclear fraction. Protein concentrations of the cytoplasmic and nuclear fractions were determined and samples were analysed by SDS PAGE and immunoblotting. Proper separation of cytosolic and nuclear fractions was analysed by immunoblotting for tubulin as a cytosolic marker and SAM68 as a nuclear marker.

Swelling buffer: 10 mM HEPES pH7.7, 10 mM KCl, 2 mM MgCl, 0.1 mM EDTA

Nuclear extraction buffer: 50 mM HEPES pH 7.7, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10 % glycerol

3.2.6. *In vivo* ubiquitination and SUMOylation analysis

For *in vivo* ubiquitination and SUMOylation analysis HEK293D cells were transiently transfected using the Polyfect reagent. For ubiquitination assays HEK293D cells were transfected in 6-wells or 10 cm cell culture dishes with 0.5 µg – 1 µg pRK5-HA-ubiquitin and the expression vectors indicated in the figure. For SUMOylation assays HEK293D cells were transfected in 6-wells with 1 µg pRK5-Myc-SUMO and the indicated expression vectors. Cells were lysed as described in 3.2.4.2. using IP-lysis buffer supplemented with 10 mM N-ethylmaleimide (NEM) as an inhibitor for de-ubiquitinating and de-SUMOylating enzymes. Immunoprecipitation was conducted as described above.

3.2.7. Immunocomplex kinase assay

Immunocomplex kinase assays were essentially performed as described (Kieser, 2001). HEK293 cells were transiently co-transfected in 6-well plates with 1 - 2 µg of each of the indicated constructs and 1 µg pRK5-HA-JNK1 for JNK1 kinase assays or pcDNA3-Flag-IKKβ for IKKβ kinase assays using Polyfect transfection. For TNIK kinase assays 1 – 2 µg HA-TNIKwt or HA-TNIK-KM were transfected using the Polyfect reagent as indicated in the figure. 24 hours after transfection, cells were lysed in 650 µl IP-lysis buffer per well and HA-JNK1, Flag-IKKβ or HA-TNIK was immunoprecipitated for 1.5 – 3 hours at 4°C using 15 µl protein G Sepharose beads covalently coupled to anti-HA (3F10) (Roche) or anti-Flag (6F7) (Sigma) antibodies per sample. Beads were washed twice with IP-lysis buffer and twice with kinase reaction buffer by centrifugation at 15000g for 30 seconds at 4°C. Samples were each adjusted to 20 µl and *in vitro* kinase assays were performed for 25 minutes at 26 °C in the presence of 10 µCi γ-^{32}P-ATP and 600 ng of the substrates GST-c-Jun for JNK kinase assays, GST-IκBα for IKKβ kinase assays or His-TRAF6(310-522) and His-TRAF2(311-501) provided by Fabian Giehler for TNIK kinase assays. To study autophosphorylation of HA-TNIK no substrate was added. The reaction was stopped by adding 12.5 µl Laemmli sample buffer and denaturing the samples by heating for 5 minutes to 95°C. Samples were subsequently analyzed by SDS-PAGE and autoradiography. Radioactive signals were quantified using the Fuji FLA-5100 Phosphoimager.

Kinase reaction buffer: 20 mM Tris-HCl pH 7.4, 20 mM NaCl, 10 mM $MgCl_2$, 1 µM DTT, 2 µM ATP

3.2.8. Purification of GST fusion proteins

GST-c-Jun and GST-IκBα as substrates for kinase assays were purified by inoculating 50 ml LB medium containing 100 µg/ml ampicillin with *E.coli* bacteria from frozen stocks containing expression vectors for the GST-proteins of interest. Cells were incubated overnight at 37°C under agitation. The following day 400 ml LB medium was inoculated with 20 ml of the overnight culture and grown to an optical density between 0.6 and 0.8. Subsequently protein expression was induced with 0.2 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) and the bacteria were incubated under agitation for 4 - 5 hours at 30°C. Bacteria were subsequently centrifuged at 2000 g for 10 minutes at 4°C and the cell pellet was lysed with 20 ml GST-lysis buffer supplemented with a spatula tip of lysozyme and incubated for 10 minutes on ice.

The suspension was then sonicated three times for 10 seconds each at 30 watts and cooled on ice between the sonication steps. Lysates were subsequently centrifuged at 16000 g for 15 minutes at 4°C. The supernatant was transferred to a fresh tube and GST fusion proteins were separated by adding 700 µl resuspended glutathione Sepharose 4B beads per 10 ml supernatant and incubated overnight under agitation. Beads were then precipitated by centrifugation at 500g for 1 minute at 4°C and subsequently washed twice with 10 ml GST-lysis buffer and twice with 10 ml TBS. The GST fusion proteins were eluted from the beads by resuspending the beads in 300 µl GST-elution buffer and rotating tubes for 30 minutes at 4°C. Beads were precipitated by centrifugation at 15000 g for 30 seconds and the supernatant which contains the GST fusion proteins was transferred to a fresh tube. Elution was repeated twice, the supernatants were pooled and the protein concentrations were determined by means of the Bradford assay.

GST- lysis buffer: 1 % Triton-X100, 10 mM DTT, 1 mM PMSF in PBS

TBS buffer: 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA

GST-elution buffer: 50 mM Tris-HCl pH 8.0, 1 % Triton X-100, 10 mM glutathione

3.2.9. Analysis of phosphorylation sites by mass spectrometry

3.2.9.1. Sample preparation

HEK293D cells were transiently transfected with 2 µg HA-TNIKwt or HA-TNIK-KM in 6-well plates using the Polyfect transfection reagent. Cells were lysed and the TNIK constructs were precipitated each with 17 µl protein G Sepharose covalently coupled to HA(12CA5) antibody. Beads were subsequently washed twice with IP-lysis buffer and twice with kinase reaction buffer (see 3.2.7) by centrifugation at 15000 g for 30 seconds at 4°C and finally adjusted to a volume of 20 µl. 20 µg His-TRAF6(310-522) in kinase reaction buffer was added to each sample and the samples were incubated for 40 minutes at 26°C to allow the kinase reaction to take place. Samples were subsequently centrifuged at 15000 g for 30 seconds at 4°C, the supernatants were transferred to a fresh tube and 17 µl Ni^{2+}-NTA agarose beads (Qiagen) were added to separate His-TRAF6(310-522) by affinity purification. Precipitation was achieved for 1 hour at 4°C. Beads were then washed twice with cold PBS and twice with 50 mM Tris pH 7.4. Beads were finally resuspended in 50 µl 50 mM Tris pH 7.4 and transferred to the proteomics facility for subsequent analysis.

3.2.9.2. Mass spectrometry analysis and data processing

Phosphorylation sites within TRAF6 were determined in cooperation with Dr. Hakan Sarioglu from the proteomics facility of the Helmholtz Center Munich. The TRAF6 samples were digested with trypsin and subsequently analysed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) using an orbitrap mass spectrometer. Mass spectrometry is an analytical technique that works by ionizing chemical compounds and separating ions according to their mass-to-charge ratios (m/z).

The digested phosphopeptides were separated by reversed phase chromatography (PepMap, 15 cm x 75 μm ID, 3 μm/100Å pore size, LC Packings) operated on a nano-high-performance liquid chromatography (nano-HPLC) (Ultimate 3000, Dionex) with a non-linear 170 minutes gradient using 2 % acetonitrile in 0.1 % formic acid in water and 0.1 % formic acid in 98 % acetonitrile as eluents at a flow rate of 250 nl/min. The nano-HPLC was connected to a linear quadrupole ion trap-Orbitrap (LTQ Orbitrap XL) mass spectrometer (ThermoFisher, Bremen, Germany) equipped with a nano-electrospray ionization (nano-ESI) source. The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap-MS and linear trap quadrupole (LTQ)-MS/MS acquisition. Survey full scan MS spectra (from m/z 300 to 1500) were acquired in the Orbitrap with a resolution R = 60,000 at m/z 400. The method used allowed the sequential isolation of the most intense ions according to signal intensity. High resolution MS scans in the orbitrap and MS/MS scans in the linear ion trap were performed in parallel. Target peptides already selected for MS/MS were dynamically excluded for 30 seconds. General mass spectrometry conditions were: electrospray voltage, 1.25-1.4 kV; no sheath and auxiliary gas flow. Ion selection threshold was 500 counts for MS/MS, and an activation Q-value of 0.25 and activation time of 30 ms were also applied for MS/MS.

All MS/MS spectra were analyzed using Mascot (Matrix Science, Version: 2.3.02) which is a search engine that uses MS data to identify proteins from primary sequence databases. Mascot was set up to search the Ensembl human database (version from 25.10.2010; 77462 sequences; 34341465 residues). Oxidation of methionine, phosphorylation of serine, threonine and tyrosine were specified in Mascot as variable modifications. Phosphorylation sites were determined with the aid of PhosphoRS, which is a probability-based site localization software that assigns and calculates individual site probabilities for phosphorylated peptides (Taus et al, 2011). PhosphoRS scores were calculated with Proteome Discoverer Version 1.3 (Thermofisher).

3.3. Microscopy and immunofluorescence

Following electroporation, MEF cells were seeded onto glass cover slips and cultivated overnight. Cells were subsequently fixed with 2 % paraformaldehyde in 6-well dishes for 15 minutes, permeabilized three times with PBS-Triton X-100 in PBS for 5 minutes each time and then blocked three times for ten minutes with blocking solution. Cells were then incubated with the primary antibody in blocking solution for 2 hours at room temperature. After washing once with PBS and twice with PBS-Triton X-100, cells were blocked for 7 minutes in blocking solution. Subsequently cells were incubated in secondary antibody diluted 1 : 200 in blocking solution for 45 minutes at room temperature keeping cover slips protected from light. After washing 2 × 5 minutes with PBS-Triton X-100 and 2 × 7 minutes with PBS, nuclei were counterstained with DAPI (Hoechst, 1 µg/ml in PBS) for 90 seconds. Cover slips were subsequently washed 2 × 2 minutes with PBS and mounted on slides with 8 µl 90 % glycerol (v/v). Images were acquired with a Leica TCS SP2 confocal laser scanning microscope fitted with a 63 x 1.4 HCX Plan Apo blue objective. The acquired digital images were deconvoluted and evaluated using the Huygens Essential Suite 3.2 software. Colocalization events were analysed by signal intensity scans.

Primary antibodies: TNIK (mouse, BD Biosciences): 1 : 200 in blocking solution
LMP1 (rat, 1G6-3, E.Kremmer): 1 : 50 in blocking solution

Secondary antibodies: goat anti-mouse IgG (H+L), CY3-conjugated (Dianova)
goat anti-rat IgG (H+L), FITC-conjugated (Dianova)

PBS-Triton X: 0.15 % TritonX-100 in PBS

Blocking solution: 1 % bovine serum albumin, 0.15 % glycine in PBS

3.4. DNA methods

3.4.1. Polymerase chain reaction (PCR)

DNA was amplified *in vitro* by the polymerase chain reaction (PCR) using VENT polymerase.

Reaction mix:

50 ng DNA template
0.1 µM (final concentration) forward and reverse primer
10 µl 10x reaction buffer (ThermoPol Reaction Buffer; New England Biolabs)
2 µl dNTP mix (2 mM, Roche)
3 µl VENT polymerase (New England Biolabs)
H_2O to 20 µl

PCR was run in a Robocycler Gradient 96 using the following parameters:

1 x	initial denaturation	10 min at 95°C
33 x	denaturation	5 min at 95°C
	primer annealing	1 min at 58°C
	elongation	2.5 min at 72°C
1 x	final elongation	10 min at 72°C

3.4.2. Agarose gel electrophoresis

DNA fragments were separated according to their size by agarose gel electrophoresis. 1 % agarose gels in 1xTBE buffer were used and 0.5 μg/ml ethidium bromide was added to visualize DNA in the gel. DNA samples were mixed with 0.2 volumes of 6x loading dye and applied to the gels. Using 1xTBE as an electrophoresis buffer, DNA fragments were separated at a voltage of 5 - 7 V/cm.

10xTBE buffer: 108 g Tris, 55 g boric acid, 40 ml 0.5 M EDTA, add H_2O to 1 l

6x loading dye: 30 % glycerol, 0.25 % bromophenol blue

3.4.3. Extraction of DNA fragments from the agarose gel and purification of PCR products

The NucleoSpin® Extract II kit was used according to the manufacturer's protocol to extract DNA from agarose gels and purify PCR products from oligonucleotides and excess dNTPs. DNA binds to a silica membrane in the NucleoSpin® Extract columns in the presence of a chaotropic salt. Contaminants like salts and soluble macromolecular components are removed by washing with an ethanolic buffer. Purified DNA is finally eluted under low ionic strength conditions at a slightly alkaline pH.

3.4.4. Ligation

DNA fragments were digested with specific restriction enzymes and inserted into a vector backbone by ligation with T4 DNA ligase using 10 ng of vector and 30 – 60 ng of insert. The

amount of re-ligation of incompletely digested vector was assessed by a control reaction without ligase, while the amount of uncut vector was estimated by a control with neither ligase nor insert. The reaction mixtures were incubated overnight at 16°C and subsequently transformed into competent *E.coli* DH5α cells and plated on LB agar plates substituted with the appropriate antibiotic. The success of ligation was analysed by comparing the number of colony forming units (cfu) on plates with the controls with the number of cfu on plates with the ligation reactions.

Reaction mix: 10 ng linearized vector
30 - 60 ng insert
1.5 µl 10 x T4 ligase reation buffer (New England Biolabs)
0.5 µl T4 DNA ligase (New England Biolabs)
H_2O to 15 µl

3.4.5. Preparation and transformation of chemically-competent *E.coli* cells

A modified version of the Hanahan method (Hanahan, 1983) was applied for preparation and transformation of competent *E.coli* cells.

Preparation of competent cells:

5 ml SOB medium were inoculated with DH5α cells from frozen stock and grown overnight in a shaking incubator at 37°C. The following day 50 ml pre-warmed SOB medium was inoculated with 1 ml of the overnight DH5α cell culture in a 500 ml Erlenmeyer flask and cultivated at 37°C in a shaking incubator until reaching an OD_{600} (optical density at 600 nm) of 0.3 - 0.55. 25 ml of the cell suspension was transferred to ice-cold 50 ml polypropylene tubes and incubated for 15 min on ice. The cells were recovered by centrifugation at 3000 rpm for 5 minutes at 4°C. The medium was decanted and the cells resuspended in 8.3 ml ice-cold TFB buffer. The suspensions were stored on ice for 15 minutes and then recovered by centrifugation at 3000 rpm for 5 minutes at 4°C. The supernatant was decanted and the cells were resuspended in 2 ml ice-cold TFB buffer. After an incubation period of 15 minutes on ice, 200 µl aliquots of the cell suspension were dispensed into sterile 1.5 ml tubes.

Transformation:

For transformation of the competent cells, 7 µl DMSO and 15 µl of a ligation reaction or 0.5 - 1 µg plasmid DNA were added to the cells and incubated for 30 minutes on ice. Cells were then subjected to a heat shock for 2 minutes at 42°C. The tubes were cooled for 2 minutes on ice before adding 1 ml LB medium and incubating at 37°C for 30 - 45 minutes.

The cells were recovered by centrifugation at 3000 rpm for 5 minutes at room temperature. The supernatant was decanted and the cell pellets resuspended in 200 µl of remaining medium. The transformed cells were transferred to LB plates supplemented with the appropriate antibiotics and grown at 37°C overnight.

TFB buffer: 10 mM MES (pH 6.3, sterile-filtered), 45 mM $MnCl_2$ x 4 H_2O, 10 mM $CaCl_2$ x 2 H_2O, 100 mM KCl, 3 mM hexamine cobalt chloride, made up to 1 l with H_2O

SOB medium: LB medium with 10 mM $MgCl_2$ and 10 mM $MgSO_4$

3.4.6. Minipreparation of plasmid DNA

The TELT Microquick method (Holmes & Quigley, 1981) was applied for small scale plasmid preparation. 5 ml LB medium supplemented with the appropriate selection antibiotic was inoculated with clones containing the desired plasmid and the cells were grown overnight at 37°C on a shaking incubator. 1.5 ml aliquots of the overnight cultures were transferred to 1.5 ml tubes and centrifuged at 13000 g for 40 seconds at room temperature. The cell pellets were resuspended in 250 µl ice-cold TELT lysis buffer and incubated for 5 minutes on ice. 7 µl freshly prepared aqueous lysozyme solution (10 mg/ml) was added to the cells, samples were vortexed for 3 seconds and boiled for 2 minutes. After cooling for 5 minutes on ice, the suspensions were centrifuged at 13000 g for 10 minutes at room temperature. The pellet was removed and the plasmid DNA was precipitated with 500 µl 100 % ethanol. After incubation for 15 minutes at room temperature, precipitated DNA was recovered by centrifugation at 13000 g for 10 minutes at room temperature. The DNA pellet was washed once with 80 % ethanol, left to dry at room temperature and finally dissolved in 20 µl H_2O.

TELT lysis buffer: 50 mM Tris/HCl (pH 8.0), 62.5 mM EDTA, 0.4 % (v/v) TritonX-100, 2.5 M LiCl

3.4.7. Maxipreparation of plasmid DNA

Plasmid DNA was prepared on a preparative scale using the JETSTAR Plasmid purification System according to the manufacturer's protocol. The procedure encompasses a modified SDS/alkaline cell lysis. After neutralization plasmid DNA is isolated from the lysate using an anion exchange column and concentrated by alcohol precipitation with 70% ethanol (v/v).

3.4.8. Cloning

TAK1, TRAF6wt and the TRAF6 mutant, TRAF6(274-522) consisting of the C-terminus of TRAF6 (amino acids 274 – 522) were cloned with an N-terminal Flag-tag. TAK1 was cloned as the corresponding PCR fragment of pRK5-HA-TAK1 (V. Nikolova and A. Kieser, unpublished) using the primers JT01 and JT02 and was inserted into a pRK5 expression vector by means of ClaI/AflII cleavage. TRAF6wt and TRAF6(274-522) were cloned on the basis of pRK5-HA-TRAF6 (J. Griese and A. Kieser, unpublished). TRAF6wt was cloned using the primers JT03 and JT04, TRAF6(274-522) was cloned using the primers JT03 and JT05 and inserted into pRK5 by means of ClaI/HindIII cleavage.

Primers:

JT01:
GGGGGATCGATACCATGGACTACAAAGACGATGACGACAAGATGTCGACAGCCTCCGCCGCCTCG

JT02:

GGGGGGGCTTAAGTCATCATGAAGTGCCTTGTCGTTTCTG

JT03:

GGGGGGGATCGATACCATGGACTACAAAGACGATGACGACAAGATGAGTCTGCTAAACTGTGAAAACAGCTG

JT04:

GGGGGGGAAGCTTCTATACCCCTGCATCAGTACTTCGTGGCTGAAAACCCTCCCTCCGAAG

JT05:

GGGGGGGATCGATACCATGGACTACAAAGACGATGACGACAAGGCCCAGGCTGTTCATAGTT

4. Results

4.1. The role of TNIK in the signal transduction of the viral oncoprotein LMP1

4.1.1. TNIK interacts with the CTAR2 domain of LMP1

In order to identify new interaction partners of LMP1 in its native context, the EBV transformed human B-cell, our group established a functional proteomics screen. Primary human B-cells were transformed with a recombinant EBV which instead of wildtype LMP1 expressed HA-LMP1-liTEV-CT in which a tobacco etch virus protease cleavage site coupled to a flexible linker (liTEV) was inserted between the transmembrane domain and the C-terminal (CT) domain of LMP1. HA-LMP1-liTEV-CT was immunoprecipitated from cellular lysates and subsequent TEV cleavage allowed specific analysis of the C-terminal signalling domain of LMP1. Proteins that interacted with the C-terminal domain of LMP1 were analysed by mass spectrometry. This revealed TNIK as a new interaction partner of LMP1 (Griese, 2008).

In order to confirm this new interaction, co-immunoprecipitation experiments were performed in the EBV-transformed lymphoblastoid cell line LCL 721. Endogenous LMP1 was immunoprecipitated and co-precipitated proteins were analysed subsequently by SDS-PAGE and immunoblotting. TNIK co-precipitated specifically together with LMP1 demonstrating that TNIK and LMP1 interact in LCL 721 cells thus confirming findings obtained by the proteomics screening (figure 4.1.A).

First results obtained from co-immunoprecipitation studies in HEK293D cells using various deletion mutants of LMP1 showed that TNIK binds to the CTAR2 domain of LMP1 (Dr. Anna Shkoda, personal communication). In order to analyse if the CTAR2 domain is sufficient for the interaction a co-immunoprecipitation experiment was carried out using HEK293D cells transiently transfected with HA-LMP1-TNFR1-CTAR2. This fusion protein consists of the transmembrane part of LMP1 fused to the cytosolic part of TNFR1 in which the death domain (DD) is replaced by amino acids 371-386 of the LMP1-CTAR2 domain. The 16 C-terminal amino acids of LMP1-CTAR2 are sufficient to induce JNK and canonical NF-κB signalling and thus the HA-LMP1-TNFR1-CTAR2 fusion protein allows analysis of CTAR2 functions independent of the CTAR1 domain (Floettmann & Rowe, 1997; Kieser et al, 1999; Schneider et al, 2008). HA-LMP1-TNFR1-ΔDD served as a negative control. Endogenous TNIK was precipitated and analysis of co-precipitated proteins by immunoblotting revealed that HA-LMP1-TNFR1-CTAR2 readily bound to TNIK, while no interaction with HA-LMP1-TNFR1-ΔDD could be detected (figure 4.1.B). This experiment demonstrates that the 16 C-terminal

amino acids of the CTAR2-domain of LMP1 are not only necessary but also sufficient for the interaction with TNIK.

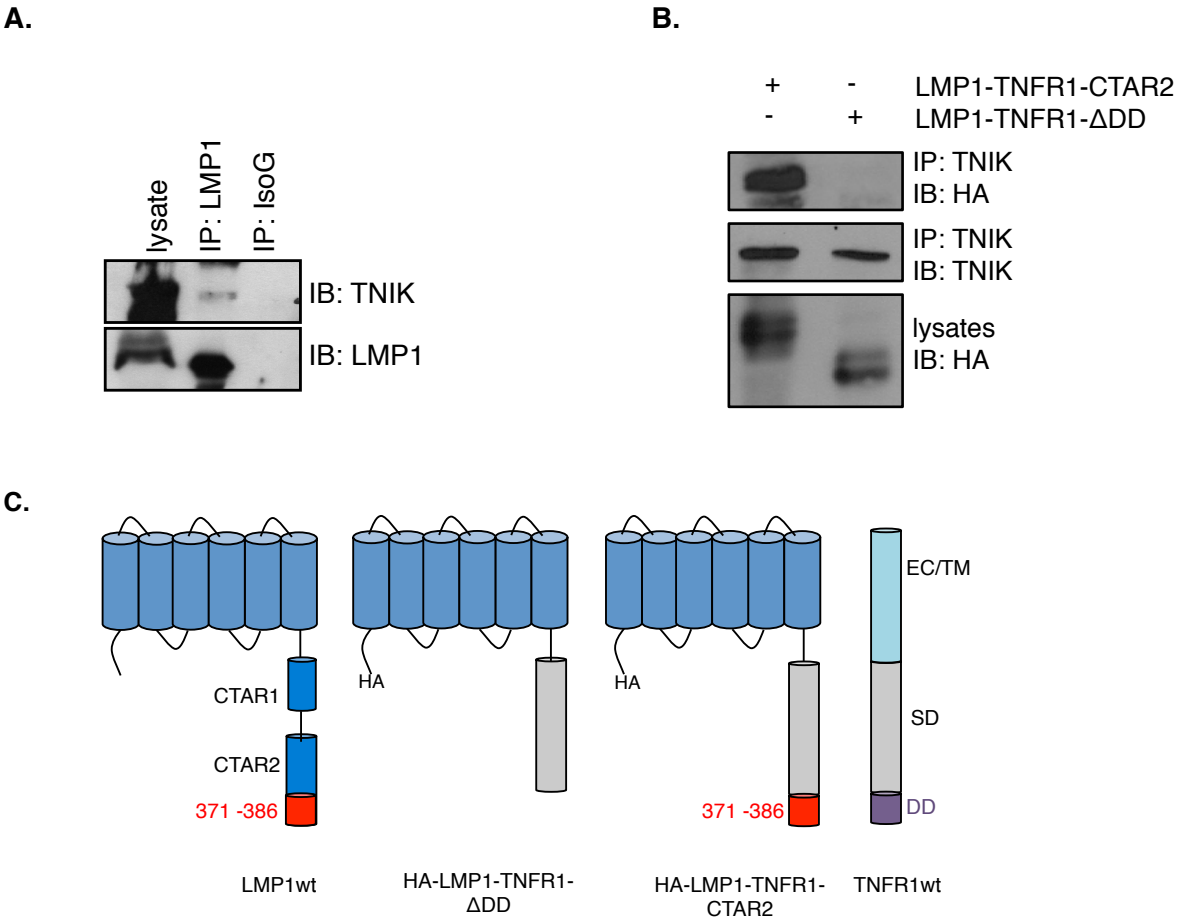


Figure 4.1. TNIK interacts with the CTAR2 domain of LMP1
A. TNIK interacts with LMP1 in lymphoblastoid cells
Endogenous LMP1 was immunoprecipitated from 721 cell lysates with the anti-LMP1(1G6-3) antibody using 3 mg of total protein. Co-precipitated TNIK was detected on immunoblots using the anti-TNIK antibody. An unrelated rat isotype IgG (IsoG) served as a negative control for immunoprecipitation. TNIK and LMP1 expression in total cell lysates was detected using anti-TNIK and anti-LMP1(1G6-3) antibodies.
B. The CTAR2 domain is sufficient for the interaction with TNIK
HEK293D cells were transfected in 15 cm cell culture dishes with 15 μg of the indicated constructs. Endogenous TNIK was immunoprecipitated from cell lysates and co-precipitation of the HA-tagged LMP1-TNFR1 fusion proteins was analysed by immunoblotting with the anti-HA(3F10) antibody. The anti-TNIK antibody was used to confirm equal TNIK precipitation in the samples.
C. Schematic diagram of LMP1-TNFR1 fusion proteins.
LMP1wt consists of 6 transmembrane helices and the C-terminal signalling domain incorporating CTAR1 and CTAR2. Amino acids 371 -386 are sufficient for the induction of JNK and NF-κB signalling. TNFR1wt consists of an extracellular and transmembrane domain (EC/TM) and an intracellular signalling domain (SD) that incorporates the death domain (DD). HA-LMP1-TNFR1ΔDD is a chimera of the LMP1 transmembrane domain and the signalling domain of TNFR1 lacking DD. HA-LMP1-TNFR1ΔDD-CTAR2 carries aa 371-386 of LMP1 instead of the DD of TNFR.

4.1.2. TNIK mediates JNK and canonical NF-κB-signalling by LMP1

Having identified TNIK as a new interaction partner of the CTAR2 domain of LMP1 the next question presenting itself regards the function of TNIK for the signal transduction of LMP1. Since TNIK is known to activate JNK signalling upon overexpression and LMP1 activates the JNK pathway via its CTAR2 domain (Eliopoulos et al, 1999a; Fu et al, 1999; Kieser et al, 1997) TNIK may be involved in JNK signalling by LMP1. This was investigated by conducting *in vitro* JNK1 kinase assays. HEK293D cells were depleted of endogenous TNIK by siRNA and the ability of LMPwt or the inactive null-mutant LMP1(Δ194-386) to activate co-transfected HA-JNK1 was analysed. In cells transfected with control siRNA LMP1wt caused a 94.5-fold induction of JNK1 as monitored by *in vitro* GST-c-Jun phosphorylation. Down-regulation of TNIK by siRNA caused a significant reduction of the induction levels of JNK1 by 72 % to 27-fold (figure 4.2.A).

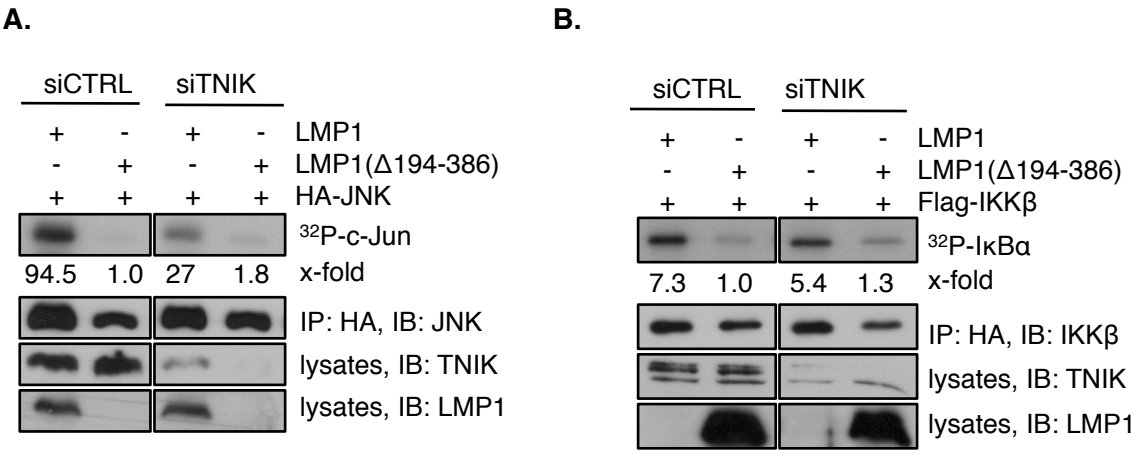


Figure 4.2. TNIK mediates JNK and canonical NF-κB signalling by LMP1
A. TNIK is essential for JNK activation by LMP1
HEK293D cells were transfected in 6-well plates with non-targeting siRNA (siCTRL) or siRNA against human TNIK (siTNIK). Cells were subsequently co-transfected with 1 μg of HA-JNK1 and 1 μg of HA-LMP1wt or HA-LMP1(Δ194-386) lacking the signalling domain of LMP1, as indicated. HA-JNK1 was immunoprecipitated from cell lysates and an immunocomplex kinase assay was performed using recombinant GST-c-Jun as a substrate. The samples were subjected to SDS-PAGE and autoradiography. GST-c-Jun phosphorylation was quantified by Phosphoimager analysis and is stated as x-fold induction. TNIK down-regulation and LMP1 expression was monitored in cell lysates by immunoblotting using anti-TNIK and anti-LMP1(1G6-3) antibodies.
B. TNIK mediates canonical NF-κB signalling by LMP1
HEK293D cells were transfected in 6-well plates with TNIK siRNA or non-targeting siRNA. Subsequently, cells were co-transfected with 1 μg each of HA-LMP1wt or HA-LMP1(Δ194-386) and 1 μg of Flag-IKKβ, as indicated in the figure. Flag-IKKβ was immunoprecipitated and IKKβ activity was monitored in immunocomplex kinase assays using GST-IκBα as a substrate. GST-IκBα phosphorylation was quantified by Phosphoimager analysis and is stated as x-fold induction. Immunoprecipitated Flag-IKKβ, expression of HA-tagged LMP1-constructs and down-regulation of TNIK was detected using the anti-IKKβ, anti-LMP1(1G6-3) and anti-TNIK antibodies respectively.

This result clearly demonstrates that TNIK plays a key role as a signalling mediator for LMP1-induced JNK signalling and identifies LMP1 as the first known receptor that engages TNIK for activation of the JNK pathway.

Another signalling pathway initiated at the CTAR2 domain of LMP1 is the canonical NF-κB pathway. To test whether TNIK also plays a role in the activation of this pathway, an *in vitro* IKKβ kinase assay was performed. HEK293D cells were transfected with control siRNA or TNIK-specific siRNA. The ability of co-expressed LMP1wt or the inactive null-mutant LMP1(Δ194-386) to activate Flag-IKKβ was analysed by monitoring the *in vitro* phosphorylation of the IKK substrate GST-IκBα. In cells transfected with control siRNA LMP1 caused a 7.3-fold induction of IKKβ, while down-regulation of TNIK reduced the induction level by 30 % to 5.4-fold (figure 4.2.B). This experiment indicates that TNIK contributes towards LMP1-induced canonical NF-κB signalling but it does not seem to be essential for LMP1-induced activation of IKKβ.

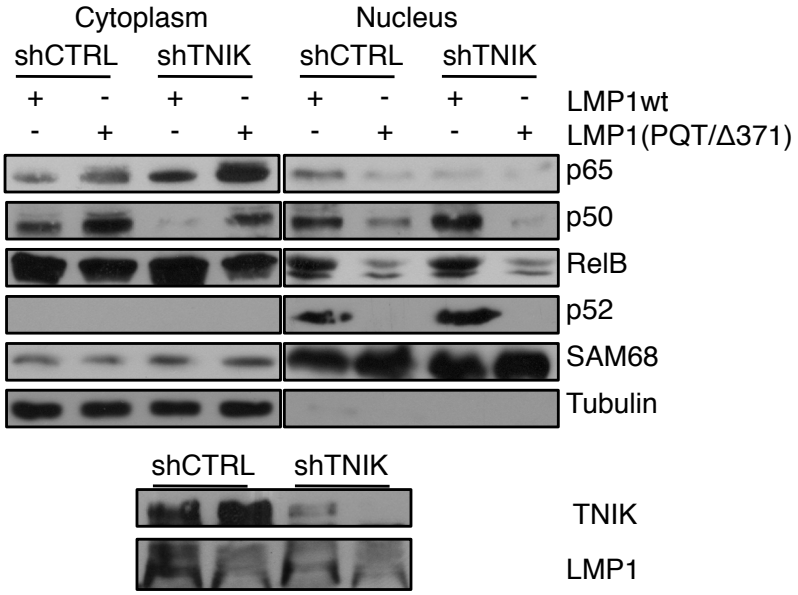


Figure 4.3. TNIK mediates induction of the canonical NF-κB pathway by LMP1 without affecting the non-canonical pathway
HEK293D cells were co-transfected in 10 cm cell culture dishes with 4 μg each of HA-LMP1wt or the mutant HA-LMP1(PQT/Δ371) which is signalling inactive regarding the NF-κB pathway, in the presence of 4 μg shRNA for human TNIK (shTNIK) or non-silencing shRNA (shCTRL) as indicated in the figure. 48 hours post transfection, cells were lysed, the cytoplasmic and nuclear fractions were separated and analysed by immunoblotting using antibodies against p65, p50, RelB, p52/p10, SAM68 and tubulin as indicated. Down-regulation of TNIK and expression of LMP1 was analysed in total cell lysates by immunoblotting with the anti-TNIK and anti-LMP1(1G6-3) antibodies respectively.

To further analyse the role of TNIK for NF-κB signalling by LMP1, the effect of TNIK down-regulation on nuclear translocations of individual NF-κB subunits by LMP1 was analysed. Activation of the canonical NF-κB pathway typically results in a nuclear translocation of the NF-κB heterodimer p65-p50, while activation of the non-canonical pathway leads to a nuclear translocation of RelB-p52. HEK293D cells were transfected with either HA-LMP1wt or the mutant HA-LMP1(PQT/Δ371) which is inactive regarding NF-κB signalling in the presence of shRNA vectors targeting TNIK or non-targeting shCTRL-vectors. In this experiment shRNA vectors were used instead of siRNA, as this method of protein down-regulation is more cost effective and thus more convenient for the large-scale protein preparations needed here.

Cells were lysed and the cytoplasmic and nuclear fractions were separated and analysed by immunoblotting. Immunoblotting for SAM68, which is a nuclear protein and tubulin, which is exclusively cytoplasmic showed the clean separation of the two fractions. Equal expression of LMP1 in each sample and the knock down of TNIK was confirmed by immunoblot analysis of total cellular lysates (figure 4.3, bottom panels). In the presence of the shCTRL-vector, as expected HA-LMP1wt induced nuclear localization of all of the analysed NF-κB subunits showing activation of both the canonical as well as the non-canonical NF-κB pathways. Down-regulation of TNIK abolished the nuclear translocation of p65, which shows a critical effect of TNIK on the activation of the canonical NF-κB pathway (figure 4.3). Localization of p50, which is usually also translocated into the nucleus upon activation of the canonical pathway, was not affected by the knock down of TNIK (figure 4.3). It is possible that p50 was translocated through CTAR1 activity, which apart from the RelB-p52 dimer also induces nuclear translocation of the p50-containing NF-κB dimers p50-p50 and p50-p52 as well as the p52-p65 heterodimer (Paine et al, 1995; Thornburg et al, 2003; Thornburg & Raab-Traub, 2007a). Levels of p52 and RelB in the nucleus remained unaffected by TNIK down-regulation showing that the non-canonical pathway did not depend on TNIK (figure 4.3). These results thus demonstrate that a down-regulation of TNIK affects the activation of the canonical NF-κB pathway by LMP1, but has no effect on the non-canonical pathway.

In summary, the results presented above show that TNIK is an important new signalling mediator of LMP1 that is critical for activation of JNK signalling and plays a role in the activation of the canonical NF-κB pathway by contributing towards activation of IKKβ and mediating the nuclear translocation of the NF-κB subunit p65.

4.2. Bifurcation of the JNK and canonical NF-κB signalling pathways at the level of TNIK

4.2.1. The canonical NF-κB pathway is induced by the kinase domain of TNIK

Since the results presented above showed that TNIK mediates activation of both JNK and canonical NF-κB signalling by the CTAR2 domain of LMP1, the next question was whether TNIK might orchestrate the bifurcation of the two pathways.

This question was addressed by dissecting the contribution of individual TNIK domains to the induction of the two pathways. For this purpose a set of HA-tagged TNIK-mutants was used comprising the full-length protein, a kinase-negative mutant (TNIK-KM), the kinase domain (KD), germinal center kinase homology domain (GCKH) as well as the ΔKD and ΔGCKH deletion mutants (figure 4.4.A). The indicated HA-TNIK constructs were co-expressed together with Flag-IKKβ in HEK293D cells. Equal expression of the HA-tagged TNIK constructs in cellular lysates and equal immunoprecipitation of Flag-IKKβ in each sample was confirmed by immunoblotting (figure 4.4.B). The ability of the individual TNIK domains to activate IKKβ upon overexpression was tested for by an *in vitro* kinase assay monitoring the phosphorylation of GST-IκBα. TNIK wildtype induced IKKβ activity by a factor of 5.5-fold. The kinase domain alone was able to activate IKKβ to the same extent as TNIK wildtype. Mutation or deletion of the kinase domain as in the constructs HA-TNIK-KM and HA-TNIK-ΔKD completely abolished the ability of TNIK to induce IKKβ. In contrast, the GCKH-domain had no effect on the activation of the NF-κB pathway. HA-TNIK-GCKH did not activate IKKβ, while the deletion of the GCKH domain as in HA-TNIK-ΔGCKH did not affect the ability of TNIK to induce IKKβ (figure 4.4.B).

Hence, these results show that the overexpressed kinase domain of TNIK is both necessary and sufficient to activate IKKβ, whereas the GCKH domain is dispensable.

Next the activation of the NF-κB pathway by TNIK was analysed more closely with regard to the individual NF-κB subunits that are translocated into the nucleus upon overexpression of TNIK. HEK293D cells were transfected with HA-TNIKwt, HA-TNIK-KD as well as with HA-LMP1wt as a positive control and pRK5 empty vector as a negative control. Cytosolic and nuclear fractions were separated and analysed by immunoblotting. Immunoblotting for SAM68, which is a nuclear protein and tubulin, which is found exclusively in the cytoplasm, showed that the two fractions were cleanly separated. Expression of the transfected constructs was confirmed by immunoblot analysis of the cytoplasmic fraction (figure 4.5, bottom).

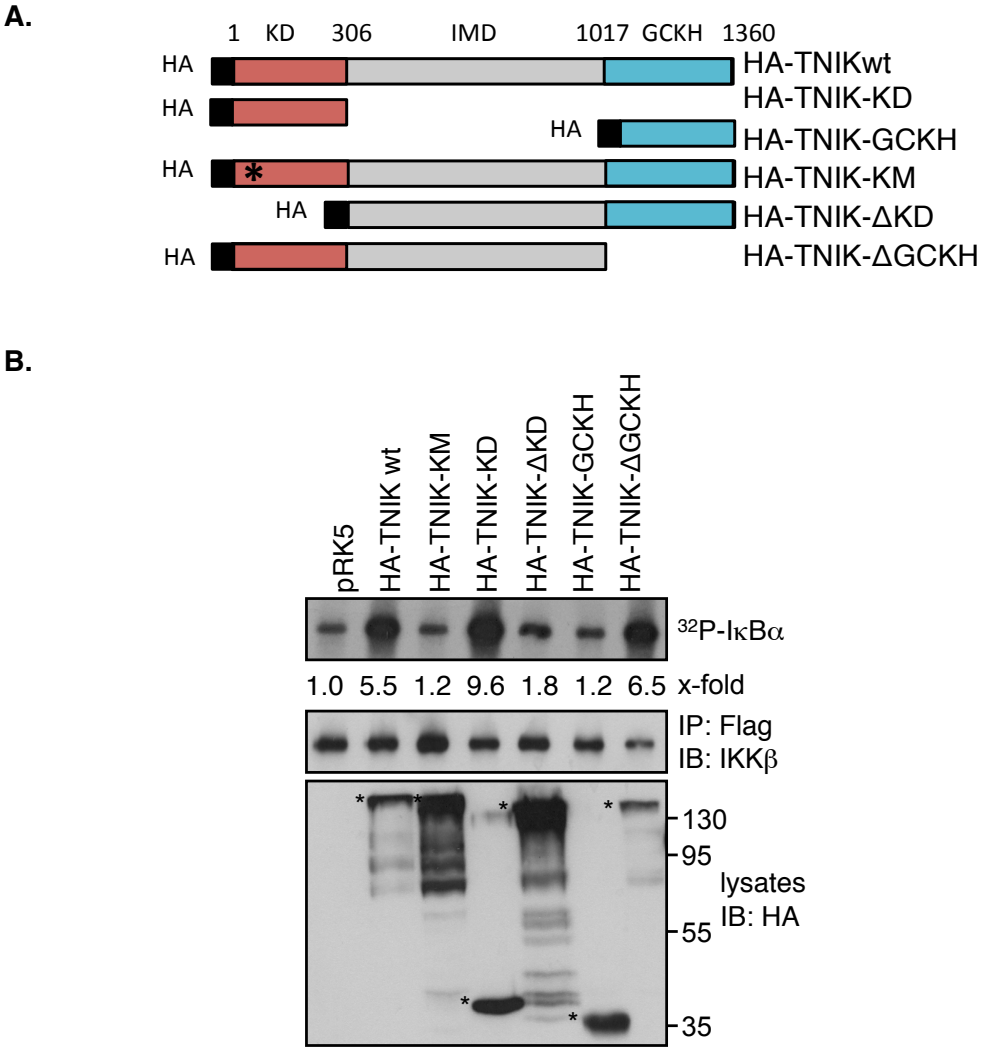
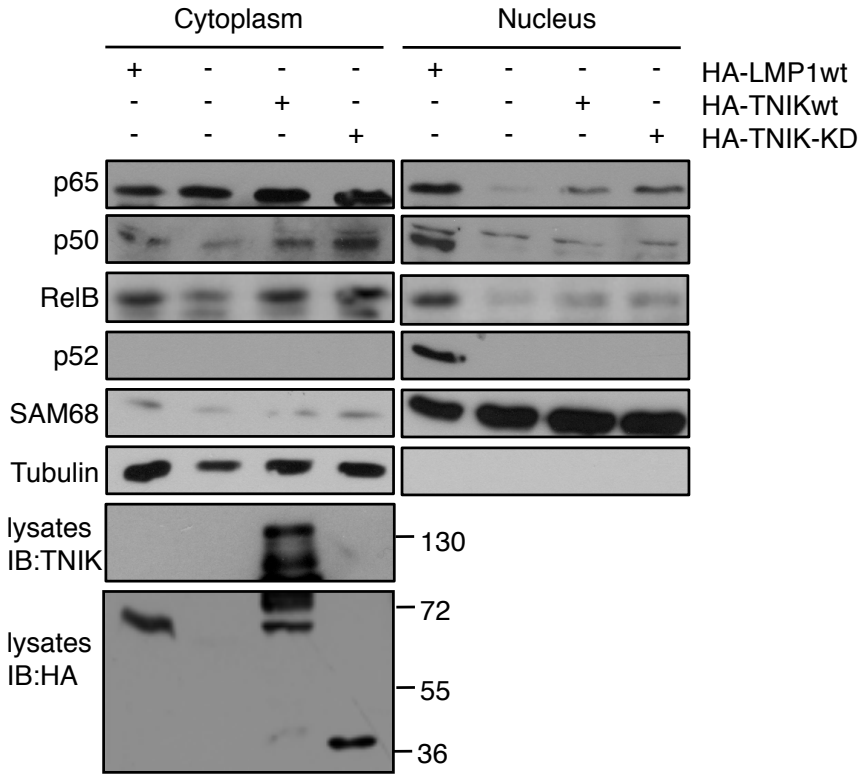


Figure 4.4. Canonical NF-κB signalling is induced by the kinase domain of TNIK
A. Schematic diagram of TNIK constructs cloned with an N-terminal HA-tag
The numbers represent amino acids of TNIK. The domains are marked: kinase domain (KD), intermediate domain (IMD) and germinal center kinase homology domain (GCKH). The asterisk indicates the K54R mutation, which abolishes kinase activity of TNIK.
B. TNIK activates IKKβ in a kinase dependent manner
HEK293D cells were transfected in 6-well plates with 2 μg of the indicated HA-tagged TNIK constructs or pRK5 empty vector and 1 μg Flag-IKKβ. Flag-IKKβ was immunoprecipitated and IKKβ activity was monitored in immunocomplex kinase assays. GST-IκBα phosphorylation was quantified by phosphoimager analysis and is given as x-fold induction. Immunoprecipitation of Flag-IKKβ and expression of HA-tagged TNIK-constructs were detected using the anti-IKKβ and anti-HA(3F10) antibodies. Asterisks mark the TNIK constructs, molecular masses are given in kDa.

Immunoblotting for the individual NF-κB subunits showed that, as expected, no nuclear translocation of any of the analysed NF-κB subunits occurred in the negative control. LMP1wt increased levels of all of the analysed NF-κB components in the nucleus showing that LMP1 activated the canonical as well as the non-canonical NF-κB pathway. Overexpression of TNIKwt and TNIK-KD increased levels of p65 in the nucleus while not

affecting the nuclear translocation of the other NF-κB subunits (figure 4.5). As the TNIK constructs did not induce nuclear translocation of p52-RelB an effect of TNIK overexpression on the non-canonical pathway can be excluded.



4.5. TNIK induces nuclear translocation of the NF-κB subunit p65
HEK293D cells were transfected in 10 cm cell culture dishes with 4 *μ*g pRK5 empty vector and 4 *μ*g each of the indicated constructs. Transfection of pRK5 empty vector served as a negative control. Cells were lysed and cytoplasmic and nuclear fractions were separated and analysed by immunoblotting for p65, p50, RelB, p52/p10, SAM68 and tubulin. Expression of the HA-TNIK constructs and HA-LMP1 was analysed by immunoblotting total cell lysates using the anti-TNIK and anti-HA(3F10) antibodies respectively.

Results presented in 4.2.1.1 showed that TNIK overexpression activated IKKβ which typically leads to a degradation of IκBα and liberation of p65-p50. p65-p65 NF-κB homodimers also exist and are translocated into the nucleus upon degradation of IκBα, but are much less abundant in the cell than p65-p50 heterodimers (Hoffmann et al, 2006). The fact that the overexpression of TNIKwt and TNIK-KD had no effect on the subcellular localization of p50 therefore suggests that TNIK exerts a specific effect on the nuclear translocation of p65. These results show that TNIK specifically activates the canonical NF-κB pathway by inducing a nuclear shift of p65, while not affecting non-canonical NF-κB signalling.

4.2.2. TNIK activates the JNK pathway via its GCKH-domain

In order to map the TNIK domain responsible for activation of the JNK pathway an in *in vitro* JNK1 kinase assay was performed using HEK293D cells transfected with the HA-JNK1 and the indicated HA-tagged TNIK constructs.

Comparable expression of the HA-TNIK constructs in cellular lysates and equal immunoprecipitation of HA-JNK1 in each sample was confirmed by immunoblot analysis. HA-TNIKwt, the kinase mutant (HA-TNIK-KM) and the GCKH-domain of TNIK induced JNK1 activity to a similar extent demonstrating that TNIK required the GCKH-domain but not its kinase activity for JNK signalling (figure 4.6). This finding is in accordance with results presented in a previous study (Fu et al, 1999).

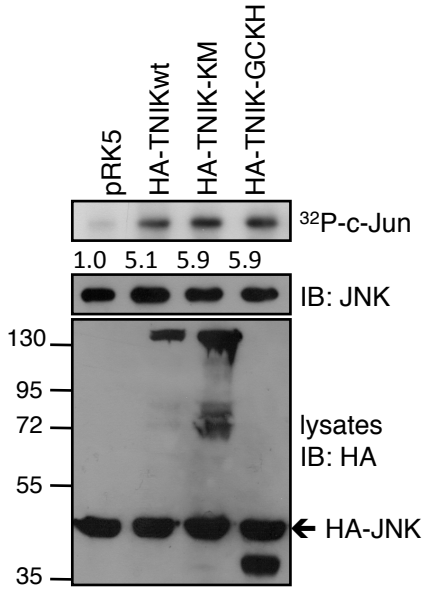


Figure 4.6. The GCKH domain of TNIK mediates activation of the JNK pathway
HEK293D cells were transfected in 6-well plates with 0.5 μg of the indicated HA-tagged TNIK constructs or pRK5 empty vector and 1 μg HA-JNK1. HA-JNK1 was immunoprecipitated and *in vitro* kinase assays were performed. HA-JNK1 activity is detected as radioactive phosphorylation of GST-c-Jun, quantified by phosphoimager analysis and stated as x-fold induction. Immunoprecipited HA-JNK1 and expressed HA-TNIK constructs were detected with the anti-JNK and anti-HA(3F10) antibodies respectively.

Taken together, the results presented above demonstrate that distinct domains of TNIK mediate activation of IKKβ and JNK respectively which indicates that TNIK might be the point of bifurcation of the canonical NF-κB- and JNK-pathways in the context of LMP1-induced signalling.

4.3. Characterization of the LMP1-induced TNIK signalling complex

4.3.1. TRAF6 is a novel interaction partner of TNIK

4.3.1.1. LMP1 induces the interaction between TNIK and TRAF6

In order to better understand the role of TNIK in signalling by LMP1, the next step was to analyse the TNIK signalling complex at the CTAR2 domain of LMP1 and elucidate how TNIK binds to LMP1. The fact that TNIK was shown to interact with TRAF2 suggests that TNIK can interact with TRAF family members (Fu et al, 1999). However, TRAF2 interacts with the consensus TRAF binding site within the CTAR1 domain of LMP1 and shows no affinity for CTAR2 (Devergne et al, 1996; Kaye et al, 1996; Kieser, 2007; Sandberg et al, 1997). TRAF6 is the only known TRAF family member that is recruited by the CTAR2 domain and is an essential signalling adapter for JNK and NF-κB signalling by LMP1 (Kieser, 2007; Luftig et al, 2003; Schultheiss et al, 2001; Wan et al, 2004). TRAF6 thus presented itself as a prime candidate to couple TNIK to LMP1.

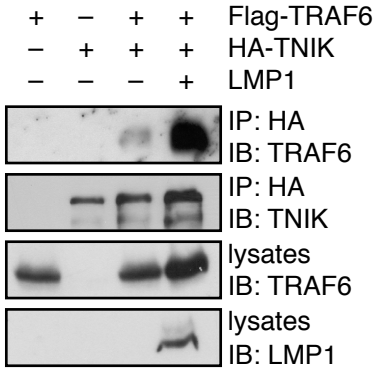


Figure 4.7. LMP1 induces an interaction between TNIK and TRAF6
HEK293D cells were transfected in 10 cm cell culture dishes with 0.5 μg each of Flag-TRAF6 and HA-TNIK, both in the presence and absence of 4 μg co-transfected pSV-LMP1wt, as indicated. Cells were lysed and HA-TNIK was immunoprecipitated using the anti-HA(12CA5) antibody. Immunoprecipitates and cell lysates were analysed by immunoblotting, using anti-TNIK, anti-TRAF6 and anti-LMP1(1G6-3) antibodies, as indicated.

In order to examine whether TNIK interacts with TRAF6, a co-immunoprecipitation experiment in HEK293D cells was conducted. HA-TNIK and Flag-TRAF6 were expressed in the presence and absence of co-transfected LMP1 and HA-TNIK was immunoprecipitated. Immunoblot analysis of the protein complexes showed comparable precipitation of HA-TNIK in each sample (figure 4.7). Flag-TRAF6 co-precipitated together with HA-TNIK, but the two proteins were associated only at a low level in the absence of LMP1. However, co-expression of LMP1 significantly induced the binding of TRAF6 to TNIK (figure 4.7).

Therefore this experiment identifies TRAF6 as a novel interaction partner of TNIK and the fact that LMP1 strongly enhanced this interaction, points to an important role of this interaction for LMP1-induced signalling.

4.3.1.2. TRAF6 mediates the interaction between TNIK and LMP1

So far it had not been investigated whether TNIK and LMP1 interact in a direct or indirect manner. Since TNIK binds to TRAF6 and TRAFs typically couple signalling molecules to upstream receptors, it was tested whether TRAF6 might be upstream of TNIK and mediate the interaction between TNIK and LMP1. An immunofluorescence experiment was carried out, comparing the co-localization of transiently transfected TNIK and LMP1 in wildtype mouse embryonic fibroblasts (MEFwt) and in TRAF6-deficient MEF cells (TRAF6 -/- MEFs). The cell nuclei of both cell types were round and well defined with clearly visible nucleoli, which showed that the stained cells were viable (figure 4.8.A, top panel). LMP1 showed a speckled staining within the cytosol, in the perinuclear region and at the plasma membrane (Fig. 4.8.A, second panel) which is in line with findings in a previous study that reported LMP1 to be distributed between the plasma membrane and intracellular cytosolic membranes (Lam & Sugden, 2003). In the wildtype cells TNIK was recruited into LMP1 clusters and co-localized with LMP1 to a large extent indicating that the two proteins interact in these cells (figure 4.8.A, third and fourth panel, left). Strikingly, in the TRAF6-deficient cells co-localization of TNIK and LMP1 was completely lost and TNIK was evenly distributed throughout the cytoplasm (figure 4.8.A, third and fourth panel, right). LMP1 did not induce nuclear translocation of TNIK as was described in the context of Wnt signalling (Mahmoudi et al, 2009). Thus TNIK appears to function as a cytoplasmic signalling mediator for LMP1. Additionally, a grey scale line scan analysis of the microscopic images shows the distribution of TNIK and LMP1 in the cells. The peaks were highly overlapping in the wildtype cells, which shows co-localization of the two proteins. In contrast, the peaks did not coincide in the TRAF6 knock out cells (figure 4.8.B, upper panel). Quantification of the experiment showed that co-localization of TNIK and LMP1 occured in 75 % of wildtype cells, but only in 12.5 % of the TRAF6-deficient cells (figure 4.8.B, bottom panel). The residual 12.5 % co-localization of TNIK and LMP1 that was observed in the TRAF6-deficient cells might be due to coincidental overlaps of the overexpressed proteins or clustering of proteins in the endoplasmic reticulum but probably does not constitute residual interaction between TNIK and LMP1 in the absence of TRAF6. Hence, this experiment demonstrates that TNIK and LMP1 interact indirectly and that this interaction is mediated by TRAF6.

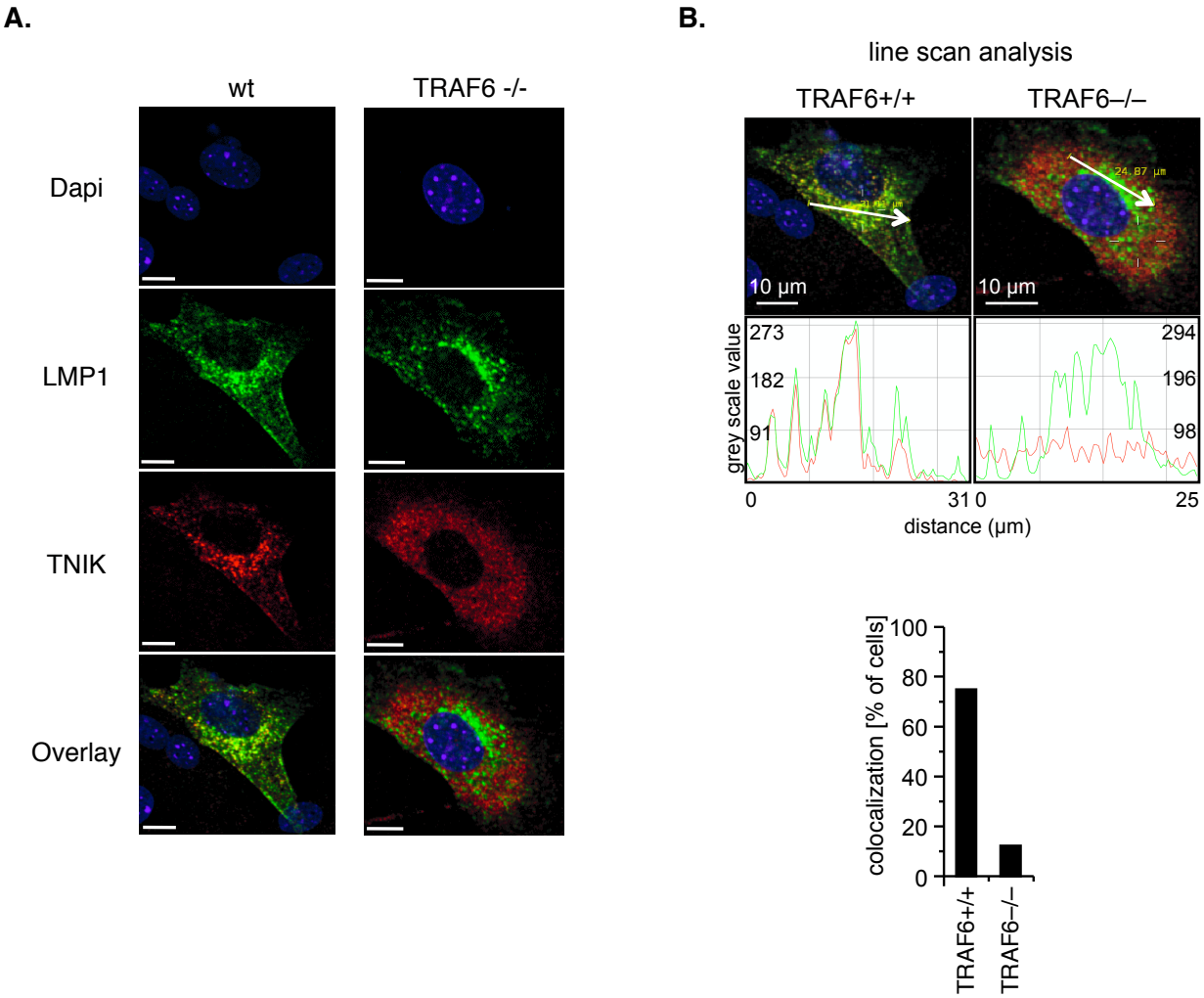


Figure 4.8. TRAF6 mediates the interaction between TNIK and LMP1
A. Co-localization of TNIK and LMP1 depends on TRAF6
MEFwt and MEF TRAF6 -/- cells were electroporated with expression vectors for Flag-TNIK and LMP1. Cells were subsequently seeded on coverslips and immunostained for TNIK (red) and LMP1 (green). Nuclei were counterstained with Dapi (blue).
B. Distribution of TNIK and LMP1 in wildtype and TRAF6 -/- MEF cells
A line scan analysis displays signal intensities of LMP1 (green) and TNIK (red) along the indicated arrows. Peaks of LMP1 and TNIK overlap only in the wildtype cells, but not in the TRAF6-/- cells. The graph shows the percentage of cells with colocalization of TNIK and LMP1 on the y-axis. Scalebar = 10 μm

4.3.1.3. TRAF6 interacts with the GCKH and intermediate domains of TNIK

Next the interaction between TNIK and TRAF6 was analysed more closely with regard to the TNIK domains that mediate the interaction. Co-immunoprecipitation experiments were carried out using HEK293D cells co-transfected with Flag-TRAF6 and the HA-tagged TNIK-mutants in the presence of LMP1 as an inducer. Immunoblot analysis of the protein complexes showed that the HA-tagged TNIK mutants were precipitated at comparable levels and revealed an interaction between Flag-TRAF6 and HA-TNIK-ΔKD as well as HA-TNIK-

GCKH, whereas no interaction between HA-TNIK-KD and TRAF6 was detected. This showed that each of the HA-TNIK constructs that incorporated a GCKH domain readily interacted with TRAF6, while the kinase domain was dispensable for this interaction. HA-TNIK-ΔGCKH showed a weak interaction with Flag-TRAF6, which shows that the intermediate domain additionally contributed some binding affinity to the interaction (figure 4.9.). Thus this experiment demonstrates that the interaction between TRAF6 and TNIK is mainly mediated by the GCKH-domain of TNIK.

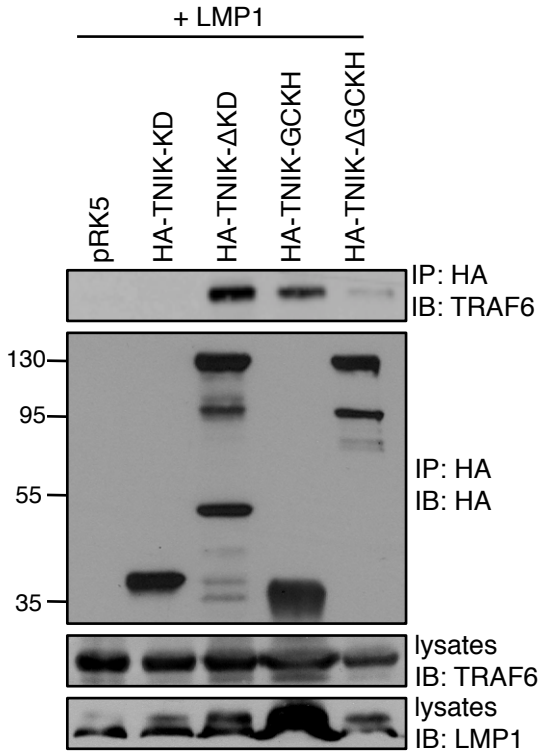


Figure 4.9. The interaction between TRAF6 and TNIK is mainly mediated by the GCKH domain of TNIK
HEK293D cells were transfected in 10 cm cell culture dishes with 0.5 μg each of Flag-TRAF6 and the HA-TNIK constructs or pRK5 empty vector in the presence of 4 μg co-transfected pSV-LMP1wt as indicated. Cells were lysed and subjected to immunoprecipitation using the anti-HA(12CA5) antibody. Immunoprecipitates and cell lysates were analysed by immunoblotting using the anti-HA(12CA5), anti-TRAF6 and anti-LMP1(1G6-3) antibodies, as indicated.

Next, the interaction was analysed with regard to the TRAF6 domain that mediates binding to TNIK. A co-immunoprecipitation experiment was conducted using HEK293D cells transfected with expression vectors for the HA-tagged TNIK constructs and the TRAF6 mutant Flag-TRAF6(274-522) in the presence of LMP1 as an inducer. Flag-TRAF6(274-522) consists of the C-terminal part of TRAF6 and lacks the N-terminal RING and Zinc finger motifs that confer ubiquitin ligase activity to TRAF6 (see 1.7.1). The HA-tagged TNIK constructs were

immunoprecipitated and immunoblot analysis of the protein complexes showed that comparable amounts of the individual TNIK constructs were precipitated in each sample.

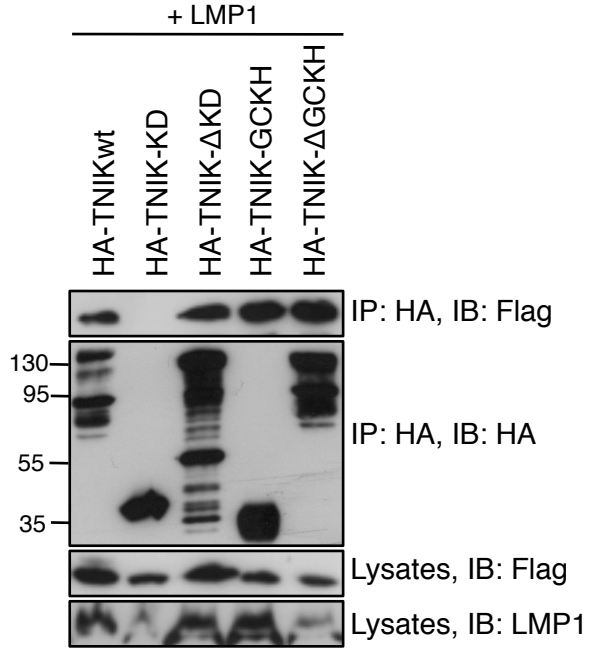


Figure 4.10. The TRAF6(274-522) mutant lacking the RING domain binds to the intermediate and GCKH domains of TNIK
HEK293D cells were transfected in 15 cm cell culture dishes with 2 μg Flag-TRAF6(274-522), 2.5 μg each of the HA-TNIK constructs or pRK5 empty vector in the presence of 5 μg of co-transfected pSV-LMP1wt. Cells were lysed and the HA-TNIK constructs were immunoprecipitated with the anti-HA(12CA5) antibody. Immunoprecipitates and cell lysates were analysed by immunoblotting using the anti-HA(12CA5), anti-Flag(M2) and anti-LMP1(1G6-3) antibodies as indicated.

The Flag-TRAF6(274-522)-mutant co-precipitated together with HA-TNIKwt as well as the HA-TNIK-ΔKD and HA-TNIK-ΔGCKH deletion mutants showing that binding of Flag-TRAF6(274-522) to TNIK was not affected by deletion of either the kinase domain or the GCKH-domain. This shows that the TRAF6 mutant interacted with the intermediate domain of TNIK. Flag-TRAF6(274-522) also co-precipitated together with HA-TNIK-GCKH showing an additional interaction with the GCKH domain, while no interaction with the kinase domain was detected (figure 4.10). Thus the TRAF6(274-522) mutant bound to the GCKH and intermediate domains of TNIK with equal affinities and thus differed in its binding pattern to TRAF6 wildtype which mainly interacted with the GCKH domain (see figure 4.9).

As the TRAF6(274-522)-mutant lacks its ubiquitin ligase activity, the observed differences in the binding patterns of the TRAF6 mutant and TRAF6 wiltype could be due to ubiquitination events. Results obtained from *in vitro* pull-down experiments using purified proteins in our

laboratory showed that the C-terminus of TRAF6 directly interacts with the intermediate domain of TNIK while no interaction between the GCKH domain and TRAF6 was detected (Fabian Giehler, personal communication). This indicates that the interaction between TRAF6 and the GCKH domain that was detected in HEK293D cells is indirect and that ubiquitination events might facilitate an intramolecular shift of TRAF6 from the intermediate domain to the GCKH domain. Dynamic processes might thus be involved in the interaction between TNIK and TRAF6.

4.3.2. TAK1 interacts constitutively with TNIK

The MAP3K TAK1 acts in concert with TRAF6 to activate JNK and canonical NF-κB signalling by a number of cellular receptors and the CTAR2 domain of LMP1 Thus, we hypothesized that TAK1 might also be a part of the TNIK-TRAF6 complex although the role of TAK1 in the activation of the canonical NF-κB pathway by LMP1 is currently under debate (Uemura et al, 2006; Wan et al, 2004; Wu et al, 2006) (see 1.8).

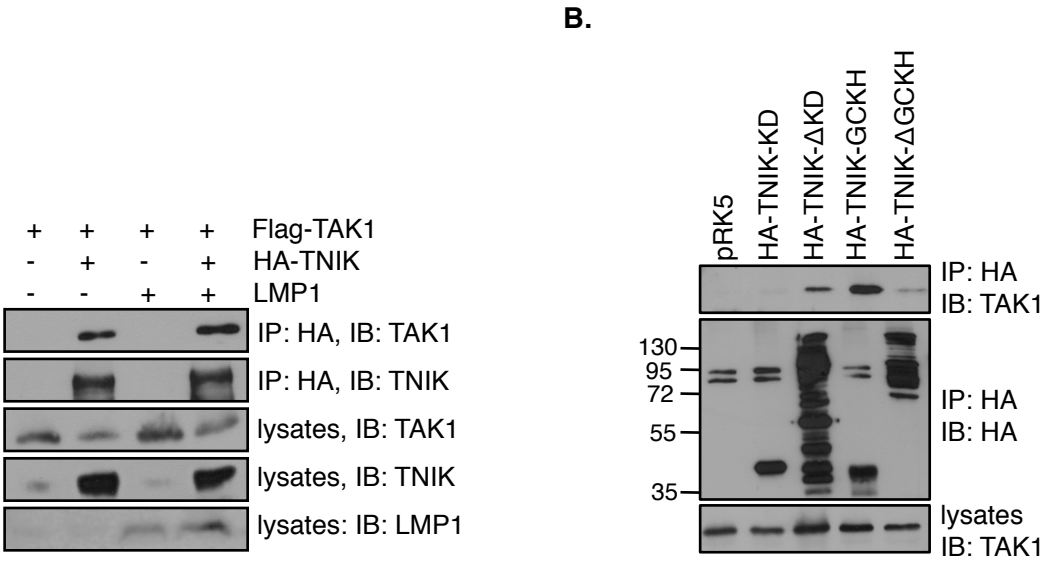


Figure 4.11. TAK1 is a novel interaction partner of TNIK
A. TAK1 interacts constitutively with TNIK
HEK293D cells were transfected in 15 cm cell culture dishes with 5 μg HA-TNIKwt and 2 μg Flag-TAK1 both in the presence and absence of 5 μg co-transfected pSV-LMP1wt, as indicated. HA-TNIKwt was immunoprecipitated with the anti-HA(12CA5) antibody. Protein complexes were separated by SDS-PAGE and analysed by immunoblotting for TNIK and TAK1. Cell lysates were immunoblotted using anti-TNIK, anti-TAK1 and anti-LMP1(CS1-4) antibodies.
B. TAK1 binds to the GCKH and intermediate domains of TNIK
HEK293D cells were transfected in 10 cm cell culture dishes with 2 μg of the indicated HA-TNIK constructs and 1 μg Flag-TAK1. HA-TNIK constructs were immunoprecipitated with the anti-HA(12CA5) antibody. Immunoprecipitates and cell lysates were analysed by immunoblotting with anti-HA(3F10) and anti-TAK1 antibodies.

A potential interaction between TAK1 and TNIK was tested for by a co-immunoprecipitation experiment in HEK293D cells transfected with HA-TNIK and Flag-TAK1. HA-TNIK was immunoprecipitated and analysis of immunoprecipitates revealed that the two proteins readily interacted, while co-transfection of LMP1 did not exert a significant effect on the strength of this interaction (figure 4.11.A). The slight induction of the TNIK-TAK1 interaction by LMP1 in this experiment was not observed in other experiments. Hence, this result demonstrates that TAK1 is a new interaction partner of TNIK and that the two proteins interact constitutively.

Subsequently this interaction was examined more closely with regard to the TNIK domains that mediate binding to TAK1. HEK293D cells were co-transfected with expression vectors for the HA-tagged TNIK mutants and Flag-TAK1. Immunoblot analysis of the protein complexes showed comparable immunprecipitation of the HA-TNIK constructs. Flag-TAK1 readily co-precipitated together with HA-TNIK-GCKH and HA-TNIK-ΔKD showing that the GCKH domain was sufficient to mediate the interaction between TNIK and TAK1, while the kinase domain did not contribute towards the interaction. A weak interaction between TAK1 and HA-TNIK-ΔGCKH was detected demonstrating that the deletion of the GCKH domain weakened the interaction but did not completely abolish it. Since the kinase domain did not contribute towards the interaction, the weak secondary interaction that was observed between TAK1 and HA-TNIK-ΔGCKH can be attributed to the intermediate domain of TNIK (figure 4.11.B).

Taken together these findings show that the GCKH-domain is the main interaction site for TAK1, while the intermediate domain contributes to the interaction, possibly by an indirect mechanism.

4.3.3. TNIK forms an inducible complex with IKKβ

IKKβ is another important player in the induction of canonical NF-κB signalling by LMP1 that depends on the TRAF6-TAB2-TAK1-complex for its activation (Uemura et al, 2006; Wu et al, 2006). Since results presented previously showed that TNIK induces the canonical NF-κB pathway via IKKβ (see 4.2.1.1), an interaction between the two proteins was analysed. A co-immunoprecipitation experiment was conducted using HEK293D cells co-transfected with expression vectors for HA-TNIK and Flag-IKKβ. HA-TNIK was immunoprecipitated and immunoblot analysis of lysates and protein complexes showed expression of the transfected constructs and comparable immunoprecipitation of HA-TNIK in the samples. In the absence of an inducer HA-TNIK did not interact with co-transfected Flag-IKKβ.

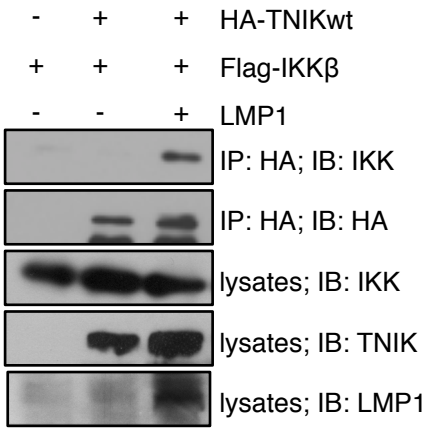


Figure 4.12. LMP1 induces an interaction of TNIK and IKKβ
HEK293D cells were co-transfected in 10 cm cell culture dishes with 3 μg each of Flag-IKKβ and HA-TNIKwt as well as 2 μg of pSV-LMP1wt as indicated. HA-TNIKwt was immunoprecipitated from cell lysates using the anti-HA(12CA5) antibody and immunocomplexes were subsequently analysed by SDS-PAGE and immunoblotting using anti-HA(12CA5) and anti-IKKβ antibodies. Expression of HA-TNIK, Flag-KKβ and LMP1 in cell lysates was detected with the anti-HA(12CA5), anti-IKKβ and anti-LMP1(1G6-3) antibodies as indicated.

However, the two proteins interacted readily in the presence of LMP1 (figure 4.12). This result thus clearly identifies IKKβ as a novel interaction partner of TNIK that binds in response to LMP1 activity.

4.3.4. LMP1 induces the interaction between TNIK and TRAF2

Since it is known that TNIK interacts with TRAF2 and LMP1 engages TRAF2 for signalling (Devergne et al, 1996; Fu et al, 1999; Sandberg et al, 1997; Song & Kang, 2010), this interaction was analysed in the context of LMP1-induced signal transduction. A co-immunoprecipitation experiment was carried out in HEK293D cells transfected with HA-TNIK and TRAF2 in the presence and absence of LMP1. HA-TNIK was immunoprecipitated and analysis of immunoprecipitates showed that comparable amounts of TNIK were precipitated in each sample. A low amount of TRAF2 co-precipitated together with TNIK in the absence of LMP1, while co-expression of LMP1 significantly induced co-precipitation of TRAF2 (figure 4.13). This result demonstrates a weak constitutive interaction of TNIK and TRAF2, which is significantly induced by LMP1 and suggests a role of this interaction for LMP1-induced signalling.

In order to gain further insight into the role of the TNIK-TRAF2 interaction for signalling by LMP1, it was investigated which domain of LMP1 induces this interaction. The experiment was thus repeated using a set of different HA-tagged LMP1-mutants as potential inducers.

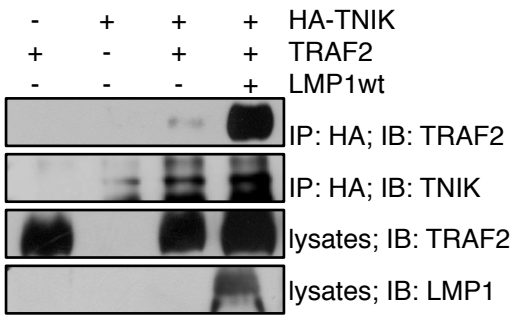


Figure 4.13. LMP1 induces an interaction between TNIK and TRAF2
HEK293D cells were transfected in 10 cm cell culture dishes with 0.5 μg each of HA-TNIKwt, TRAF2 and 3 μg of HA-LMP1wt. Cells were lysed and TNIK was precipitated using the anti-TNIK antibody. Lysates and immunoprecipitations were analysed by immunoblotting with the anti-TRAF2, anti-TNIK and anti-LMP1(1G6-3) antibodies.

Immunoblot analysis of cellular lysates showed equal expression levels of TRAF2 and each of the LMP1-mutants in the samples. Analysis of immunoprecipitations showed comparable precipitation of HA-TNIK in each sample and co-precipiation of TRAF2 upon co-expression of HA-LMP1 wildtype and HA-LMP1(PQT). The CTAR1 mutant, LMP1(PQT) induced the interaction between TNIK and TRAF2 to the same extent as LMP1 wildtype, whereas mutation of the CTAR2 domain as in LMP1(Y384G), LMP1(Δ371) and the CTAR1/CTAR2 double mutant, LMP1(PQT/ Δ371) abolished the ability of LMP1 to induce the interaction (figure 4.14).

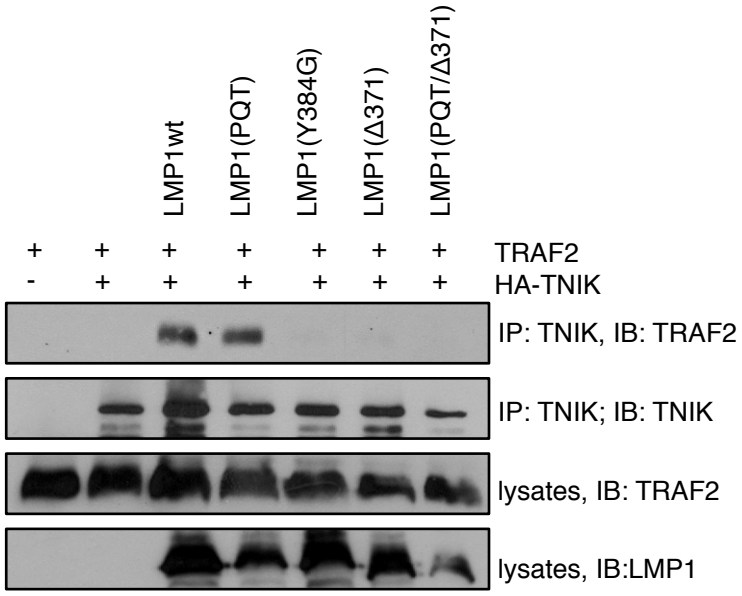


Figure 4.14. The interaction between TNIK and TRAF2 is induced by the CTAR2 domain of LMP1
HEK293D cells were transfected in 10 cm cell culture dishes with 0.5 μg each of HA-TNIKwt and TRAF2 as well as 3 μg each of the HA-LMP1 constructs as indicated. Cells were lysed and HA-TNIKwt was immunoprecipitated. Immunoprecipitates and lysates were subsequently analysed by immunoblotting with the anti-TRAF2, anti-TNIK and anti-LMP1(1G6-3) antibodies.

This result demonstrates that the CTAR2 domain of LMP1 induces binding of TNIK to TRAF2. The role of TRAF2 in CTAR2-mediated signalling is currently under debate and might be cell type-specific (see 1.7.2). While a recent study showed a role of TRAF2 for the induction of the canonical NF-κB pathway (Gewurz et al, 2012), TRAF2 does not bind to the CTAR2 domain (Kieser, 2007). Thus TNIK and TRAF2 might interact within a cytoplasmic complex. The contribution of TRAF2 to TNIK-mediated signalling in the context of LMP1 is subject of future studies.

4.3.5. TNIK is an organizer of the signalling complex at the CTAR2 domain of LMP1

Having demonstrated that TNIK interacts with the major components of the signalling complex at the CTAR2 domain of LMP1 and that TRAF6 lies upstream of TNIK, the next question was, whether TNIK might function as a complex organizer that facilitates the recruitment of downstream interaction partners to LMP1. Analysis of this question focussed on the TAK1-TAB2 complex.

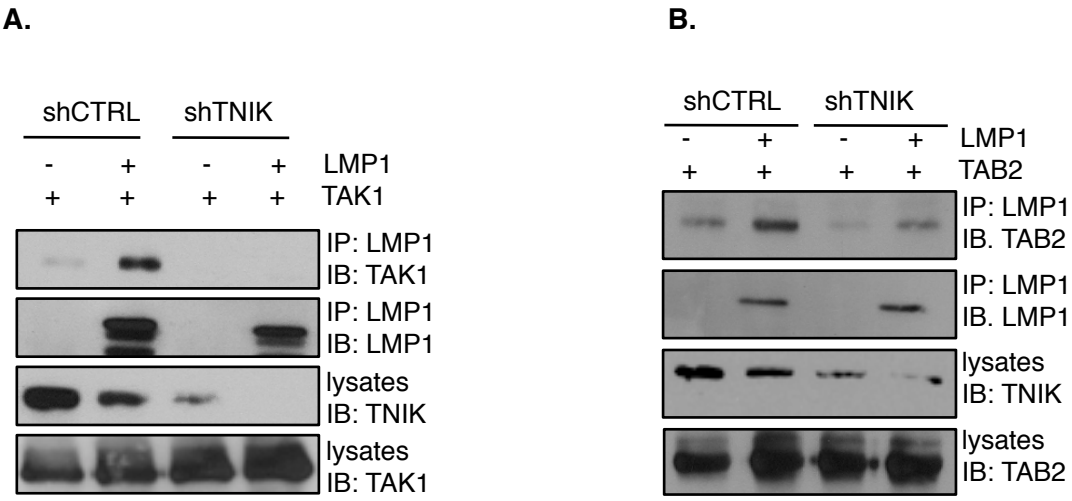


Figure 4.15. TNIK mediates association of the TAK1-TAB2 complex with LMP1
A. Down-regulation of TNIK abolishes the interaction between LMP1 and TAK1
HEK293 cells were co-transfected in 15 cm cell culture dishes with 5 μg Flag-TAK1, 3 μg HA-LMP1wt and 7 μg each of shRNA for human TNIK (shTNIK) or non-silencing shRNA (shCTRL). Cells were lysed and LMP1 was immunoprecipitated using the anti-LMP1(1G6-3) antibody. Protein complexes and cell lysates were analysed using anti-TAK1, anti-LMP1 and anti-TNIK antibodies.
B. TNIK mediates the interaction between LMP1 and TAB2
HEK293 cells were co-transfected in 15 cm cell culture dishes with 5 μg HA-TAB2, 3 μg LMP1wt and 7 μg each of shTNIK or shCTRL vectors. Cells were lysed and LMP1 was precipitated with the anti-LMP1(1G6-3) antibody. Immunoprecipitates and lysates were analysed by immunoblotting with the anti-TAB2(3744S), anti-LMP1(1G6-3) and anti-TNIK antibodies.

Previous results obtained in this study showed an interaction between TNIK and TAK1 (see 4.3.2). Additionally, an interaction between TNIK and TAB2 in the presence of LMP1 has been demonstrated (Dr. Anna Shkoda, personal communication). If TNIK was required for the recruitment of the TAK1-TAB2 complex to LMP1, the knockdown of endogenous TNIK should abolish binding of TAK1 and TAB2 to LMP1. This was investigated using HEK293D cells depleted of endogenous TNIK using TNIK-specific shRNA vectors. As a control, cells were transfected with non-targeting shRNA-control vectors. In this experiment shRNA was used instead of siRNA to achieve a knockdown of endogenous TNIK as this method of protein knock-down is more cost effective and thus more convenient for the large scale protein preparations needed here. LMP1 and either Flag-TAK1 or HA-TAB2 were co-expressed. Immunoblot analysis of cellular lysates showed expression of Flag-TAK1 and HA-TAB2 as well as the down-regulation of TNIK. LMP1 was immunoprecipitated and analysis of the protein complexes revealed that both TAK1 and TAB2 readily co-precipitated together with LMP1 in the presence of shCTRL vectors. The knockdown of endogenous TNIK abolished binding of TAK1 and TAB2 to LMP1 (figure 4.15). This result thus showed that TNIK mediates binding of the downstream TAK1-TAB2 complex to LMP1 and indicates a role for TNIK as an essential organizer of the signalling complex at the CTAR2 domain of LMP1.

4.4. The role of TNIK in signal transduction of the cellular CD40 receptor

4.4.1. TNIK interacts with CD40

The previous findings have demonstrated that TNIK is an essential mediator of the JNK and canonical NF-κB signalling pathways of the viral oncoprotein LMP1. LMP1 induced an interaction between TNIK and the major signalling mediators of the CTAR2 domain including TRAF6, the TAK1-TAB2 complex and IKKβ. The next task was to identify a cellular receptor engaging TNIK for the activation of JNK and NF-κB signalling. Given that LMP1 is a functional mimic of the CD40 receptor that is mainly expressed by B-cells, a role of TNIK for signalling by CD40 was investigated.

First a potential interaction between TNIK and CD40 was analysed by co-transfecting HEK293D cells with expression vectors for HA-TNIKwt and CD40. Overexpression of CD40 is sufficient for activation of the receptor in this cell line through transient formation of receptor multimers without the need to stimulate with CD40L (Davies et al, 2005; Rothe et al, 1995).

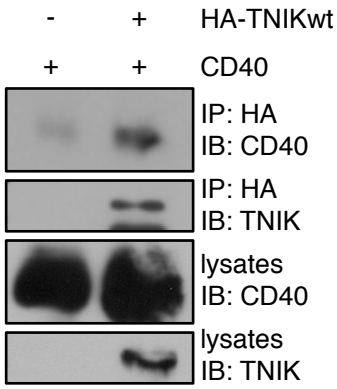


Figure 4.16. TNIK interacts with CD40
HEK293D cells were transfected in 10 cm cell culture dishes with 2 μg each of HA-TNIKwt and CD40. HA-TNIKwt was precipitated with the anti-HA(12CA5) antibody and immunoprecipitates and lysates were analysed by immunoblotting for TNIK and CD40.

HA-TNIKwt was immunoprecipitated and subsequent immunoblot analysis revealed that CD40 readily interacted with TNIK (figure 4.16). This experiment identifies TNIK as a new interaction partner of the cellular CD40 receptor.

4.4.2. TNIK mediates JNK- and canonical NF-κB-signalling by CD40

Next the role of TNIK in CD40-mediated signalling was analysed. To test, whether TNIK mediates JNK and canonical N-κB signal transduction, HEK293D cells were depleted of endogenous TNIK by TNIK-specific siRNA. Expression vectors for CD40 and either HA-JNK1 (figure 4.17.A) or Flag-IKKβ (figure 4.17.B) were co-transfected. Activation of JNK or IKKβ was examined by *in vitro* kinase assays monitoring the phosphorylation of the substrates c-Jun and IκBα respectively (figure 4.17). Immunoblot analysis of cellular lysates showed comparable expression levels of CD40 and down-regulation of TNIK by siRNA (figure 4.17, bottom panels). Equal immunoprecipitation of HA-JNK1 and Flag-IKKβ in each sample was confirmed by immunoblotting (figure 4.17, second panel from the top). Down-regulation of TNIK by siRNA significantly reduced the ability of CD40 to activate either JNK1 or IKKβ. In the presence of control siRNA, CD40 caused a 1.6-fold induction of JNK1 and a 10-fold induction of IKKβ. The knockdown of TNIK reduced induction levels of JNK activity to 0.9, which is at the level of the base induction that is caused by the negative control HA-LMP1(Δ194-386). In the case of IKKβ activity the knockdown of TNIK reduced the induction levels by 92 % to 1.7-fold (figure 4.17).

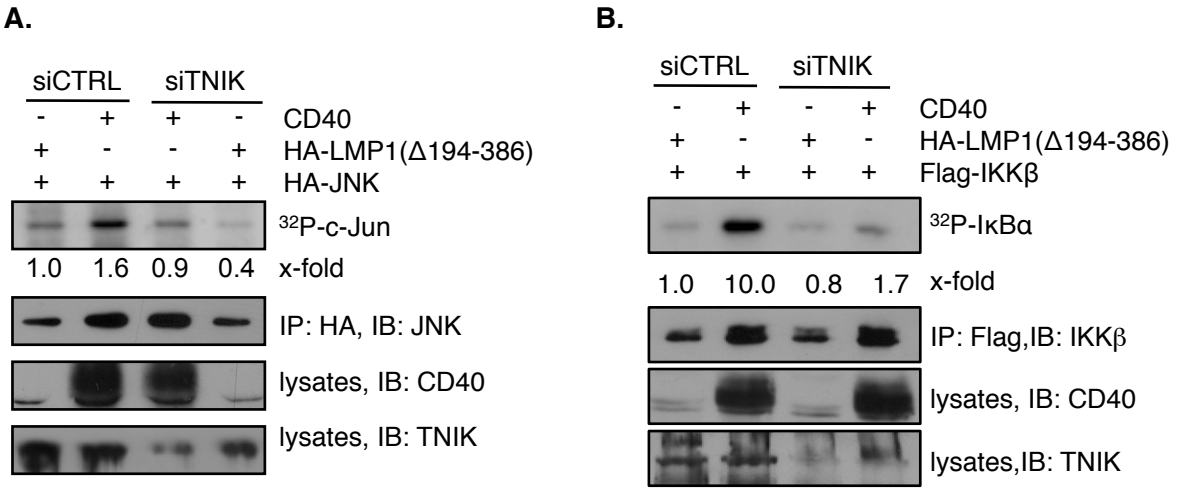


Figure 4.17. TNIK mediates JNK and canonical NF-κB signalling by CD40
A. Down-regulation of TNIK impairs CD40 mediated JNK signalling
HEK293D cells were transfected in 6-well plates with TNIK siRNA or non-targeting siRNA. Subsequently cells were co-transfected with 2 μg of CD40 or the signalling-inactive HA-LMP1(Δ194-386) and 1 μg of HA-JNK1, as indicated. HA-JNK1 activity was monitored in immunocomplex kinase assays using GST-cJun as a substrate. GST-cJun phosphorylation was quantified by Phosphoimager analysis and is stated as x-fold induction. Immunoprecipitated HA-JNK1, expression of CD40 and down-regulation of TNIK was detected using the anti-JNK, anti-CD40 and anti-TNIK antibodies.
B. Activation of the canonical NF-κB pathway by CD40 depends on TNIK
HEK293D cells were transfected in 6-well plates with TNIK siRNA or non-targeting siRNA. Cells were subsequently co-transfected with 2 μg of expression vectors for CD40 or the signalling-inactive HA-LMP1(Δ194-386) and 1 μg of Flag-IKKβ as indicated in the figure. Flag-IKKβ activity was monitored in immunocomplex kinase assays using GST-IκBα as a substrate. GST-IκBα phosphorylation was quantified by Phosphoimager analysis and is stated as x-fold induction. Immunoprecipitated Flag-IKKβ, expression of CD40 and down-regulation of TNIK was detected using the anti-IKKβ, anti-CD40 and anti-TNIK antibodies.

Taken together, these results demonstrate that TNIK is critical for JNK and canonical NF-κB signalling by CD40 thus identifying TNIK as a novel component of the CD40 signalling complex.

4.4.3. Characterization of the CD40-induced TNIK signalling complex

4.4.3.1. CD40 induces an interaction of TNIK with TRAF6, TRAF2 and IKKβ

Results presented previously in this thesis have shown that LMP1 induces an interaction of TNIK with TRAF6, TRAF2 and IKKβ (see figures 4.7, 4.12 and 4.13). These proteins are also engaged for signalling by CD40. Both TRAF6 and TRAF2 are critical for canonical NF-κB and JNK signal transduction by CD40, while IKKβ mediates induction of the canonical NF-κB pathway (Karin & Gallagher, 2009). To examine whether CD40 induces a similar TNIK signalling complex as LMP1, co-immunoprecipitation experiments were carried out in HEK293D cells.

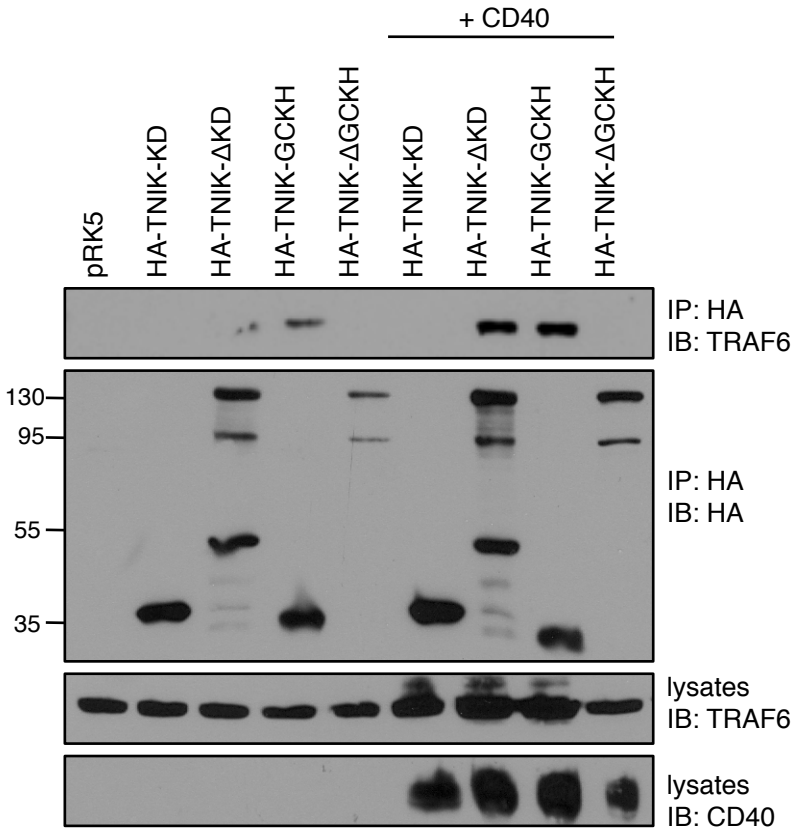


Figure 4.18. CD40 induces an interaction between TRAF6 and the GCKH domain of TNIK
HEK293D cells were co-transfected in 10 cm cell culture dishes with 0.5 μg Flag-TRAF6, 1 μg each of the HA-TNIK mutants and 3 μg CD40 as indicated. The HA-TNIK mutants were immunoprecipitated and protein complexes and lysates were analysed by immunoblotting using anti-HA(12CA5), anti-TRAF6 and anti-CD40 antibodies.

CD40 induced an interaction between TNIK and TRAF6 in B-cells (see 4.4.3.2 and figure 4.20.B). The interaction between TNIK and TRAF6 with regard to the TNIK domains that mediated the interaction was analysed in HEK293D cells. Flag-TRAF6 and the HA-tagged TNIK constructs were co-expressed in 293D cells in the presence and absence of CD40 as an inducer. As expression levels of HA-TNIKwt were significantly lower than that of the other HA-TNIK mutants, the interaction between TNIK wildtype and TRAF6 could not be analysed in this experiment. The HA-TNIK constructs were immunoprecipitated and analysed by immunoblotting. This showed comparable precipitation of each of the HA-TNIK mutants and revealed that Flag-TRAF6 co-precipitated together with HA-TNIK-ΔKD and HA-TNIK-GCKH showing that the GCKH domain is sufficient to mediate the interaction with Flag-TRAF6, while the kinase domain is dispensable. No interaction took place with HA-TNIK-KD and HA-TNIK-ΔGCKH which lack the GCKH domain. Thus the GCKH domain of TNIK mediated the interaction with TRAF6. Co-expression of CD40 enhanced the binding of TRAF6 to TNIK, but had no effect on the binding pattern (figure 4.18). In contrast to LMP1 no interaction with HA-

TNIK-ΔGCKH was detected (see figure 4.9). This could be due to the fact that the much weaker interaction between HA-TNIK-ΔGCKH was not detected in this experiment. Hence, this experiment demonstrates that TRAF6 interacts with the GCKH domain of TNIK and that this interaction is enhanced by CD40. A potential additional interaction with the intermediate domain, however, cannot be excluded.

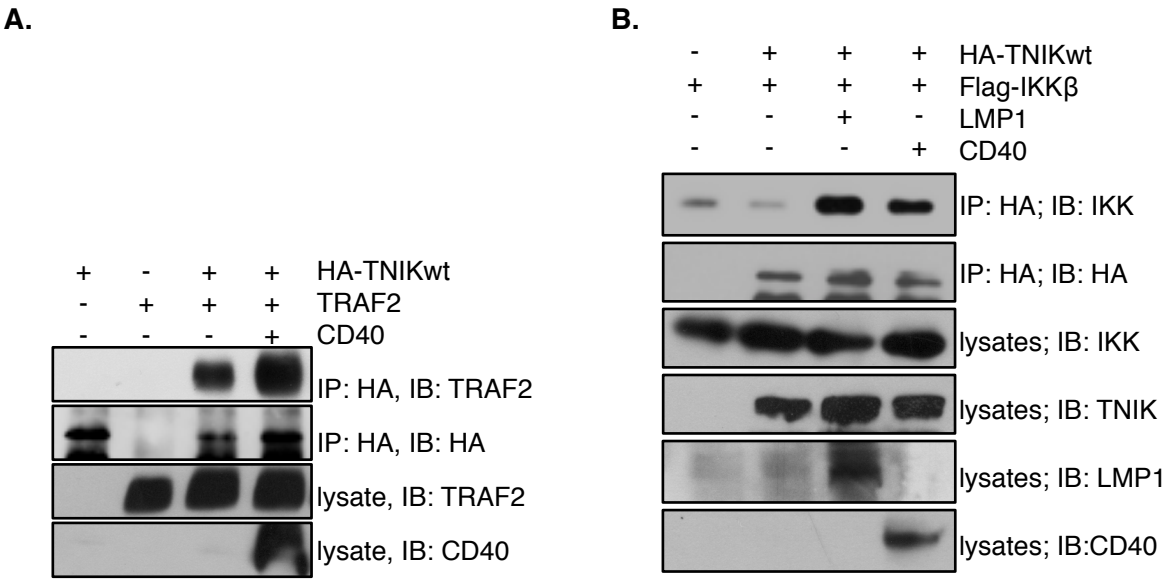


Figure 4.19. CD40 induces binding of TRAF2 and IKKβ to TNIK
A. CD40 induces an interaction between TNIK and TRAF2
HEK293D cells were transfected in 10 cm cell culture dishes with 1 μg each of HA-TNIKwt and TRAF2 in the presence of 3 μg of co-transfected CD40. HA-TNIKwt was immunoprecipitated and immunoprecipitates and lysates were analysed by immunoblotting using the anti-HA(3F10), anti-TRAF2 and anti-CD40 antibodies.
B. The interaction between TNIK and IKKβ is induced by CD40
The experiment shown here is the same as in figure 12. HEK293D cells were co-transfected in 10 cm cell culture dishes with 3 μg each of Flag-IKKβ and HA-TNIKwt as well as 2 μg each of pSV-LMP1 and CD40 as indicated. HA-TNIKwt was immunoprecipitated from cell lysates using the anti-HA(12CA5) antibody and immunocomplexes and lysates were subsequently analysed by immunoblotting using anti-HA(12CA5), anti-IKKβ, anti-LMP1(1G6-3) and anti-CD40 antibodies as indicated.

In order to examine whether CD40 induces the interaction between TNIK and TRAF2 as well as between TNIK and IKKβ as has been shown for LMP1 (see 4.3.3 and 4.3.4), HEK293D cells were transfected with expression vectors for HA-TNIKwt and either TRAF2 or Flag-IKKβ in the presence and absence of CD40 as a potential inducer. Immunoblot analysis of cellular lysates showed expression of the transfected constructs and analysis of immunoprecipitates revealed that comparable amounts HA-TNIKwt were precipitated in each sample.

TRAF2 co-precipitated together with HA-TNIKwt in the absence of an inducer showing that TRAF2 and TNIK are constitutively associated. The co-expression of CD40 enhanced this interaction (figure 4.19A). Thus CD40, like LMP1 enhances the interaction between TNIK and TRAF2 (see 4.3.4). No interaction of Flag-IKKβ with HA-TNIKwt was detected in the absence of an inducer, but Flag-IKKβ readily co-precipitated together with TNIK upon co-expression of CD40 or LMP1 (figure 4.19B). This shows that both LMP1 and CD40 induce an interaction between TNIK and IKKβ.

Taken together, these findings show that the composition of the TNIK signalling complex with regard to TRAF6, TRAF2 and IKKβ that is induced by CD40 is similar to that induced by the viral counterpart LMP1.

4.4.3.2. Analysis of the signalling complex in B-cells

Having analysed the TNIK signalling complexes with overexpressed proteins in the epithelial cell line HEK293D, the next task was to investigate the CD40-induced signalling complex with endogenous proteins in B-cells.

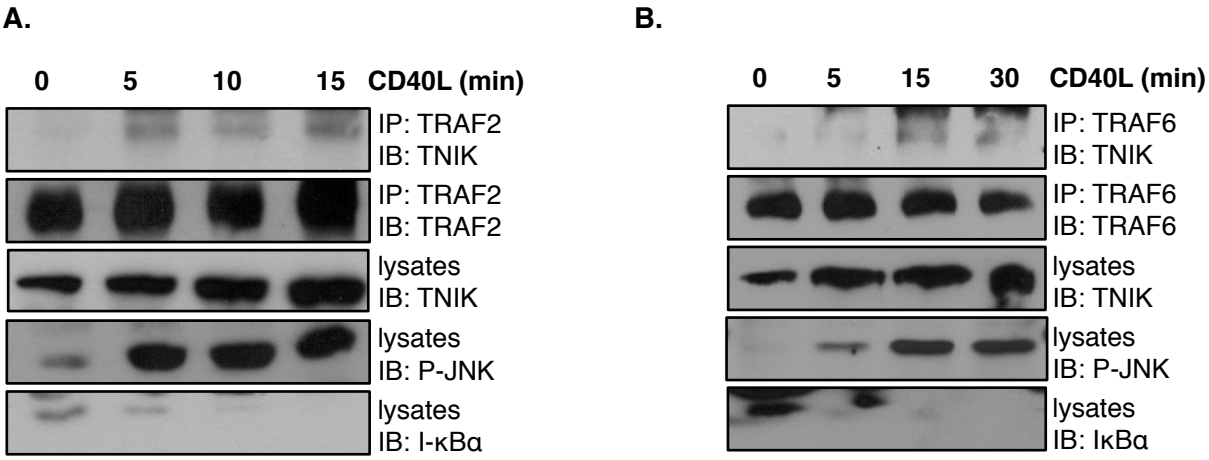


Figure 4.20. Analysis of the CD40-induced signalling complex in B-cells
A. Recruitment of TNIK and CD40 to TRAF2 after stimulation of CD40
BL41 cells were stimulated with 600 ng/ml CD40L for 0, 5, 10 and 15 minutes using 1.5 x 10^8 cells per time point. Cells were lysed, protein concentrations in the samples adjusted to 10 mg/ml and endogenous TRAF2 was precipitated with the anti-TRAF2 antibody. Protein complexes and lysates were resolved by SDS PAGE and analysed by immunoblotting using anti-TRAF2, anti-TNIK, and anti-CD40 antibodies. Activation of the JNK and canonical NF-κB pathways was analysed by immunoblotting lysates with the anti-phospho-JNK and anti-IκBα antibodies.
B. CD40 stimulation induces the interaction between TNIK and TRAF6
BL41 cells were stimulated with 500 ng/ml CD40L for 0, 5, 15 and 30 minutes using 1.0 x 10^8 cells per time point. Cells were lysed, protein concentrations in the samples adjusted to 6.0 mg/ml and endogenous TRAF6 was immunoprecipitated using the anti-TRAF6 antibody. Immunoprecipitates and lysates were analysed by immunoblotting using antibodies against TRAF6, TNIK, CD40, phospho-JNK and IκBα.

To accomplish this, CD40 was induced in the B-cell line BL41 by stimulating the cells with recombinant soluble human CD40L. At the indicated time points the stimulation was stopped and the signalling complex was analysed by co-immunoprecipitation studies. Activation of JNK and canonical NF-κB signalling was analysed by monitoring JNK phosphorylation and degradation of I-κBα respectively in cellular lysates. Immunoprecipitation of TRAF2 and TRAF6 revealed that TNIK associated with both TRAFs after 5 minutes of stimulation with CD40L (figure 4.20 A and B, top panels). After 30 minutes TNIK and TRAF6 started to dissociate again (figure 4.20 B, top panel). Both the JNK and the canonical NF-κB pathways were induced after 5 minutes of stimulation which coincided with the binding of TNIK to TRAF2 and TRAF6 (figure 4.20 A and B, bottom panels). This indicates that the association of TNIK with either TRAF protein is an important step in the induction of both pathways.

Immunoprecipitation of TNIK revealed that CD40 interacted with TNIK in non-stimulated cells and rapidly dissociated from the receptor upon induction of CD40 with CD40L (figure 4.21). The interaction of TNIK with TRAF2 and TRAF6 could not be detected in this experiment, which is probably due to technical reasons as the interactions analysed here are hard to detect with endogenous proteins in B-cells.

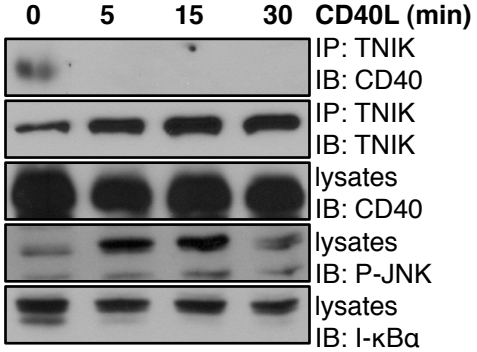


Figure 4.21. TNIK and CD40 interact constitutively in B-cells
BL41 cells were stimulated with 600 ng/ml CD40L for 0, 5, 15 and 30 minutes using 1.5 x 10^8 cells per time point. Cells were lysed, protein concentrations in the samples adjusted to 7 mg/ml and endogenous TNIK was precipitated using the anti-TNIK antibody. Protein complexes and lysates were analysed by immunoblotting using anti-TNIK, anti-CD40, anti-phospho-JNK and anti-I-κBα antibodies. The upper band in the bottom panel is a non-specific band.

Results presented above show that TNIK interacts with TRAF2 and TRAF6 after 5 minutes of stimulation (figure 4.20) but TNIK cannot be detected at the receptor at this time point (4.21, top panel). This suggests the existence of cytoplasmic signalling complexes involving TNIK, TRAF2 and TRAF6, which is in line with findings presented in a previous study describing the

translocation of CD40-induced signalling complexes to the cytoplasm as an essential initiating step in signal transduction by CD40 (Matsuzawa et al, 2008).

4.5. Post-translational modifications within the TNIK signalling complex

4.5.1. The role of phosphorylation

4.5.1.1. The role of the kinase activity of TNIK for the activation of IKKβ

Post-translational modifications of proteins, such as phosphorylation, ubiquitination and SUMOylation play an important role in the activation of many cellular signal transduction pathways. Phosphorylation is the best-studied secondary modification and is involved in a wide variety of cellular processes, including induction of NF-κB signalling (Hunter, 2007).

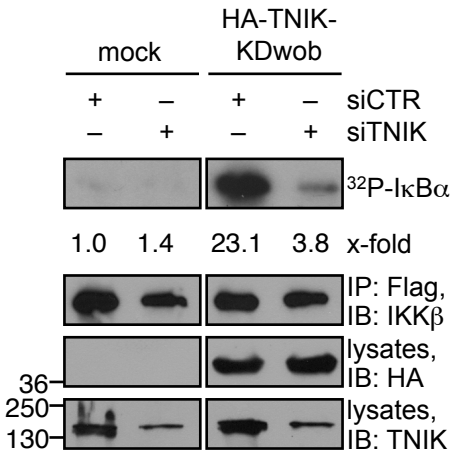


Figure 4.22. The kinase domain of TNIK requires endogenous TNIK to induce IKKβ
HEK293 cells were transfected in 6-wells with 2 μg HA-TNIK-KDwob, 1 μg Flag-IKKβ and either TNIK siRNA or none targeting siRNA. Flag-IKKβ was immunoprecipitated with the Flag(6F7) antibody and IKKβ activity was analysed as GST-IκBα phosphorylation in an immunocomplex kinase assay. GST-IκBα phosphorylation was quantified by phosphoimager analysis and is stated as x-fold induction. Immunoprecipitated Flag-IKKβ and expression of HA-TNIK-KDwob was detected by immunoblotting using anti- IKKβ and anti-HA(3F10) antibodies.

Results presented previously in this thesis have shown that TNIK activated canonical NF-κB signal transduction through its kinase activity (see 4.2.1). Notably, the kinase domain itself was able to activate IKKβ to the same extent as TNIK wildtype (see figures 4.4 B and 4.5). Since IKKβ is activated through phosphorylation (Mercurio et al, 1997) it was tested whether TNIK might accept IKKβ as a substrate. However, experiments using recombinant human IKKβ as a potential substrate for the kinase activity of TNIK provided no evidence that TNIK

phosphorylates IKKβ (data not shown). Additionally, none of the other components of the TNIK-signalling complex, such as TRAF6 or the TAK1-TAB2 complex that are involved in the activation of NF-κB signalling, interacted with the kinase domain but bound to the intermediate and GCKH domains of TNIK (see figures 4.9 and 4.11). Since the GCKH domain was dispensable for the induction of IKKβ (see figure 4.4.B), it was hypothesized that the kinase domain might phosphorylate either TNIK or one of the signalling molecules that bind to TNIK. Autophosphorylation of TNIK has been described (Fu et al, 1999; Taira et al, 2004) and about 40 phosphorylation sites of TNIK have been detected by high throughput screenings and are located mainly within the intermediate domain of TNIK (www.phosphosite.org).

If the hypothesis was true then the exogenously expressed kinase domain should not be able to induce IKKβ in the absence of endogenous TNIK. In order to investigate whether the kinase domain itself is able to activate IKKβ, HEK293D cells were depleted of endogenous TNIK using siRNA and co-transfected with expression vectors for Flag-IKKβ and HA-TNIK-KDwob which expresses the wildtype TNIK kinase domain and is not targeted by the TNIK-specific siRNA due to silent wobble mutations at the nucleotide level. Co-transfection of pRK5 empty vector served as a negative control and is designated as mock transfection (figure 4.22, left panel).

Immunoblot analysis of cellular lysates showed expression of HA-TNIK-KDwob and down-regulation of TNIK by siRNA (figure 4.22, bottom panels). Equal immunoprecipitation of Flag-IKKβ in each sample was confirmed by immunoblotting (figure 4.22, second panel from the top). *In vitro* IKKβ kinase assays were performed, which revealed that in the absence of endogenous TNIK the kinase domain of TNIK lost its ability to activate IKKβ. The knock-down of TNIK reduced induction levels of IKKβ by 87.3 % from 23.1-fold to 3.8-fold (figure 4.22, top panel). Hence, this result demonstrates that the kinase domain depends on endogenous TNIK to induce NF-κB signalling. Thus, the kinase activity of TNIK might either be needed to phosphorylate TNIK itself or one of its interaction partners.

4.5.1.2. Autophosphorylation activity of TNIK is enhanced by LMP1 and CD40

As autophosphorylation of TNIK might be involved in the activation of the canonical NF-κB pathway, it was examined whether LMP1 or CD40 enhance the capacity of TNIK to autophosphorylate. HA-TNIKwt was expressed in the presence or absence of either LMP1 or CD40. Expression of LMP1 and CD40 and cellular lysates and equal immunoprecipitation of HA-TNIKwt was confirmed by immunoblotting (figure 4.23, bottom panels).

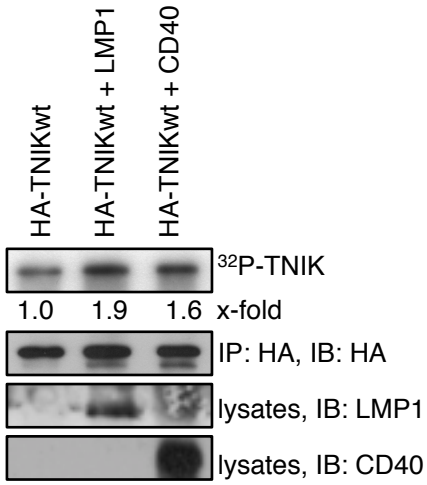


Figure 4.23. LMP1 and CD40 induce the capacity of TNIK to autophosphorylate *in vitro*
HEK293D cells were transfected in 6-wells with 1 μg each of HA-TNIKwt, pSV-LMP1 and CD40. HA-TNIKwt was immunoprecipitated with the anti-HA(12CA5) antibody and subjected to a immunocomplex kinase assay. Autophosphorylation of TNIK was quantified by phosphoimager analysis and is stated as x-fold induction. Immunoprecipiation of HA-TNIKwt and expression of LMP1 and CD40 was analysed by immunoblotting using the anti-HA(12CA5), anti-LMP1(1G6-3) and anti-CD40 antibodies.

The autophosphorylation activity of TNIK was analysed by an *in vitro* kinase assay in which the immunoprecipitated HA-TNIKwt was incubated in the presence of γ-^{32}P-ATP without adding a substrate. This revealed that the phosphorylation activity of TNIK was enhanced by the presence of an inducer. LMP1 caused a 1.9-fold induction and CD40 a 1.6-fold induction of the phosphorylation of TNIK (figure 4.23, top panel).

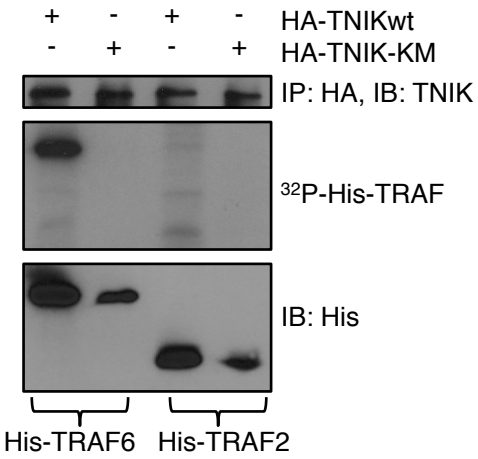
This experiment demonstrates that the autophosphorylation activity of TNIK is enhanced by either LMP1 or CD40 and indicates that autophosphorylation of TNIK is involved in signalling by these receptors.

4.5.1.3. TNIK phosphorylates TRAF6

Since direct phosphorylation of IKKβ by TNIK could not be detected (see 4.5.1.1), it was analysed whether a target for the kinase activity of TNIK other than TNIK itself existed. TRAF2 and TRAF6 both bind to TNIK and are important signalling mediators of LMP1- and CD40-induced signal transduction (see 1.7.2 and 1.7.3). Phosphorylation of TRAF proteins has been detected in high and low throughput screenings (www.phosphosite.org). Phosphorylation of TRAF2 at serine and threonine residues within the N-terminal RING and Zinc finger domains mediates activation of the JNK and canonical NF-κB pathways in response to TNFα and oxidative stress (Blackwell et al, 2009; Thomas et al, 2009; Zhang et

al, 2009). However, the role of TRAF6 phosphorylation has not been revealed so far. In order to investigate whether TRAF2 or TRAF6 constitute potential targets for the kinase activity of TNIK, HEK293D cells were transfected with expression vectors for HA-TNIKwt or the kinase mutant HA-TNIK-KM. The HA-tagged TNIK proteins were immunoprecipitated and subjected to an *in vitro* TNIK kinase assay using the purified His-tagged TRAF-domains of TRAF2 and TRAF6, His-TRAF2(311-501) and His-TRAF6(310-522), provided by Fabian Giehler, as potential substrates.

A.



B.

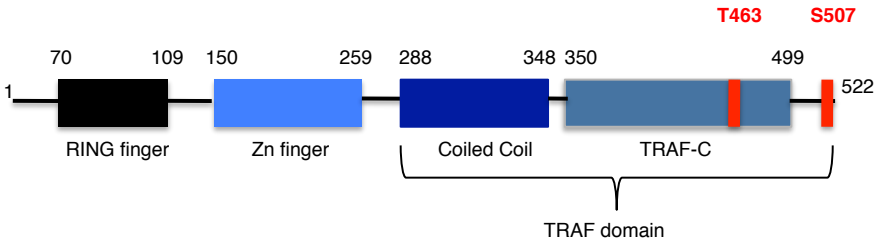


Figure 4.24. TNIK phosphorylates TRAF6
A. Phosphorylation of TRAF6, but not TRAF2 by TNIK
HEK293D cells were transfected in 6-wells with 2 µg each of HA-TNIKwt and HA-TNIK-KM. HA-TNIK was immunoprecipitated with the anti-HA(12CA5) antibody and subjected to an immunocomplex kinase assay using His-TRAF6(310-522) and His-TRAF2(311-501) as substrates. Substrate phosphorylation was analysed by autoradiography. Equal amounts of substrate and HA-TNIK in the samples was confirmed by immunoblotting using anti-His and anti-TNIK antibodies respectively.
B. Schematic diagram of TRAF6
The numbers represent amino acids. The two detected phosphorylation sites targeted by TNIK are marked in red.

Strikingly, TNIK accepted TRAF6 as a target and a high amount of phosphorylated His-TRAF6(310-522) was detected. In contrast, His-TRAF2(311-501) was not phosphorylated.

HA-TNIK-KM served as a negative control and did not phosphorylate either substrate (figure 4.24.A). This experiment revealed TRAF6 as a novel target of the kinase activity of TNIK and thus makes TNIK the first known kinase that phosphorylates TRAF6.

In order to discover at which sites within the TRAF6 protein phosphorylation takes place a liquid chromatography-mass spectrometry (LC-MS) approach was pursued in co-operation with Dr. Hakan Sarioglu from the proteomics facility of the Helmholtz Center Munich. HA-TNIKwt and HA-TNIK-KM were transiently expressed in HEK293D cells and subjected to immunoprecipitation and an in vitro kinase assay in the presence of cold ATP and His-tagged TRAF6(310-522) as a substrate. His-TRAF6(310-522) was isolated with Ni^{2+}-agarose beads and then forwarded to the proteomics facility for subsequent trypsin digestion and analysis of phosphorylation sites using the Proteome Discoverer software (Thermo Scientific).

This revealed two phosphorylation sites within the C-terminus of TRAF6 at threonine 463 and serine 507 (figure 4.24.B). The site at serine 507 has been detected recently by mass spectrometry in a T-cell line (www.phosphosite.org). However, the biological function of phosphorylation of TRAF6 at either site has not been revealed so far and will be the subject of future studies.

4.5.2. The role of ubiquitination

4.5.2.1. TNIK mediates auto-ubiquitination of TRAF6

Ubiquitination of proteins has received increasing attention in the past years and is critical for many cellular functions including the activation of NF-κB and MAPK signalling pathways. Auto-ubiquitination of TRAF6 facilitates the recruitment of the TAK1-TAB complex to receptors such as IL1R, TNFR or TLR, and mediates the activation of the JNK and NF-κB pathways. It is however unclear how auto-ubiquitination of TRAF6 is induced by upstream receptors and the mechanisms leading to auto-ubiquitination of TRAF6 have not been studied in the context of LMP1- or CD40-induced signalling. The involvement of so far unknown factors in this process has been suggested in past publications (Adhikari et al, 2007). The fact that TRAF6 interacts with TNIK and that this interaction plays an important role in signalling by LMP1 and CD40 suggested a potential role of TNIK in the auto-ubiquitination of TRAF6. A previous study showed that ubiquitination of TRAF6 is induced by LMP1 (Song et al, 2006). It has been suggested that ubiquitination of TRAF6 also occurs in the context of CD40-induced signalling. However, this has not been shown to date.

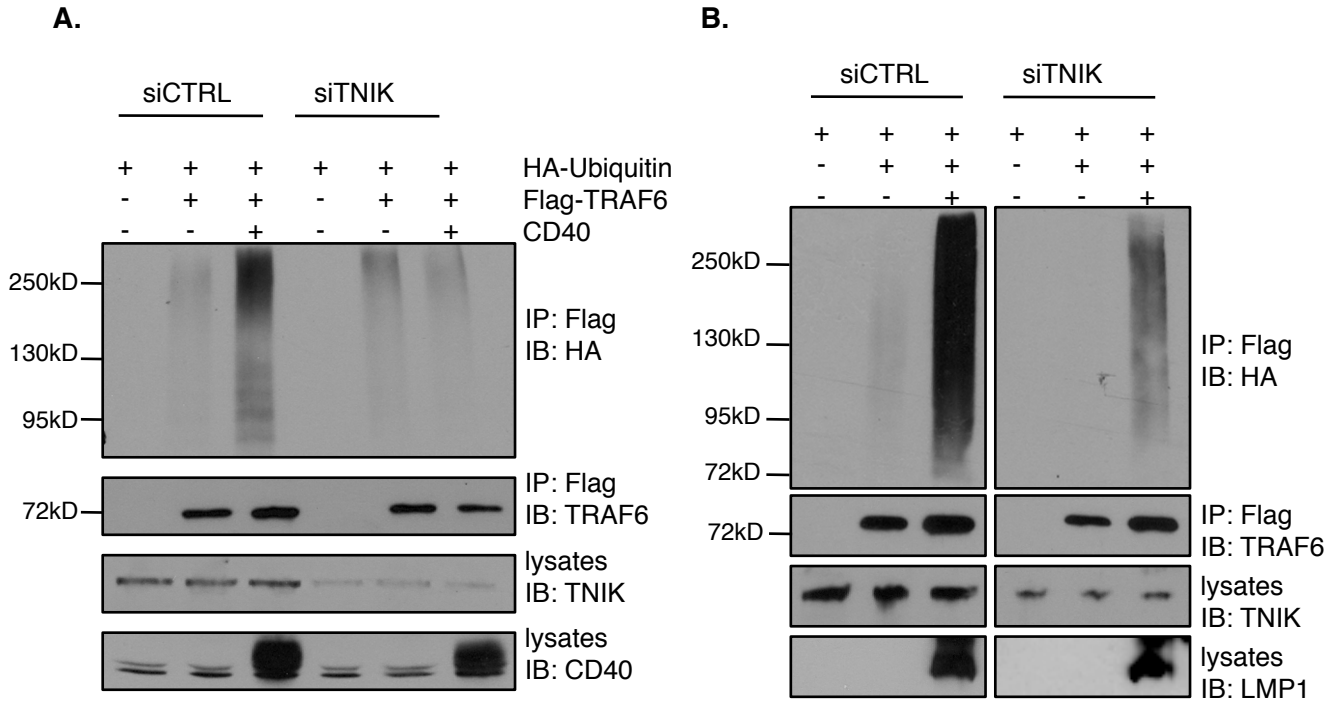


Figure 4.25. TNIK is essential for LMP1- and CD40-induced auto-ubiquitination of TRAF6
A. TNIK mediates auto-ubiquitination of TRAF6 induced by CD40
HEK293D cells were transfected in 6-wells with siTNIK or non-targeting siCTRL. Subsequently, cells were co-transfected with 1 μg HA-ubiquitin, 0.5 μg Flag-TRAF6wt and 1 μg CD40. Flag-TRAF6 was immunoprecipitated with the anti-Flag(6F7) antibody and immunoprecipitates and lysates were analysed by immunoblotting using the anti-HA(12CA5), anti-TRAF6, anti-TNIK and anti-CD40 antibodies as indicated.
B. TNIK is essential for auto-ubiquitination induced by LMP1
The experiment was conducted as described in A. but cells were transfected with 1 μg HA-LMP1wt instead of CD40. Immunoprecipitates and lysates were analyzed by immunoblotting using the anti-HA(12CA5), anti-TRAF6, anti-TNIK and anti-LMP1(1G6-3) antibodies as indicated.

A potential role of TNIK in the ubiquitination of TRAF6 was investigated by co-transfecting HEK293D cells with expression vectors for HA-ubiquitin, Flag-TRAF6 and either LMP1 or CD40 together with TNIK-specific siRNA or non-targeting control siRNA. Expression of LMP1 and CD40 as well as down-regulation of TNIK by siRNA was detected by immunoblotting (figure 4.25.A and B, bottom panels). Flag-TRAF6 was immunoprecipitated and ubiquitination was detected by immunoblotting using an antibody directed against the HA-tag of HA-ubiquitin. In the presence of control siRNA both LMP1 and CD40 significantly induced auto-ubiquitination of TRAF6, which results in HA-ubiquitin chains that can be seen as a smear of high molecular weight bands (figure 4.25.A and B, top panels). The ubiquitination of TRAF6 that was detected here represents auto-ubiquitination of TRAF6, as ubiquitination of a TRAF6(C70A) mutant that harbours a point mutation within its RING-finger domain and is devoid of ubiquitin ligase activity is not detectable in this type of assay (Andrea Obermeier, personal communication). Thus the contribution of a different ubiquitin ligase can be

excluded. Strikingly, the knockdown of endogenous TNIK completely abolished the ability of either receptor to induce auto-ubiquitination of TRAF6 (figure 4.25). Thus this experiment demonstrates an essential role of TNIK for auto-ubiquitination of TRAF6. The exact contribution of ubiquitination of TRAF6 to the induction of NF-κB and JNK signalling is currently under debate and a necessary but insufficient role of TRAF6 auto-ubiquitination in the activation of NF-κB has been suggested (Lamothe et al, 2007; Megas et al, 2011; Walsh et al, 2008). However, the role of TRAF6 ubiquitination for LMP1- and CD40-induced signalling has not been studied to date.

4.5.2.2. The kinase activity of TNIK inhibits ubiquitination of TRAF6

Having discovered a crucial function of TNIK as a mediator of auto-ubiquitination of TRAF6, it was analysed whether auto-ubiquitination of TRAF6 is induced by the overexpression of TNIK. HEK293D cells were co-transfected with HA-ubiquitin, Flag-TRAF6 and HA-TNIKwt, as well as with HA-TNIK-KM, HA-TNIKΔKD and the intermediate domain of TNIK as potential inducers of TRAF6 auto-ubiquitination.

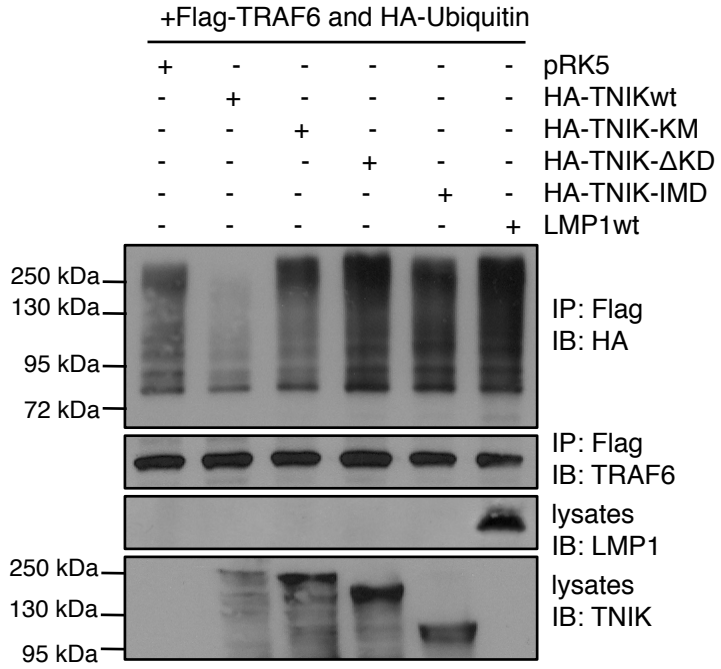


Figure 4.26. The kinase activity of TNIK inhibits ubiquitination of TRAF6
HEK293D cells were transfected in 10 cm cell culture dishes with 0.5 μg each of HA-ubiquitin and Flag-TRAF6wt as well as 1 μg each of the HA-TNIK constructs and pSV-LMP1. Flag-TRAF6wt was immunoprecipitated using the anti-Flag(6F7) antibody and immunoprecipitates and lysates were analysed by immunoblotting with the anti-HA(3F10), anti-TRAF6 and anti-LMP1(1G6-3) antibodies.

Co-expression of LMP1wt served as a positive control. Expression of the HA-TNIK constructs and LMP1 was detected by immunoblotting of cellular lysates (figure 4.26, bottom panels). Flag-TRAF6 was immunoprecipitated and analysed by immunoblotting. Surprisingly, this revealed that the co-expression of HA-TNIKwt exerted a negative effect on the ubiquitination of TRAF6. In contrast, co-expression of HA-TNIK-KM, HA-TNIK-ΔKD and HA-TNIK-IMD that each lack kinase activity induced ubiquitination of TRAF6 to a similar extent as LMP1wt (figure 4.26, upper panel) which suggests a negative effect of the kinase activity of TNIK on the ubiquitination of TRAF6. The co-expression of the intermediate domain of TNIK was sufficient to induce ubiquitination of TRAF6. This suggests that the interaction between the intermediate domain of TNIK and TRAF6 which was shown by co-immunoprecipitation studies presented in this thesis (see 4.3.1.3) and experiments conducted *in vitro* with purified proteins (Fabian Giehler, personal communication) induces ubiquitination of TRAF6.

Post-translational modifications of proteins often act in combination and crosstalk between phosphorylation and ubiquitination occurs at several levels with phosphorylation positively or negatively regulating ubiquitination events (Hunter, 2007). This experiment shows that the intermediate domain of TNIK is sufficient to induce ubiquitination of TRAF6, whereas the kinase activity of TNIK exerts a negative effect. This suggests that the phosphorylation of TRAF6 might be involved in a negative feedback mechanism to shut down signalling events mediated by ubiquitinated TRAF6.

4.5.3. The role of SUMOylation

4.5.3.1. TNIK is SUMOylated in response to LMP1 and CD40 activity

Post-translational modification of proteins by SUMOylation is an important regulator of protein function and is involved in a wide range of cellular processes, including the regulation of NF-κB and JNK signalling pathways. SUMO is conjugated to a lysine residue of a target protein within the consensus site ΨKxE, where Ψ is a large hydrophobic amino acid, K is a lysine residue, x can be any amino acid and E is a glutamic acid residue (Geiss-Friedlander & Melchior, 2007). Given the important role of SUMOylation in a large number of cellular processes, computational prediction of SUMOylation sites has received much attention in the past years. The SUMOsp 2.0. algorithm was used to predict SUMOylation sites within TNIK (Ren et al, 2009) and revealed 6 potential SUMOylation sites (figure 4.27.A and table 4.1).

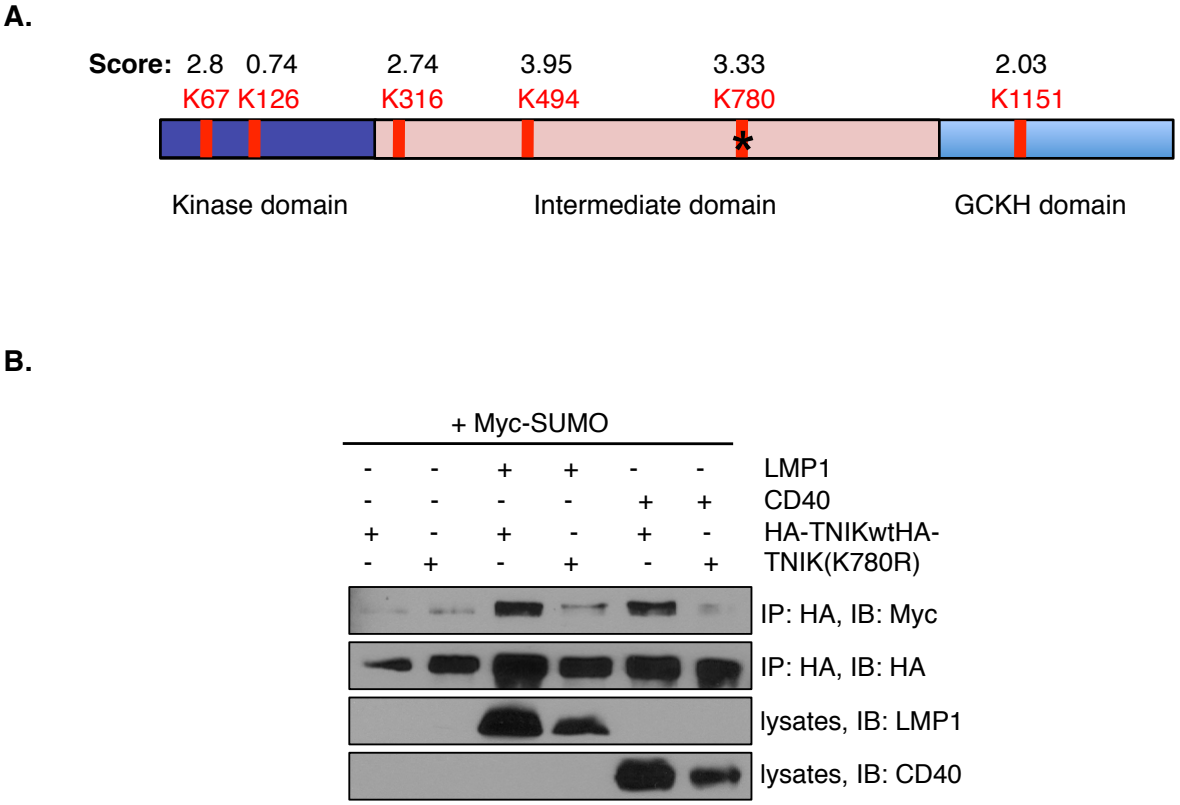


Figure 4.27. SUMOylation of TNIK
A. Potential SUMOylation sites within TNIK
Schematic diagram of TNIKwt. Lysine residues within potential SUMOylation sites (red) and the corresponding scores (black) as predicted by the SUMOsp 2.0. algorithm (Ren et al, 2009) are marked. The asterisk indicates the K708R mutation.
B.TNIK is SUMOylated in response to LMP1 and CD40 activity
HEK293D cells were transfected in 6-wells with 1 μg Myc-SUMO, 1 μg pSV-LMP1, 1.5 μg CD40 and 0.5 μg each of HA-TNIKwt and HA-TNIK(K780R). HA-TNIK was precipitated using the anti-HA(12CA5) antibody and SUMOylation was analysed by immunoblotting for Myc. Expression of LMP1 and CD40 in lysates was analysed by immunoblotting using the anti-LMP1(1G6-3) and anti-CD40 antibodies, as indicated.

Another search was conducted using the eukaryotic linear motif (ELM) resource that predicts potential sites for post translational protein modifications not only according to sequence prediction but additionally takes into account the structural and biochemical context of a site (elm.eu.org). Functional sites must be accessible and thus cannot lie inside globular domains but reside in exposed loops on a protein. This second search predicted only one potential SUMOylation site within the intermediate domain of TNIK at K780. Therefore the site at K780 was chosen as an initial target for mutational analysis of SUMOylation of TNIK.

In order to investigate whether SUMOylation of TNIK occurs in the cell and whether this modification plays a role in signalling by LMP1 and CD40, HEK293D cells were transfected with expression vectors for Myc-SUMO, HA-TNIKwt and the TNIK-mutant HA-TNIK(K780R) as well as LMP1 and CD40 as potential inducers. The HA-TNIK constructs were

immunoprecipitated and expression of LMP1 and CD40 as well as comparable precipitation of TNIK was confirmed by immunoblotting.

position	peptide	score
67	EEI**K$_{67}$**QEI	2.853
126	NTL**K$_{126}$**EEW	0.739
316	RGE**K$_{316}$**DET	2.735
494	RQL**K$_{494}$**QER	3.948
780	AKV**K$_{780}$**PEE	3.332
1151	KVV**K$_{1151}$**YER	2.033

Table 4.1. Potential SUMOylation sites within TNIK as predicted by the SUMO sp 2.0. algorithm (Ren et al, 2009)

Analysis of immunoprecipitations by immunoblotting for Myc showed that while no SUMOylation could be detected in the absence of an inducer, both LMP1 and CD40 led to a SUMOylation of TNIKwt. In contrast, mutation of K780 abolished the SUMOylation of TNIK (figure 4.27.B). This experiment thus revealed that modification of TNIK by SUMOylation takes place in response to LMP1 and CD40 activity and that K780 is a major SUMOylation site within TNIK.

4.5.3.2. SUMOylation of TNIK affects activation of the canonical NF-κB pathway

SUMOylation can have various effects on the target protein including a change in the activity of the protein and is often involved in the modulation of cellular signalling pathways. To analyse whether SUMOylation of TNIK has an effect on its ability to activate the canonical NF-κB pathway, HEK293D cells were co-transfected with Flag-IKKβ and HA-TNIKwt as well as the SUMO-mutant HA-TNIK(K780R). Expression of the HA-TNIK constructs and equal immunoprecipitation of Flag-IKKβ was confirmed by immunoblot analysis of cellular lysates and immunoprecipitates respectively (figure 4.28). IKKβ kinase assays were subsequently performed and showed that HA-TNIKwt caused a 1.6-fold induction of IKKβ. Mutation of the SUMOylation site at K780 completely abolished the ability of TNIK to activate IKKβ (figure 4.28).

Hence, the experiment showed that SUMOylation of TNIK at K780 is a new secondary modification of TNIK that is involved in the ability of TNIK to activate canonical NF-κB signalling.

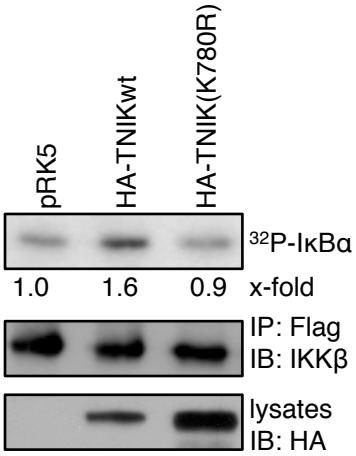


Figure 4.28. SUMOylation of TNIK affects its abliity to activate the canonical NF-κB pathway
HEK293D cells were transfected in 6-wells with 1 μg Flag-IKKβ and 2 μg each of HA-TNIKwt and HA-TNIK(K780R). Flag-IKKβ was immunoprecipitated and activation of IKKβ was analysed by an immunocomplex kinase assay. Phosphorylation of GST-IκBα was quantified by phosphoimager analysis and is stated as x-fold induction. Precipitation of Flag-IKKβ and expression of HA-TNIKwt and HA-TNIK(K780R) in lysates was analyzed by immunoblotting using the anti-IKKβ and anti-HA(12CA5) antibodies.

Future studies will address the mechanism underlying the involvement of SUMOylation of TNIK in the activation of the canonical NF-κB pathway. It is possible that SUMOylation of TNIK leads to a conformational change and activation of TNIK or SUMOylation might facilitate the binding of downstream signalling molecules that are involved in NF-κB signalling.

5. Discussion

LMP1 is the primary oncogene of EBV and a constitutively-active viral pseudoreceptor that mimics the cellular CD40 receptor in an amplified and sustained manner and induces an efficient signalling complex at its C-terminal domain (see 1.2.2). However, despite intense research the composition of the signalling complex as well as the mechanism through which induction of the signalling pathways is organized, is still poorly understood. The existence of still unidentified components of the signalling complex has thus been hypothesized. In order to identify new interaction partners of LMP1, our laboratory conducted a functional proteomics screen that revealed the germinal center kinase family member TNIK as a new interaction partner of LMP1 that mediates signalling by the CTAR2 domain of LMP1. TNIK is an ubiquitously expressed serine/threonine protein kinase that activates the JNK pathway upon overexpression (Fu et al, 1999). Various functions have been described for TNIK, which seem to be cell-type specific to a certain extent. TNIK plays an important role during embryonic development, regulates neurite growth in the brain, is involved in the organization of the cytoskeleton in epithelial cells and activates Wnt target genes in intestinal crypt cells (Fu et al, 1999; Kawabe et al, 2010; Mahmoudi et al, 2009; Satow et al, 2010; Taira et al, 2004). However, many open questions regarding TNIK remain. The physiological role of TNIK in the haematopoietic system, a receptor engaging TNIK for JNK activation and the role of the interaction with TRAF2 remain largely unknown.

It was the aim of this thesis to characterize the role of TNIK in the signal transduction of LMP1 and its cellular counterpart, the co-stimulatory CD40 receptor. This was achieved by studying LMP1- and CD40-induced signal transduction in cells depleted of endogenous TNIK by TNIK-specific siRNA or shRNA. The mechanisms of TNIK-mediated signal transduction as well as the composition of the signalling complexes at the intracellular domains of LMP1 and CD40 was investigated by co-immunoprecipitation studies conducted with HEK293D cells, EBV transformed LCLs and the EBV-negative B-cell line BL41. Finally, the role of the post-translational protein modifications, phosphorylation, ubiquitination and SUMOylation for TNIK-mediated signalling by LMP1 and CD40 was analysed.

5.1. The role of TNIK in LMP1-induced signalling

When the germinal center kinase family member TNIK was first revealed as a new interaction partner of the viral oncoprotein LMP1, hardly anything was known about the biological functions of this kinase. TNIK had initially been discovered in a yeast two-hybrid screen for proteins that interact with TRAF2 and Nck (Fu et al, 1999). TNIK activated the JNK pathway upon overexpression and had an inhibitory function on cell spreading by inducing the disassembly of actin fibres (Fu et al, 1999).

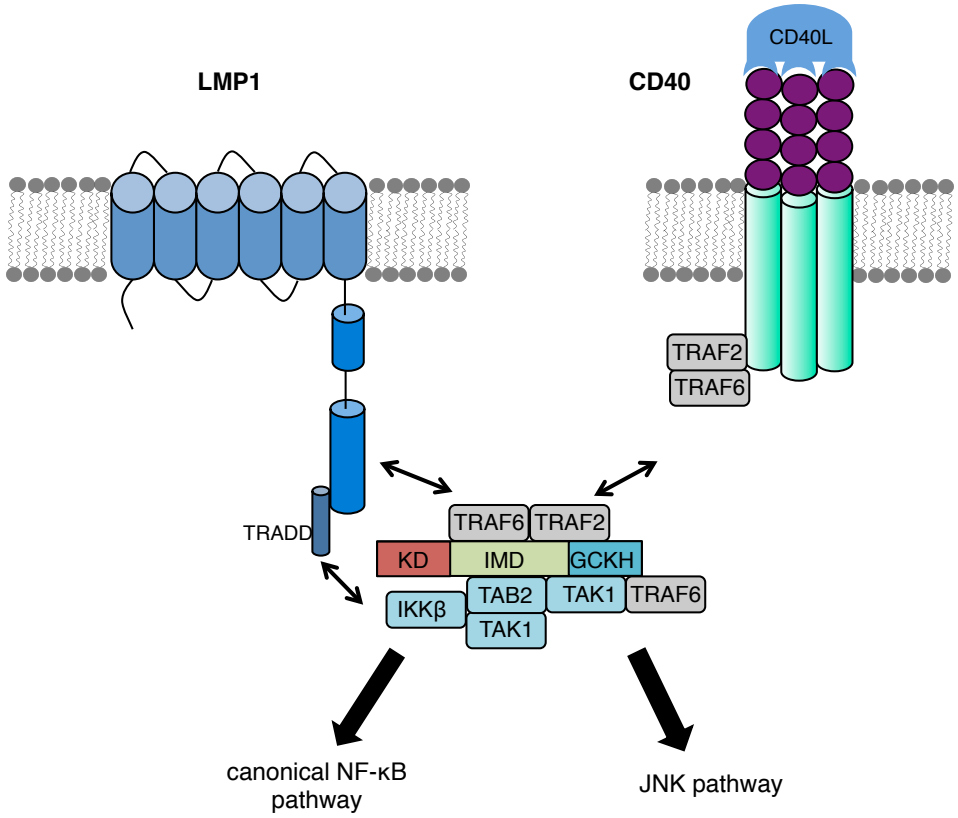


Figure 5.1. Schematic overview of the role of TNIK in signalling by LMP1 and CD40
TNIK interacts with TRAF6, TRAF2, TAK1, TAB2 and IKKβ and mediates activation of the canonical NF-κB and JNK pathways by LMP1 and CD40. The kinase domain of TNIK mediates activation of IKKβ and induction of the canonical NF-κB pathway, while the GCKH domain facilitates induction of JNK signalling. In the context of LMP1-induced signalling TRADD stabilizes the signalling complex and thus additionally enhances activation of the NF-κB pathway. The interaction between TNIK and the CTAR2 domain of LMP1 is mediated by TRAF6. It has not been investigated to date how TNIK interacts with CD40. Since CD40 induces the recruitment of TRAF2 and TRAF6 to TNIK, interaction between TNIK and CD40 might be mediated by these adapter proteins. Please confer to the text for a more detailed explanation.

The high expression level of TNIK in cells of the hematopoietic system of mice (Dr. Vigo Heissmeyer, Katharina Vogel, personal communication) strongly suggested a role of TNIK in

the lymphoid compartment. Data presented in this thesis demonstrate TNIK to be a novel key player in canonical NF-κB and JNK signalling by the viral oncoprotein LMP1 and the B-cell surface receptor CD40 (figure 5.1). This study reveals for the first time a physiological role of TNIK in lymphocytes and indicates a function of TNIK as a novel oncoprotein for EBV and CD40-associated malignancies.

The interaction between TNIK and LMP1 that had initially been discovered in our laboratory by a functional proteomics screen was verified in this thesis by co-immunoprecipitation studies with endogenous proteins in EBV-transformed LMP1-expressing LCLs. Experiments conducted subsequently in HEK293D cells in combination with results obtained previously in this laboratory (Dr. Anna Shkoda, personal communication) showed that TNIK interacts with the CTAR2 domain of LMP1 and that the CTAR2 domain is both necessary and sufficient to mediate this interaction. The next step was to analyse a potential role of TNIK for LMP1-induced signal transduction. Two important signal transduction pathways initiated at the CTAR2 domain are the JNK and canonical NF-κB signal transduction pathways (see 1.2.2). To analyse a potential role of TNIK in these signalling pathways, JNK and IKKβ kinase assays were conducted in HEK293D cells depleted of endogenous TNIK by siRNA.

Results of JNK kinase assays showed that the knockdown of TNIK resulted in a significant reduction of LMP1-induced activation of JNK, thus revealing TNIK as an essential mediator of the JNK pathway by LMP1. This discovery makes LMP1 the first known receptor to engage TNIK for the activation of JNK signalling.

Results of IKKβ kinase assays showed that the knockdown of TNIK resulted in a decrease in LMP1-mediated activation of IKKβ. However, in a parallel experiment the down-regulation of TNIK almost completely blocked IKKβ activation by LMP1 in HEK293D cells (Dr. Anna Shkoda, personal communication). In siRNA experiments conducted for this thesis the effects of TNIK knockdown on IKKβ activation by LMP1 were less pronounced but always present which might be due to a less efficient knockdown of TNIK in my set of experiments. The full body of evidence obtained in our laboratory strongly suggests an important role of TNIK in activation of the canonical NF-κB pathway by LMP1 because the knockdown of TNIK inhibited degradation of IκBα in LCLs, the overexpression of the kinase-mutant, TNIK-KM, efficiently blocked CTAR2-mediated activation of NFκB by LMP1 (Dr. Anna Shoda, personal communication) and results presented in this thesis showed that TNIK overexpression activates IKKβ.

To gain further insight into the role of TNIK for canonical NF-κB signalling by LMP1 the effect of TNIK on the nuclear translocations of NF-κB subunits was investigated in HEK293D cells. The CTAR2 domain of LMP1 induces the canonical NF-κB pathway, which leads to a

translocation of p65-p50 NF-κB dimers into the nucleus (Herrero et al, 1995; Huen et al, 1995; Mitchell & Sugden, 1995). The CTAR1 domain activates the non-canonical NF-κB pathway and increases nuclear levels of the p52-RelB complex (Atkinson et al, 2003) (see 1.8). In epithelial cells CTAR1 activity can additionally result in nuclear translocations of the alternative p50-p50, p52-p50 and p52-p65 NF-κB dimers (Paine et al, 1995; Song & Kang, 2010; Thornburg & Raab-Traub, 2007b), which does not allow a clear discrimination between CTAR1 and CTAR2 activity in epithelial cells on the basis of the levels of canonical NF-κB subunits in the nucleus. However, the nuclear translocation of p52 and RelB can be exclusively allocated to the activation of the non-canonical NF-κB pathway by the CTAR1 domain of LMP1 (Atkinson et al, 2003). Since the knockdown of TNIK exerted no effect on the ability of LMP1 to translocate p52 and RelB, a role of TNIK for activation of CTAR1-induced non-canonical signalling can be excluded. The knockdown of TNIK completely abolished the ability of LMP1 to induce nuclear translocation of the NF-κB subunit p65 indicating an essential role of TNIK for the activation of the canonical NF-κB pathway by LMP1. No effect on the nuclear translocation of p50 was detected. This might be attributed to increased nuclear levels of alternative p50-containing NF-κB complexes in response to CTAR1 activity (Paine et al, 1995; Song & Kang, 2010; Thornburg et al, 2003; Thornburg & Raab-Traub, 2007b). Future experiments which engage the overexpression of LMP1 mutants to analyse the effect of the knockdown of TNIK on the contribution of either CTAR to the nuclear translocation of individual NF-κB subunits in the cells used here will help to clarify the findings obtained in this study.

The functions of TNIK for the activation of the canonical NF-κB pathway were further analysed by conducting experiments with overexpressed TNIK. Overexpression of TNIK induced IKKβ activity in kinase assays, but exclusively increased nuclear levels of p65 without affecting nuclear levels of any of the other NF-κB subunits. While p65-p65 NF-κB complexes occur in the cell and are translocated into the nucleus upon IKKβ-induced IκBα degradation, these complexes are much less abundant than the p65-p50 complexes and have a lower affinity for IκBα (Hoffmann et al, 2006; Malek et al, 2003). Hence, these results suggest that TNIK does not solely act as an upstream inducer of IKKβ, which typically induces degradation of I-κBα and subsequent nuclear translocation of p65-p50 subunits, but additionally enhances the nuclear translocation of p65 downstream of IKKβ. As overexpression of the kinase domain of TNIK increased nuclear levels of p65 to the same extent as TNIK wild-type, phosphorylation of p65 by TNIK might be involved. p65 is phosphorylated at several sites by a number of different kinases which can result in a nuclear

translocation of p65 independently of the IKK complex and I-κBα degradation (Gewurz et al, 2012; He et al, 2006; Kasyapa et al, 2009; Kobayashi et al, 2003) (see 1.6.3).

In the context of Wnt signal transduction TNIK is translocated into the nucleus and phosphorylates and thus activates the transcription factor TCF4 (Mahmoudi et al, 2009; Shitashige et al, 2010). It is thus conceivable that TNIK enhances activation of the canonical NF-κB pathway in a similar manner by phosphorylating the NF-κB subunit p65 either in the cytoplasm to induce nuclear translocation and enhance transcriptional activity of p65 or in the nucleus to prevent nuclear export of p65 (Cao & Karin, 2003; He et al, 2006; Kasyapa et al, 2009; Kobayashi et al, 2003; Lee et al, 1999a). Future experiments will have to show, whether TNIK phosphorylates p65 and whether this takes place in the cytoplasm or in the nucleus. The co-expression of TNIK and LMP1 in MEF cells did not result in a nuclear translocation of TNIK in immunofluorescence studies, which suggests that potential effects of TNIK on the nuclear translocation of p65 occur in the cytoplasm.

The hypothesis presented above would involve a role for TNIK both upstream of IKKβ, where it functions as an organizer of the LMP1-induced signalling complex that leads to the activation of IKKβ as well as downstream of IKKβ, where TNIK additionally enhances the nuclear translocation of p65 likely by phosphorylation of p65.

Results obtained by overexpression of TNIK mutants in HEK293D cells gave further insight into the mechanism of TNIK-induced activation of the canonical NF-κB and JNK signalling pathways. These experiments revealed that different domains of TNIK mediate activation of IKKβ and JNK respectively with the kinase domain inducing IKKβ and the GCKH domain inducing JNK. While activation of JNK upon overexpression of the GCKH domain of TNIK has been described in a previous study (Fu et al, 1999), this thesis shows for the first time that overexpression of TNIK mediates activation of IKKβ and thus reveals TNIK as a novel inducer of the canonical NF-κB pathway.

Experiments conducted with purified proteins in a previous study showed that TAK1 phosphorylates and thus activates IKKβ *in vitro* in the presence of TAB1/TAB2 and the ubiquitin conjugating enzyme complex Ubc13/Uev1A and the ubiquitin ligase TRAF6 without the need of TNIK (Wang et al, 2001). However, protein concentrations in a cell are much lower than in a test tube using purified proteins. Therefore, it is likely that the activation of IKKβ in the cell additionally requires TNIK that binds the individual signalling molecules and brings them into close proximity of each other and thus facilitates the activation of IKKβ.

The fact that distinct domains of TNIK mediate activation of canonical NF-κB and JNK signalling indicates that the two pathways bifurcate at the level of TNIK. Hence, these results

show that TNIK organizes the signalling complex at the CTAR2 domain and orchestrates activation of the canonical NF-κB and JNK pathways thus contributing towards the understanding of CTAR2-induced signal transduction (figure 5.1).

5.2. The LMP1-induced TNIK signalling complex

Despite the vast number of research papers dealing with LMP1-induced signal transduction, the exact composition of the signalling complex at the C-terminus is still poorly understood (see 1.8). Results presented in this thesis demonstrate that TNIK interacts with the major signalling molecules that are required for the activation of the JNK and canonical NF-κB pathways. TRAF6, TAK1 and IKKβ were identified as new interaction partners (figure 5.1).

TRAF6 is an important signalling mediator of the two major pathways that are initiated at the CTAR2 domain of LMP1: the canonical NF-κB pathway and the JNK pathway (Kieser, 2007; Luftig et al, 2003; Schultheiss et al, 2001; Wan et al, 2004; Wu et al, 2006). Results presented in this study identified TRAF6 as a new interaction partner of TNIK. TNIK and TRAF6 are associated at a low level constitutively and co-expression of LMP1 significantly enhanced the interaction between TNIK and TRAF6, which indicates an important role of this interaction for LMP1-induced signalling. A role of this interaction was revealed by immunofluorescence studies conducted in MEF cells demonstrating that TRAF6 mediates the interaction between TNIK and LMP1. Thus, this finding shows that TRAF6 plays an essential role to mediate the interaction between TNIK and LMP1 and is in line with the fact that both proteins play central roles in CTAR2-induced signalling.

Additionally, while previous experiments showed that TNIK and LMP1 interacted, the question whether this interaction occurs in a direct or indirect manner had not been addressed before. Results presented in the present study reveal for the first time that the two proteins interact in an indirect manner via TRAF6. This finding is in line with the concept of TRAF proteins typically coupling signalling molecules to receptors (see 1.7.1).

A more detailed analysis of the interaction between TNIK and TRAF6 by co-immunoprecipitation experiments in HEK293D cells showed that TRAF6 mainly binds to the GCKH domain of TNIK, while only a very low amount of TRAF6 interacts with the intermediate domain of TNIK. This shows in contrast to experiments conducted in our laboratory using purified proteins to analyse protein-protein interactions *in vitro* that TRAF6 specifically interacts with the intermediate domain of TNIK, while no interaction between TNIK and the GCKH or kinase domain was detected (Fabian Giehler, personal

communication). Hence, this finding shows that a direct interaction between the intermediate domain of TNIK and TRAF6 takes place. However, while the *in vitro* experiments used a TRAF6 protein comprising only the C-terminal domain of TRAF6, the experiment conducted in this thesis analysed the interaction between TRAF6 wildtype and TNIK. Hence, the different binding patterns might either be attributed to binding mediated by the N-terminal RING and Zn-finger domains of TRAF6 or by other proteins that are present in the cell but absent in the *in vitro* experiments.

Co-immunoprecipiation experiments analysing the interaction between the TRAF6 deletion-mutant TRAF6(274-522) which consists of the C-terminal TRAF domain of TRAF6 and the individual TNIK domains, gave further insight into the TNIK-TRAF6 interaction. TRAF6(274-522) bound to the intermediate and GCKH domains of TNIK with equal affinities. Since TRAF6(274-522) lacks the N-terminal domain that confers ubiquitin ligase activity to TRAF6, the differences in the binding patterns of TRAF6 wild-type and TRAF6(274-522) might be due to ubiquitination events. The binding of TRAF6 to the GCKH domain might thus be facilitated either by binding of TRAF6 in a mechanism involving auto-ubiquitination or by inducing the ubiquitination of a different signalling molecule to which TRAF6 then binds (see 5.5.2). A likely candidate in this context is TAK1. TRAF6 ubiquitinates TAK1 in response to stimuli derived by receptors of the Il-1R, TLR or TNFR families and ubiquitination of TAK1 is a prerequisite for the induction of JNK and NF-κB signalling (Fan et al, 2010; Hamidi et al, 2012; Yamazaki et al, 2009). Results presented in this thesis additionally show that TNIK mediates auto-ubiquitination of TRAF6 (discussed in 5.5.2). Taken together, these findings suggest that the initial interaction between TNIK and TRAF6 might be direct and occur at the intermediate domain. Subsequent activation of TRAF6 by TNIK then induces its auto-ubiquitination and results in a rearrangement within the complex that leads to the interaction of TRAF6 with the GCKH domain of TNIK.

TAK1 was also identified as a new interaction partner of TNIK that mainly interacted with the GCKH domain of TNIK, while the interaction between TAK1 and the intermediate domain of TNIK was significantly weaker. This binding pattern is thus reminiscent of the TRAF6-TNIK interaction shown in this thesis. It is thus probable that the interaction of both proteins with TNIK brings the two proteins into close proximity of each other and facilitates ubiquitination and thus activation of TAK1 by TRAF6. Up to now it has not been examined, whether TNIK and TAK1 can interact directly as is the case with TNIK and TRAF6. It is possible that the weaker interaction that was observed between TAK1 and the intermediate domain of TNIK constitutes an indirect interaction. Data obtained in this laboratory showed that LMP1 induces an interaction between the intermediate domain of TNIK and the TAK1 binding protein TAB2

(Dr. Anna Shkoda, personal communication). As TAB2 facilitates the binding of TAK1 to upstream interaction partners, usually TRAF6 (Kanayama et al, 2004), it is possible that TAK1 binds via TAB2 to the intermediate domain of TNIK. It remains to be shown whether the interaction between TAK1 and the GCKH domain is direct or indirect. A common feature, however, of members of the germinal center kinase family is that they bind MAP3K through their GCKH domains and that this interaction leads to an activation of the MAP3K (Dan et al, 2001). To date it has not been elucidated how the activation of the MAP3K by the binding to the GCKH domain of GCK family members is conferred. It has been suggested that the interaction with the GCKH domain leads to a conformational change that induces auto-phosphorylation of the MAP3K which is thus activated (Dan et al, 2001). Recent findings have shown that the ubiquitination of TAK1 by TRAF6 is essential for its activation (Fan et al, 2010; Hamidi et al, 2012) and suggests that in the case of TNIK it is the binding and thus close proximity of both TAK1 and TRAF6 that lead to the activation of TAK1.

Additionally, experiments conducted with HEK293D cells depleted of endogenous TNIK by shRNA showed that TNIK facilitates binding of TAK1 and TAB2 to LMP1. This provides additional evidence that TNIK lies upstream of the TAK1-TAB2 complex and indicates a role of TNIK as a complex organizer and adapter protein that facilitates binding of downstream interaction partners to the CTAR2 domain. This finding thus contributes towards the understanding of complex formation at the CTAR2 domain.

In contrast to TRAF6 and TAB2, TAK1 interacted constitutively with TNIK and co-expression of LMP1 did not significantly enhance this interaction. This makes TAK1 the only one of the studied interaction partners of TNIK that is pre-associated with TNIK.

IKKβ was also identified as a new interaction partner of TNIK. TNIK and IKKβ did not interact constitutively but an interaction was readily detected in the presence of LMP1. As the establishment of a co-immunoprecipitation protocol for the detection of the interaction between TNIK wildtype and IKKβ was time-consuming, the domain of TNIK to which IKKβ binds could not be studied in the present thesis and is subject of future studies. TRADD also interacts with the CTAR2 domain of LMP1 and mediates activation of the canonical NF-κB pathway by facilitating the recruitment of IKKβ (Schneider et al, 2008). Hence, TRADD might additionally stabilize the signalling complex that forms at TNIK and might enhance the interaction between TNIK and IKKβ.

The first study on TNIK had identified TRAF2 as an interaction partner of TNIK (Fu et al, 1999). However, the biological functions of this interaction remain largely unknown. A recent study suggested a role of TRAF2 as an adapter that links TNIK to CD27-mediated Wnt signalling in leukaemia stem cells (Schurch et al, 2012). Data presented in this thesis

indicate an additional role of this interaction for LMP1-induced signalling. TRAF2 binds to the consensus TRAF binding site within the CTAR1 domain but is not recruited by CTAR2 (see 1.7.2). Since results obtained in this thesis showed that TNIK interacts with the CTAR2 domain and mediate signalling by the CTAR2 domain of LMP1 (see 5.1), it was surprising to find that LMP1 induced an interaction between TNIK and TRAF2.

There have been some controversies regarding the role of TRAF2 for CTAR2-induced canonical NF-κB and JNK signalling (see 1.7.2). While studies engaging TRAF2-deficient cell lines reported no role of TRAF2 for activation of the canonical NF-κB and JNK pathways (Luftig et al, 2003; Wan et al, 2004; Xie et al, 2004), a recent study employing a genome wide siRNA screen identified TRAF2 as a mediator of CTAR2-induced activation of the canonical NF-κB pathway (Gewurz et al, 2012). The controversies regarding the contribution of TRAF2 towards CTAR2-induced signalling might reflect cell type-specific differences and it is possible that due to partial redundancy TRAF2 deficiency might be rescued by a different TRAF family member in the TRAF2-deficient cell lines (see 1.7.2).

The finding that LMP1 significantly induced an interaction between TNIK and TRAF2 indicates a role of this interaction for LMP1-mediated signalling. Interestingly, only LMP1 mutants with an intact CTAR2 domain induced the interaction and deletion or mutation of the 16 C-terminal amino acids of the CTAR2 domain that are essential for activation of JNK and canonical NF-κB signalling failed to induce an interaction between TNIK and TRAF2. In contrast, mutation of the TRAF binding site within the CTAR1 domain had no effect on the induction of the TNIK-TRAF2 interaction. This indicates that the interaction between TNIK and TRAF2 is involved in signalling by the CTAR2 domain of LMP1. However, as TRAF2 is not recruited to the CTAR2 domain (see 1.7.2), the interaction between TNIK and TRAF2 must occur in the cytoplasm. The role of this cytoplasmic signalling complex remains to be investigated and TRAF2 might have an accessory but non-essential function. However, since results of a recent study demonstrate a role of TRAF2 for LMP1-induced canonical NF-κB signalling (Gewurz et al, 2012) and results presented in this thesis show a role of TNIK for this pathway, it is possible that TRAF2 enhances activation of this pathway by TNIK.

In summary, results presented in this study show that an activation-dependent complex consisting of TNIK, TRAF6, TRAF2, TAK1 and IKKβ forms at the CTAR2 domain of LMP1.

5.3. The role of TNIK in CD40-induced signalling

Having established a role of TNIK as an essential signalling mediator of the viral oncoprotein LMP1, the next task was to find a cellular receptor that engages TNIK for signal transduction. CD40 is a co-stimulatory receptor that is found expressed mainly on B-cells and is critical for the function of the adaptive immune response (Kawabe et al, 2011). LMP1 mimics functions of the CD40 receptor and both receptors induce similar signalling complexes that facilitate the activation of an overlapping set of signal transduction pathways (see 1.3.3). Therefore a role of TNIK in CD40-induced signal transduction seemed feasible. The first indication that TNIK might be involved in functions of CD40 was the finding that overexpressed TNIK and CD40 interacted in HEK293D cells. A potential role of TNIK for canonical NF-κB and JNK signalling was subsequently analysed by IKKβ and JNK kinase assays performed with HEK293D cells depleted of endogenous TNIK by siRNA. Results of these experiments clearly showed that the knockdown of TNIK significantly reduced the ability of CD40 to activate IKKβ and JNK, thus revealing TNIK as a critical mediator of CD40-induced activation of the canonical NF-κB and JNK pathways. This finding is in line with results obtained with B-cells in this laboratory showing that the knockdown of endogenous TNIK abrogated canonical NF-κB and JNK signalling upon stimulation of CD40 with CD40L (Dr. Anna Shkoda, personal communication).

Taken together, TNIK was identified as a novel mediator of canonical NF-κB and JNK signalling by CD40 indicating important roles for TNIK both for the normal function of the immune system as well as for malignancies that are associated with deregulated CD40-induced signalling (figure 5.1).

5.4. The dynamic CD40-induced TNIK signalling complex

Experiments conducted with endogenous proteins in the course of this thesis showed that activation of CD40 induces an interaction between TNIK and TRAF6. This interaction was analysed more closely with regard to the TNIK domains that mediate this interaction by a co-immunoprecipitation experiment in HEK293D cells, which showed that TRAF6 interacted with the GCKH domain. Co-expression of CD40 enhanced the interaction but had no effect on the TNIK domain to which TRAF6 binds. In contrast to results obtained in the context of LMP1, no interaction between TRAF6 and the intermediate domain was detected. However, since this interaction was much weaker and was thus hard to detect by immunoprecipitation experiments, it is probable that the interaction between the intermediate domain takes place

in the context of CD40-induced signalling but was not detected in the experiments conducted for this thesis.

Furthermore, co-immunoprecipiation studies in HEK293D cells showed that CD40 induces an interaction of TNIK with TRAF2 and IKKβ. Co-expression of TNIK and TRAF2 in HEK293D cells showed that the proteins were associated at a low level constitutively, while co-expression of CD40 enhanced this interaction indicating that this interaction plays a role for signalling by CD40. TNIK and IKKβ did not interact constitutively, but as was shown for LMP1, the co-expression of CD40 induced an interaction between TNIK and IKKβ. Thus, the results obtained by co-immunoprecipitation experiments with HEK293D cells show that like LMP1, CD40 induces the association of TNIK with TRAF6, TRAF2 and IKKβ (figure 5.1).

To gain further insight into the dynamics of complex formation in B-cells, the CD40-induced signalling complex was analysed by co-immunoprecipitation experiments with the EBV-negative B-cell line BL41. BL41 cells were stimulated with CD40L. As TRAF2 and TRAF6 interact with TNIK and both TRAFs play important roles for JNK and canonical NF-κB signalling by CD40 (Davies et al, 2005; Fu et al, 1999; Grech et al, 2004; Hostager, 2007; Nguyen et al, 1999; Rowland et al, 2007) (see 1.7.3), analysis of the signalling complex in B-cells focussed on the dynamic association of TRAF2, TRAF6, TNIK and CD40.

Current concepts of CD40-induced JNK and canonical NF-κB signalling involve the formation of distinct signalling complexes. Activation of the canonical NF-κB pathway is induced by a receptor-bound signalling complex at the membrane while activation of the JNK pathway depends on the translocation of signalling complexes into the cytosol (Matsuzawa et al, 2008). As results obtained in this study showed that TNIK is essential for the activation of both the canonical NF-κB as well as the JNK pathway, TNIK should be present in the NF-κB as well as the JNK inducing signalling complexes. Results obtained by co-immunoprecipitation experiments in BL41 cells showed that TRAF2 and TRAF6 started to associate with TNIK after 5 minutes of CD40 stimulation which coincides with the initiation of the JNK and canonical NF-κB pathways. This indicates that the binding of TRAF2 and TRAF6 to TNIK is important for the initiation of these pathways.

The analysis of the interaction between TNIK and CD40 revealed that TNIK is pre-associated with CD40 in non-stimulated cells. Stimulation of CD40 with CD40L resulted in the rapid disassociation of TNIK from the receptor. This might reflect a role of TNIK for the activation of cytoplasmic signalling complexes that have been described in the context of CD40-induced JNK signal transduction (Matsuzawa et al, 2008). Matsuzawa et al. showed that upon activation of CD40 two distinct signalling complexes form at the intracellular domain of the receptor, a TRAF6-TAK1 complex and a TRAF2-MEKK1 complex. The release of the

signalling complexes into the cytoplasm, which depends on the degradation of TRAF3, is the prerequisite for activation of the MAP3K TAK1 and MEKK1 and subsequent induction of the JNK pathway. However, how kinase activation in the cytoplasm is conferred is not known. Results presented in this study show that TNIK interacts with TAK1 in HEK293D cells (see 5.2). While the interaction between TNIK and TAK1 remains to be shown in B-cells, activation of CD40 in BL41 cells induced the interaction of TNIK with TRAF6. It is thus possible that TNIK in complex with TRAF6 associates with and activates the kinase TAK1 in the cytoplasm and thus induces JNK signalling. A role of TNIK in the activation of the second TRAF2-MEKK1 signalling complex remains to be shown. However, since association of TRAF2 with TNIK is induced upon activation of CD40 and related members of the GCK family, such as NIK and NESK, have been shown to activate MEKK1 (Nakano et al, 2000; Su et al, 1997), it is possible that TNIK also mediates activation of the TRAF2-MEKK1 complex.

In contrast to activation of the JNK pathway in the cytoplasm according to Matsuzawa et al., the canonical NF-κB pathway is induced by a receptor bound complex (Matsuzawa et al, 2008). As TNIK was shown to be essential for both JNK and canonical NF-κB signalling by CD40 in this thesis, TNIK should also be present in the NF-κB inducing signalling complex. TNIK interacted with CD40 in non-stimulated cells. Stimulation of CD40 by binding of CD40L might then lead to the activation of TNIK and subsequently induce binding of TRAF6, TRAF2 and IKKβ to TNIK which constitutes the NF-κB activating signalling complex and leads to the induction of the canonical NF-κB pathway.

TNIK dissociated rapidly from the CD40 receptor and was no longer detected at the receptor after 5 minutes of stimulation with CD40L. According to Matsuzawa et al. activation of the JNK and canonical NF-κB pathways is not only spatially (cytoplasmic versus receptor bound) but also temporally separated with activation of IKKβ occurring as early as 2 minutes after receptor stimulation, while activation of JNK has a delayed time course (Matsuzawa et al, 2008). Hence, TNIK might mediate activation of IKKβ early after CD40 activation and might subsequently facilitate activation of JNK in the cytoplasm at a later time point.

The earliest time point analysed in this study was at 5 minutes of CD40 stimulation. A more detailed analysis of the time course of complex formation is thus necessary to verify the hypothesis presented above. Additionally, analysis of the subcellular localization of signalling complexes by conducting co-immunoprecipitation experiments separately from membrane and cytoplasmic fractions will gain further insight into the role of TNIK for CD40-mediated signalling.

It remains to be shown, whether the interaction between TNIK and CD40 is direct or indirect. Given that LMP1 mimics CD40 in its engagement of signalling molecules and TNIK interacts

via TRAF6 with LMP1, the interaction between TNIK and CD40 might also be mediated by a TRAF family member. As CD40 induced the binding of TRAF2 and TRAF6 to TNIK, either TRAF might facilitate binding of TNIK to CD40. However, while TNIK and CD40 are associated in non-stimulated cells, the interaction of TNIK with TRAF2 and TRAF6 was detected only after 5 minutes of stimulation, which argues against the hypothesis of either TRAF mediating the interaction between TNIK and CD40. However, experiments obtained by co-immunoprecipitation studies conducted with overexpressed proteins in HEK293D cells showed that TNIK interacts weakly with both TRAF2 and TRAF6 in the absence of an inducer. It is thus also possible that the interaction of TNIK with TRAF2 and TRAF6 in non-stimulated cells occurs but was not detected with endogenous proteins in BL41 cells.

Future studies analysing the interaction between TNIK and CD40 using purified proteins will show, whether TNIK and CD40 can interact directly. Furthermore, experiments conducted in TRAF2- or TRAF6-deficient cell lines or analysis of the interaction of TNIK with different CD40 mutants harbouring mutations within the TRAF6 or TRAF1,2,3 binding sites will clarify how TNIK interacts with CD40.

5.5. Activation of the TNIK signalling complex – the role of post-translational modifications

5.5.1. The role of phosphorylation

Post-translational modifications increase the functional scope of the modified protein and are involved in many cellular signal transduction pathways (see 1.6). In order to understand how signal transduction by LMP1 and CD40 is regulated, post-translational modifications of the individual components of the receptor-induced signalling complexes were investigated. Phosphorylation is the best studied type of post-translational protein modification and a common feature of many cellular signal transduction pathways with a large amount of proteins being phosphorylated in a cell at one time and more than 500 different kinases known in humans (Manning et al, 2002). Phosphorylation can alter the activity of a protein, modify protein-protein interactions or have positive or negative effects on other kinds of post-translational protein modifications (Hunter, 2007; Karin & Ben-Neriah, 2000). Results presented in this thesis show that the kinase activity of TNIK is critical for the induction of the canonical NF-κB pathway and mediates activation of IKKβ as well as nuclear translocation of p65 demonstrating that phosphorylation events are involved in TNIK-induced NF-κB signalling.

The exogenously expressed kinase domain was unable to induce IKKβ in the absence of endogenous TNIK. This indicates that the kinase domain phosphorylates either TNIK itself or one of the signalling molecules that bind to TNIK in order to initiate NF-κB signalling. One possibility is that TNIK directly phosphorylates IKKβ, possibly at a site different to the one targeted by TAK1. However, experiments conducted with purified IKKβ as a potential substrate did not reveal phosphorylation of IKKβ by TNIK. Moreover, TAK1 has been shown to phosphorylate and thus activate IKKβ (Wang et al, 2001). It is thus probable that this also happens in the context of TNIK-dependent signalling with TNIK mediating the activation of TAK1.

The kinase activity of TNIK might be required for autophosphorylation of TNIK. TNIK contains multiple phosphorylation sites within its intermediate domain (www.phosphosites.org) and results obtained by TNIK kinase assays showed that the autophosphorylation activity of TNIK is induced by LMP1 and CD40, which indicates that phosphorylation of TNIK plays a role for signalling by these receptors. As the kinase mutant of TNIK was not analysed as a control in the TNIK kinase assays, it is possible that the phosphorylation of TNIK did not constitute autophosphorylation, but was induced by an unknown kinase that binds to TNIK. However, as the immunoprecipitates containing TNIK are subjected to stringent wash conditions, it seems unlikely that significant amounts of a co-precipitated kinase remain present in the kinase reactions. Hence, it is probable that the phosphorylation that was detected in the kinase assays constitutes autophosphorylation of TNIK.

Autophosphorylation of TNIK has been described in previous studies and seems to have differential effects depending on the cell type and context. In the context of Wnt signalling phosphorylation of TNIK at serine 764 leads to nuclear translocation and activation of TCF-LEF transcription factors (Mahmoudi et al, 2009; Shitashige et al, 2010), while phosphorylation of TNIK can also increase its localization in the cytoskeletal compartment, where TNIK induces the disassembly of F-actin (Taira et al, 2004). In the context of LMP1- and CD40-induced signalling, auto-phosphorylation of TNIK might increase its localization at the signalling domains of the receptors. Phosphorylation of TNIK might also facilitate binding of the TAK1/TAB2/IKKβ signalling complex either by creating new binding sites or by the induction of a conformational change in TNIK. Mass spectrometry identification of phosphorylation sites within TNIK after activation by LMP1 or CD40 and subsequent mutation of the respective phosphorylation sites will shed light on the contribution of TNIK phosphorylation to signal transduction in future studies.

In the quest to find a target for the kinase activity of TNIK other than TNIK itself, it was tested whether TNIK might accept TRAF2 or TRAF6 as potential targets. Immunocomplex kinase

assays using the purified His-tagged TRAF-domains of TRAF2 and TRAF6 provided by Fabian Giehler as potential substrates for TNIK, revealed that while TNIK did not accept TRAF2 as a substrate, it strongly phosphorylated TRAF6. This makes TNIK the first known kinase that phosphorylates TRAF6. The sites at which TNIK phosphorylates TRAF6 were analysed by an LC-MS approach in cooperation with Dr. Hakan Sarioglu from the proteomics facility of the Helmholtz Center Munich. This analysis revealed two phosphorylation sites within TRAF6 at threonine 463 and serine 507. Threonine 463 resides within the TRAF-C domain of TRAF6 while serine 507 lies at the far C-terminus of TRAF6. The TRAF-C domain mediates interaction with receptors or other signalling molecules while the function of the C-terminal amino acids 499-522 has not been described so far (see 1.7.1).

Phosphorylation of TRAF6 at serine 507 as well as three other phosphorylation sites (threonine 322, tyrosine 326 and tyrosine 353) within the TRAF-domain of TRAF6 have been detected by mass spectrometry screenings (www.phosphosite.org). The phosphorylation site at threonine 463 has not been detected so far. The phosphorylation sites at threonine 322 and threonine 507 were detected by a mass spectrometry screening in T-cell leukaemia-derived Jurkat cells (www.phosphosite.org). Phosphorylation of TRAF6 at tyrosine 326 was detected by mass spectrometry in HEK293 cells expressing the exogenous fibroblast growth factor (FGFR1) (Kasyapa et al, 2009). FGFR1 is a tyrosine kinase that acts as a cell-surface receptor for fibroblast growth factors and is essential for the regulation of embryonic development, cell proliferation, differentiation and migration (Coumoul & Deng, 2003). Phosphorylation at tyrosine 353 has emerged in a screen of phosphotyrosine kinase activity in lung cancer cell lines (Rikova et al, 2007).

TRAF2 is not phosphorylated by TNIK, but phosphorylation of TRAF2 has been described in the literature. Ten phosphorylation sites have been detected within TRAF2, the majority of which reside within the N-terminal RING- and Zn-finger domains. Phosphorylation at serine and threonine residues within the N-terminal RING and Zn-finger domains of TRAF2 is associated with the activation of the JNK and NF-κB pathways (Blackwell et al, 2009; Thomas et al, 2009; Zhang et al, 2009). Phosphorylation of TRAF2 at serine 11 and serine 55 is induced by TNFα and cellular stresses and is essential for prolonged IKK activation and full JNK activation, but inhibits the prolonged phase of JNK activation that leads to apoptosis. TRAF2 phosphorylation is thus associated with cell survival (Blackwell et al, 2009; Thomas et al, 2009; Zhang et al, 2009). Consistent with this finding, constitutively-phosphorylated TRAF2 was found in some malignant cancer cell lines and in Hodgkin's lymphoma (Thomas et al., 2009). A study employing an RNAi screen to study the role of phosphatases for the regulation of the NF-κB pathway in astrocytes showed that TRAF2 is

phosphorylated at threonine 117 and that phosphorylation is required for activation of the NF-κB pathway in response to TNF treatment (Li et al, 2006). Phosphorylation at the C-terminus of TRAF2 has been detected, but to date the biological role remains unknown. Phosphorylation at threonine 431 within the C-terminal TRAF domain was detected by a mass spectrometry analysis of Jurkat T-cells. TRAF2 phosphorylated at serine 274 which lies between the N-terminal Zn-finger domains and the coiled coil domain was detected in Jurkat T-cells as well as in the breast adenocarcinoma cell line MCF-7 (www.phosphosite.org).

The biological function of TRAF6 phosphorylation remains elusive and is subject of future studies. This study identifies TNIK as the first kinase that phosphorylates TRAF6 at two sites within its C-terminus. The important role of the TNIK-TRAF6 interaction for LMP1- and CD40-induced signalling suggests that TRAF6 phosphorylation might be involved in signalling by these receptors and might play a role for normal and malignant B-cell functions.

5.5.2. The role of ubiquitination and SUMOylation

A general concept of JNK and NF-κB signalling involves auto-ubiquitination of TRAF6 with non-degradative K63-linked ubiquitin chains. These chains serve as a scaffold to facilitate binding and activation of the downstream TAK1-TAB complex (Kanayama et al, 2004; Skaug et al, 2009; Wang et al, 2001). Thus, ubiquitination of TRAF6 constitutes an important initiating step in signal transduction. However, this concept has been challenged by recent publications suggesting a necessary but insufficient role of TRAF6 ubiquitination for the activation of signal transduction (Lamothe et al, 2007; Megas et al, 2011; Walsh et al, 2008). TRAF6 ubiquitination is induced by LMP1 (Song et al, 2006), but the role of TRAF6 ubiquitination for LMP1- and CD40-induced signalling has not been studied so far.

Results presented in this thesis show that depletion of HEK293D cells of endogenous TNIK abolished the ability of either LMP1 or CD40 to induce ubiquitination of TRAF6. The ubiquitination of TRAF6 likely represents auto-ubiquitination since a TRAF6(C70A) mutant of TRAF6 which is devoid of ubiquitin ligase activity was not ubiquitinated in response to LMP1 and CD40 activity (Andrea Obermeier, personal communication).

Up to now the mechanisms leading to the induction of ubiquitin ligase activity of TRAF6 are still poorly understood and have not been studied in the context of LMP1- or CD40-induced signalling. Evidence has been provided that the ubiquitin ligase activity of TRAF6 is promoted by induced oligomerization of TRAF6. A study showed that in the context of IL-1R-induced signalling the TRAF-interacting protein with a forkhead-associated domain (TIFA)

activates IKK by promoting the oligomerization and ubiquitin ligase activity of TRAF6 (Ea et al, 2004; Takatsuna et al, 2003). A similar mechanism of TRAF6 activation was found in T-lymphocytes. The mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) is an essential mediator of NF-κB activation in response to T-cell receptor stimulation. *In vitro* assays showed that MALT1 forms oligomers that bind TRAF6 and activate its ubiquitin ligase activity (Sun et al, 2004). A different study using TAB2-deficient MEF cells showed that IL-1R-induced ubiquitination of TRAF6 depends on TAB2 (Kishida et al, 2005). However, the mechanism through which TAB2 induces ubiquitination of TRAF6 has not been revealed in this study. This thesis identifies TNIK as a new inducer of the ubiquitin ligase activity of TRAF6. It remains to be shown how TNIK activates TRAF6, but it is probable that similar to the mechanisms that have been described for TIFA and MALT1, binding of TRAF6 to TNIK might result in oligomerization and thereby induce auto-ubiquitination.

Experiments studying the ability of TNIK wild-type and TNIK mutants to induce ubiquitination of TRAF6 demonstrated that the intermediate domain was sufficient to induce ubiquitination of TRAF6. This suggests that the interaction between TRAF6 and the intermediate domain of TNIK is sufficient to induce ubiquitination of TRAF6. Results obtained by co-immunoprecipitation experiments in HEK293D cells showed that the interaction between TNIK and TRAF6 was mainly mediated by the GCKH domain of TNIK and that the TRAF6(274-522) mutant that is devoid of ubiquitin ligase activity interacted equally with the intermediate and GCKH domains (see 5.2). These results suggest that binding of TRAF6 to the intermediate domain of TNIK mediates auto-ubiquitination of TRAF6 and that an intramolecular shift of TRAF6 to the GCKH domain of TNIK occurs (figure 5.2).

Additionally, this experiment showed that the kinase-mutant of TNIK (TNIK-KM), the deletion mutant lacking the kinase domain (TNIK-ΔKD) as well as the intermediate domain of TNIK, each induced ubiquitination of TRAF6 while TNIK wild-type exerted an inhibitory effect on TRAF6 ubiquitination suggesting that the kinase activity of TNIK inhibits ubiquitination of TRAF6. Post-translational modifications often work in combination with one sort of modification at a given site affecting another modification (Hunter, 2007). In the case of TRAF6, phosphorylation of TRAF6 by TNIK might constitute a negative feedback loop that shuts off TRAF6-mediated signal transduction. More studies are needed to determine the exact contribution of secondary modifications of TRAF6 to LMP1- and CD40-induced signalling.

SUMOylation is a type of post-translational protein modification that is involved in multiple cellular processes. In contrast to ubiquitination, SUMOylation occurs at consensus sites and

can be predicted *in silico*. A search for potential SUMOylation sites within TNIK using the SUMOsp 2.0. algorithm (Ren et al. 2009) came up with six potential SUMOylation sites within the protein. One of these sites at K780 within the intermediate domain of TNIK was located within an accessible structure of the TNIK protein (elm.eu.org). Experiments with HEK293D cells showed that SUMOylation of TNIK occurs in the cell and is induced by LMP1 and CD40. The site at K780 within the intermediate domain was identified as a SUMOylation site of TNIK and mutation of this site completely abolished the SUMOylation of TNIK in response to LMP1 and CD40 activity. An IKKβ kinase assay showed that mutation of the SUMOylation site at K780 abolished the ability of TNIK to induce IKKβ and thus demonstrated that SUMOylation of TNIK is critical for the induction of the canonical NF-κB pathway. SUMOylation alters the activity of target proteins and modifies protein-protein interactions. In the context of TNIK, SUMOylation might induce an activated state of the protein or might facilitate interaction of downstream signalling molecules that are involved in NF-κB signalling.

5.5.3. Model of the TNIK-associated dynamic signalling complex

Taken together, the findings presented in this thesis suggest the following model for the role of TNIK in LMP1- and CD40-induced signal transduction:

Upon LMP1 or CD40 activation, TNIK associated with TAK1 is recruited to the intracellular signalling domains of the receptors. TRAF6, TAK1-TAB2 and IKKß are recruited to TNIK. In the case of LMP1, TRAF6 mediates the interaction between TNIK and the CTAR2 domain. TRADD which also interacts with CTAR2 (Izumi & Kieff, 1997; Schneider et al, 2008) might stabilize the signalling complex and thus enhances activation of the canonical NF-κB pathway. TRAF2 is also recruited to TNIK and might act in concert with TRAF6 to mediate activation of the canonical NF-κB and JNK pathways (see 1.7.2 and 1.7.3). In the case of LMP1-induced signalling, TRAF2 might have a non-essential role within a cytoplasmic signalling complex.

In addition to its function as a scaffolding protein that organizes complex formation at the intracellular signalling domains of LMP1 and CD40 and acting as an adapter to mediate binding of downstream signalling molecules, TNIK modifies the activity of its interaction partners either through binding or through phosphorylation. The interaction of TRAF6 with the intermediate domain of TNIK induces auto-ubiquitination of TRAF6. This results in a rearrangement within the complex, which leads to an intramolecular shift of TRAF6 to the GCKH domain of TNIK. TNIK additionally phosphorylates TRAF6, which might constitute a

negative feedback loop that inhibits the auto-ubiquitination and activation of TRAF6. TAK1 might be activated either through the interaction with TNIK that induces an activated conformation of TAK1 or through ubiquitination by TRAF6 (see 5.2).

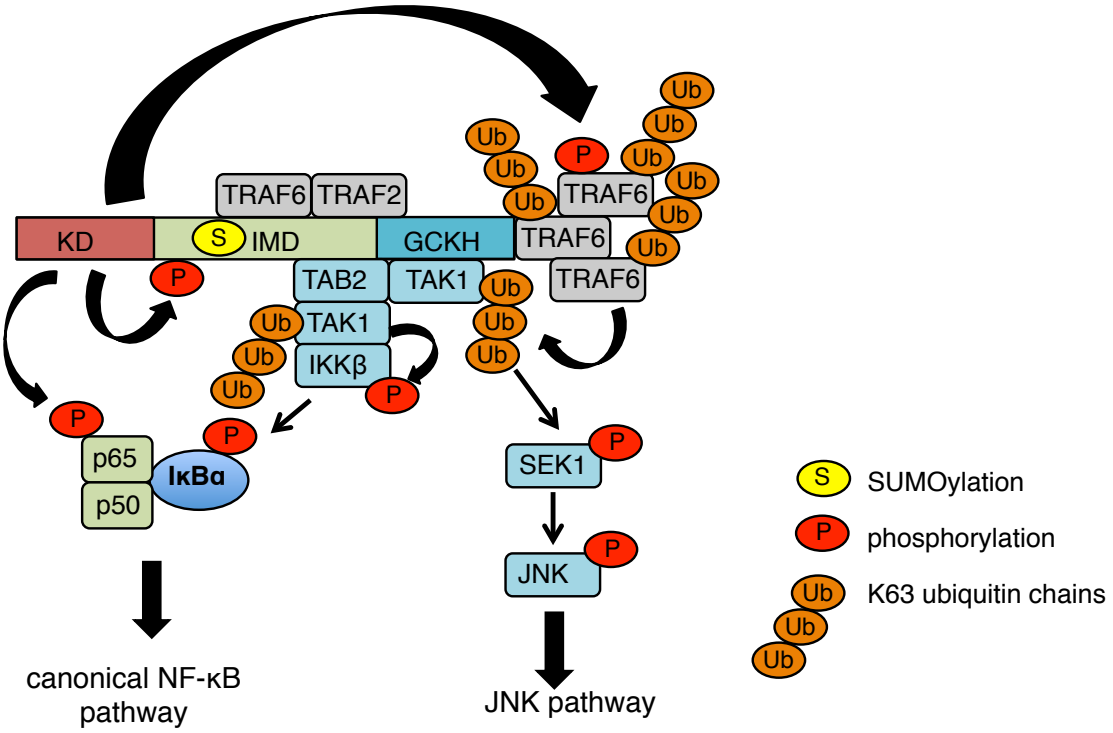


Figure 5.2. Model of the signalling complex at TNIK
The signalling complex is depicted in its activated state. The kinase domain of TNIK phosphorylates itself and TRAF6. Additionally, TNIK is SUMOylated within the intermediate domain. Binding of TRAF6 to the intermediate domain of TNIK induces its ubiquitination with K63-linked ubiquitin chains and results in an intramolecular shift of TRAF6 to the GCKH domain of TNIK. TAK1 interacts with the intermediate and GCKH domains of TNIK and might be activated by ubiquitination with K63-linked ubiquitin chains by TRAF6. Activated TAK1 mediates activation of canonical NF-κB and JNK signalling. Induction of the NF-κB pathway depends on the kinase domain of TNIK and is initiated by the phosphorylation IKKβ which in turn phosphorylates IκBα. This leads to the degradation of IκBα and subsequent liberation of p65-p50 NF-κB transcription factors. In addition to the role of TNIK upstream of IKKβ, TNIK might induce the nuclear translocation of p65 by phosphorylation. Induction of the JNK pathway depends on the GCKH domain of TNIK and leads to a consecutive phosphorylation and activation of SEK1 and JNK. Please confer to the text for a more detailed explanation.

The kinase domain of TNIK mediates activation of the canonical NF-κB pathway while the GCKH domain facilitates activation of the JNK pathway. Hence, TNIK constitutes the point of bifurcation of these two signalling pathways. Activation of the canonical NF-κB pathway depends on the SUMOylation of TNIK at the intermediate domain and the kinase activity of TNIK. The kinase activity might be needed to phosphorylate TNIK, which might facilitate the interaction or activation of the TAK1-TAB2-IKKβ-signalling complex. As described in previous

publications, activated TAK1 phosphorylates and thus activates IKKβ which then in turn phosphorylates IκBα which is subsequently degraded and liberates p65-p50 NF-κB transcription factors into the nucleus (see 1.4). Additionally, TNIK might enhance the nuclear translocation of p65 by phosphorylation. Induction of the JNK pathway depends on the GCKH domain of TNIK. Activated TAK1 phosphorylates and thus activates SEK1, which in turn phosphorylates JNK (see 1.5).

5.6. Potential biological role of TNIK

This thesis identifies TNIK as a novel mediator of LMP1- and CD40-mediated signal transduction and extends the knowledge of the biological functions of this kinase to the haematopoietic system. While a very recent study showed a role of TNIK in the activation of the Wnt pathway by the TNFR family member CD27 in leukaemia stem cells (Schurch et al, 2012), the physiological function of TNIK in B- and T-lymphocytes remains unknown.

The role of TNIK for CD40-induced signalling suggests a role of TNIK in immunity and inflammation (Schonbeck & Libby, 2001; Wagner, 2009). In B-cells, TNIK may be involved in physiological processes that are mediated by CD40 including enhanced cell survival, proliferation, germinal center formation, IL-6 secretion, immunoglobulin secretion, up-regulation of co-stimulatory molecules, affinity maturation and generation of long-lived plasma cells (see 1.3.1).

As TNIK mediates activation of JNK and NF-κB signalilng, a role of TNIK in physiological and pathogenic processes that are conferred by these pathways in B-cells is conceivable. CD40-induced activation of the JNK pathway mediates IgE class switching and IL-6 production (Jabara & Geha, 2005; Vanden Bush & Bishop, 2008) while CD40-mediated activation of the NF-κB pathway mediates antibody secretion and proliferation (Hsing & Bishop, 1999; Zarnegar et al, 2004). Additionally, CD40 functions that mediate the interaction between T- and B-cells, such as up-regulation of B7-1 and ICAM-1 (intracellular adhesion molecule-1, also known as CD54), depend on the activation of NF-κB (Hsing & Bishop, 1999). Results obtained in our laboratory showed that the knockdown of TNIK in B-cells reduces CD40-mediated up-regulation of ICAM-1 which is a hallmark of B-cell activation and thus shows a physiological role of TNIK in the function of the immune system (PD Dr. Arnd Kieser and Jennifer Town, unpublished data).

Aberrant CD40 signalling is associated with the development and progression of a number of autoimmune diseases and several haematopoietic and non-haematopoietic human

malignancies (see 1.3.2). CD40 mediates proliferation and survival of normal and neoplastic B-cells, primarily through the activation of NF-κB target genes, such as CD40L and the anti-apoptotic Bcl2 family member Bfl-1/A1 (D'Souza et al, 2004; Pham et al, 2005). CD40-induced NF-κB signalling is associated with oncogenic transformation of epithelial cells and protects cells from apoptosis in low-grade B-cell malignancies (Baxendale et al, 2005; Eliopoulos & Young, 2004).

Additionally, the function of TNIK in signalling by LMP1 implicates a role of this kinase in LMP1-associated diseases. LMP1 is the primary oncoprotein of EBV, which is associated with malignancies such as Hodgkin's lymphoma, Burkitt's lymphoma, gastric carcinoma and nasopharyngeal carcinoma (see 1.2.1). The LMP1-induced canonical NF-κB and JNK pathways that depend on TNIK are essential for LMP1-mediated B-cell transformation (Cahir McFarland et al, 1999; Cahir-McFarland et al, 2004; Kieser, 2007; Kutz et al, 2008). Results obtained recently in our laboratory demonstrate that the down-regulation of TNIK in LCLs retards proliferation and leads to apoptosis thus demonstrating an important role of TNIK for survival and proliferation of EBV-transformed cells (Fabian Giehler and PD Dr. Arnd Kieser, unpublished data). Hence, the essential role of TNIK for signalling by LMP1 and CD40 makes TNIK an attractive target for novel therapies for LMP1- and CD40-associated diseases.

The interaction between TNIK and TRAF6 and the role of TNIK for the activation of TRAF6 that was revealed in this thesis suggests additional functions of TNIK for other receptors that engage TRAF6 for signalling. For instance, TNIK may mediate signalling by members of the IL-1R/TLR superfamily that play critical roles for innate and adaptive immunity and engage TRAF6 for activation of the NF-κB and JNK pathways (Kobayashi et al, 2004; Wu & Arron, 2003). Additionally, TNIK might be involved in TCR-induced activation of the canonical NF-κB pathway which depends on activation of IKK by TRAF6, TRAF2, MALT1 and TAK1 (Sun et al, 2004).

5.7. Outlook

Our understanding of the biological functions of TNIK in different cell types will greatly benefit from future studies using TNIK knockout mice. As previous publications suggest a role of TNIK for embryonic development (Satow et al, 2010), a knockout of TNIK might result in severe developmental defects or even embryonic lethality. Hence, the conditional knockout of TNIK in specific tissues is desirable and would for instance allow the analysis of the role of

TNIK in the haematopoietic system. Additionally, the analysis of the contribution of exogenously overexpressed individual TNIK domains in TNIK-deficient cell lines would further enhance the understanding of the composition of the signalling complex at TNIK and the exact contribution of the individual TNIK domains to the activation of the JNK and NF-κB pathways. A problem of the analysis of the functions of individual TNIK domains in wildtype cells is that one cannot rule out the potential contribution of endogenous TNIK, which might interact with or modify exogenously expressed domains.

The understanding of the role of TNIK in CD40-induced signal transduction will benefit from a more detailed analysis of complex formation with regard to interaction partners such as the TAK1-TAB2 complex and IKKβ as well as a more detailed analysis of the spatial and temporal aspects of complex formation. Co-immunoprecipitaion experiments conducted separately from membrane and cytoplasmic fractions would allow further insight into the potential roles of TNIK within receptor-associated, membrane-proximal and cytoplasmic-signalling complexes in the context of CD40-induced signalling.

Furthermore, future studies will focus on post-translational protein modifications within the TNIK-signalling complex. Mass spectrometry analysis of phosphorylation sites within TNIK after activation by LMP1 or CD40 and subsequent mutation of the respective sites will facilitate the analysis of the biological role of TNIK phosphorylation. Additionally, a more detailed analysis of the role of SUMOylation of TNIK will show, how activation of IKKβ upon SUMOylation of TNIK is conferred. Such an analysis will focus on the SUMOylation-dependent subcellular localization of TNIK, protein-protein interactions and effects on other post-translational modifications, such as phosphorylation of TNIK.

Future work will also address the biological function of TRAF6 phosphorylation by TNIK. Analysis of the respective TRAF6 mutants will show, whether phosphorylation of TRAF6 by TNIK affects its subcellular localization, protein-protein interactions or, as indicated by results presented in this thesis, affects the ubiquitination of TRAF6. Furthermore, experiments using purified proteins will show, whether in addition to TRAF6, TNIK phosphorylates another downstream interaction partner, such as TAK1 or TAB2.

6. Summary

LMP1 acts as an efficient viral pseudoreceptor that exploits cellular signal transduction pathways to achieve onocogenic transformation of cells. The CD40 receptor that is expressed mainly on B-cells is the cellular counterpart of the viral LMP1. The two receptors engage similar signalling molecules and engage an overlapping set of signal transduction pathways. LMP1 is the main oncoprotein of EBV, which is associated with various human malignancies such as Hodkins's lymphoma, Burkitt's lymphoma, nasopharyngeal carcinoma and post-transplant lymphoproliferative diseases. CD40 has critical functions for the immune response, but deregulated CD40 activity is associated with autoimmune diseases and several haematopoietic and non-haematopoietic human malignancies. Due to the important functions of the two receptors, intense research has gone into understanding the mechanisms of signal transduction. Especially aberrant JNK and NF-κB signalling are powerful means of tumour cells that ensure survival and continuous proliferation of malignant cells. Several components of the LMP1- and CD40-induced signalling complexes leading to the activation of JNK and NF-κB have been identified to date. Nevertheless the exact composition and activation of these signalling complexes is still only incompletely understood.

Data presented in this thesis identify the germinal center kinase TNIK as a novel interaction partner of the CTAR2 domain of LMP1 and CD40 and reveals a critical role of TNIK for the induction of the JNK and canonical NF-κB signalling pathways. While the activation of JNK by overexpressed TNIK has been described in a previous publication (Fu et al, 1999), the biological function or a receptor engaging TNIK for JNK signalling has remained elusive. This thesis identifies LMP1 and CD40 as the first known receptors that engage TNIK for activation of the JNK pathway. Additionally, TNIK is identified as a novel inducer of the canonical NF-κB pathway that activates IKKβ and facilitates the nuclear translocation of the NF-κB subunit p65. Additionally, data presented in this thesis show that TNIK specifically induces the canonical NF-κB pathway but has no role for the induction of the non-canonical pathway. Distinct domains of TNIK mediate activation of the canonical NF-κB and JNK pathways with induction of NF-κB depending on the N-terminal kinase domain of TNIK and induction of JNK depending on the C-terminal GCKH domain of TNIK. This suggests that the two pathways bifurcate at the level of TNIK and reveals an essential role of TNIK as an organizer of LMP1- and CD40-induced signalling.

Analysis of the signalling complexes that are induced by LMP1 and CD40 revealed the adapter protein TRAF6, the MAP3K TAK1 and IKKβ as novel interaction partners of TNIK. Furthermore, data presented in this thesis show that while TNIK and TAK1 are constitutively-

associated, TRAF6, TRAF2 and IKKβ bind to TNIK in an activation-dependent manner. Hence, TNIK interacts with the major components of the LMP1- and CD40-induced signalling complexes leading to activation of JNK and the canonical NF-κB pathway functioning as an essential complex organizer that facilitates the recruitment and activation of the individual signalling molecules within the complex. The function of TNIK as a complex organizer is underlined by the finding that TNIK mediates the interaction of the TAK1-TAB2 complex with LMP1.

Analysis of the post-translational modifications occurring within the LMP1- and CD40-induced signalling complexes gave insight into mechanisms leading to activation of the individual components of the signalling complexes and induction of signal transduction. LMP1 and CD40 induce the autophosphorylation activity and SUMOylation of TNIK. Additionally, results presented in this thesis reveal TNIK as a novel mediator of auto-ubiquitination of TRAF6 which is an important initiating step in JNK and canonical NF-κB signalling. TNIK was identified as the first known kinase that phosphorylates TRAF6. The phosphorylation sites were identified by mass spectrometry and results obtained in this study suggest that phosphorylation of TRAF6 by TNIK might constitute a negative feedback loop to shut off activation of TRAF6.

7. Abbreviations

aa	Amino acid
AP1	Activator protein 1
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
BAFF	B-cell activating factor
Bax	Bcl-2 associated protein X
BMP	Bone morphogenic protein
CBP	cAMP response element-binding
CD40L	CD40 ligand
cIAP1/2	Cellular inhibitor of apoptosis 1/2
CNH	Citron homology
CTAR	C-terminal activation region
DD	Death domain
DISC1	Disrupted-in-schizophrenia
EBV	Epstein-Barr Virus
ERK	Extracellular-regulated kinase
GCK	Germinal center kinase
GCKH	Germinal center kinase homology
GCKR	Germinal center kinase-related
GFP	Green fluorescent protein
GLK	Germinal center kinase-like kinase
HA	Hemagglutinin
HDAC	Histone deacetylase
HEK	Human embryonic kidney
HECT	Homologous to E6-associated protein carboxyl terminus
HGK	HPG/GCK-like kinase
HPK	Haematopoietic progenitor kinase
IB	Immunoblot
ICAM-1	Intracellular adhesion molecule-1
Ig	Immunoglobulin
I-κB	Inhibitor of NF-κB
IKK	I-κB kinase
IL-1R	Interleukin-1 receptor
IRAK1	Interleukin 1-receptor associated kinase 1

JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LCL	Lymphoblastoid cell line
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry
LMP1	Latent membrane protein 1
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
MEKK1	Mitogen-activated protein kinase kinase kinase 1
MHC	Major histocompatability complex
MINK	Misshapen-NIKs-related kinase
MS	Mass spectometry
NESK	Nck-interacting kinase-like embryo specific kinase
NIK	Nck-interacting kinase or NF-κB-inducing kinase
NF-κB	Nuclear factor-κB
PAK	p21-activated kinase
PI3K	Phosphatidylinositol 3-kinase
RHD	Rel-homology domain
RING	Really interesting new gene
RSK1	Ribosomal S6 kinase 1
SAPK	Stress-activated kinase
siRNA	Small interfering-RNA
shRNA	Short hairpin RNA
SEK1	SAPK/ERK kinase 1
SH	Scr homology
STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-related modifier
TAB	TAK1-binding protein
TAK1	Transforming growth factor-β-receptor-accociated kinase 1
TCR	T-cell antigen receptor
TIFA	TRAF-interacting protein with a forkhead-associated domain
TLR	Tolllike receptor
TGFβ	Transforming growth factor β
TM	Transmembrane domain
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TNIK	TRAF2 -and Nck-interacting kinase

TRADD	Tumour necrosis factor receptor-associated death domain protein
TRAF	Tumour necrosis factor receptor-associated factor
TRAIL	Tumour necrosis factor -related apoptosis-inducing ligand

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