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Abstract (deutsch)

Das angeborene Immunsystem ist die erste Verteidigungslinie gegen eindringende Pathogene und führt seine Funktion durch eine Reihe sehr vielseitiger Funktionswege aus. Diese werden aktiviert durch das Erkennen bestimmter molekularer Strukturen, genannt „Pathogen-assoziirte molekulare Muster“, welche von vielen Pathogenen geteilt werden. Sie werden erkannt durch verschiedene membrangebundene und zytoplasmische Rezeptoren, die alle spezifisch für eine bestimmte Art von Muster sind und zur Aktivierung des angeborenen Immunsystems zur Bekämpfung der Bedrohung führen.

Der NFκB Signalweg ist einer der zentralsten und wichtigsten dieser Signalwege und verbindet immunologische Funktionen, wie die Produktion von entzündlichen Zytokinen, mit anderen Funktionen wie zelluläres Überleben, Wachstum und Apoptose. Er wird ausgelöst durch die Aktivierung einiger immunrelevanter Rezeptoren, aber auch durch andere Einflüsse, wie zum Beispiel DNA-Schäden. Unter Ruhebedingungen ist der NFκB Komplex von seinem Inhibitor, dem Inhibitor von κB (IκB), gebunden, der ihn im Zytoplasma beschlagnahmt und so daran hindert seine Funktionen als Transkriptionsfaktor auszuführen. Nach Aktivierung des Signalweges wird der IκB durch den Inhibitor von κB Kinase (IKK) Komplex phosphoryliert, was zu dessen Ubiquitin abhängigen Abbau führt und dem NFκB Komplex ermöglicht in den Zellkern zu translozieren.

Nach dieser Translokation arbeitet NFκB nicht bloß als Transkriptionsfaktor zur Verteidigung gegen angreifende Pathogene, sondern auch für Zell Homeostase, Wachstum und die Reparator von beschädigter DNA. Er ist daher nicht bloß während Infektionen wichtig, sondern auch bei Krebs und zur DNA Reparatur. Da eine Fehlregulation schreckliche Folgen haben kann, welche von Immunschwäche über Autoimmunerkrankungen bis zu ungewolltem Zelltod und Krebs reichen können, ist eine präzise Regulation durch mehrere Instanzen von ausserordentlicher Wichtigkeit.

Neben den IκB und IKK Proteinen sind viele weitere an der Regulation dieses Signalweges beteiligt. Eine sehr wichtige Protein-Familie, welche stark in die Regulation des angeborenen Immunsystems und auch des NFκB Signalweges involviert ist, ist die Familie der „Tripartite-Motif“ (TRIM) Proteine. TRIM Proteine sind eine Familie von vermeintlichen E3 Ubiquitin Ligasen und sind definiert durch ein N-terminales, dreiteiliges Sequenz Motif, welches aus einer RING Domäne, bis zu zwei B-Box Domänen und einer Coiled-Coil Domäne besteht. Es sind bereits viele TRIM Proteine die mit dem NFκB Signalweg interagieren und diesen regulieren bekannt. Jedoch wurde kürzlich ein weiterer, vielversprechender Regulator in der Form von TRIM29 entdeckt.

TRIM29 ist ein neuer und noch weitgehend unerforschter Faktor in der Regulation des angeborenen Immunsystems. Er ist ein nachgewiesener Mitspieler in der Reparatur beschädigter DNA, sowie ein Vermittler von zellulärem Überleben und Wachstum durch Regulation des p53 und des Wnt Signalweges. In Zusammenhang mit diesen Erkentnissen ist er ein nachgewiesener Biomarker und mögliches Onkogen in mehreren Formen von Krebs. Überdies haben jüngste Studien in Lungen- und Blasen-krebs Zelllinien eine Rolle von TRIM29 in zellulärem Wachstum und Zellzyklus Progression durch die Regulation des NFκB Signalweges nachgewiesen. Die genauen Mechanismen hinter dieser Regulation sind noch weitestgehend unbekannt. Jedoch auf Grund der Erkenntnisse, dass sowohl das IKK Protein NEMO als auch TRIM29 eine Funktion in der Reparatur beschädigter DNA spielen, sowie in „yeast-two-hybrid“ Screens als mögliche Interaktoren nachgewiesen wurden, hypothetisieren wir, dass TRIM29 den NFκB Signalweg durch eine Interaktion mit dem IKK Komplex reguliert.

In dieser Studie untersuchten wir ob TRIM29 mit den IKK Proteinen interagiert um den zugrundeliegenden Mechanismus einer TRIM29 abhängigen NFκB Aktivierung zu ergründen. Wir verfolgten diesen Ansatz durch Co-Immunprezipitations Experimente, um eine Interaktion zwischen TRIM29 und einem der IKK Proteine nachzuweisen. Des Weiteren versuchten wir eine Co-Lokalisierung von TRIM29 und dem IKK Protein NEMO durch Immunfluoreszenz zu bestimmen.

Um die Relevanz von TRIM29 in der Aktivierung von NFκB durch DNA schädigende oder pathogene Stimuli weiter zu bestimmen etablierten wir Western-Blot basierte Assays um die NFκB Aktivierung zwischen Wild-Typ und *Trim29* KO Zellen zu vergleichen. Zuletzt untersuchten wir den Einfluss von TRIM29 auf zelluläres Überleben gegenüber DNA schädigenden und pathogenen Bedrohungen durch MTT Assay Experimente.

Wir waren nicht in der Lage eine Interaktion zwischen TRIM29 und den IKK Proteinen oder einen NFκB aktivierenden Effekt nachzuweisen. Dennoch suggerieren die verfügbaren Daten über TRIM29 einen komplexen Einfluss auf den NFκB Signalweg, welcher zwischen verschiedenen zellulären Umgebungen variieren zu scheint und einen substantiellen Einfluss auf die Aktivierung des NFκB Komplexes hat.

Abstract

The innate immune system is the first line of defense against incoming pathogenic threats and performs its functions through a multitude of very versatile pathways. They are activated through the recognition of certain molecules and structures, called pathogen-associated molecular patterns, which are common to a multitude of invading pathogens. These are recognized by various membrane-bound or cytoplasmic receptors which all are specific for a certain kind of molecular pattern and lead to the activation of one or more immune pathways to counter the incoming threat.

The NFκB pathway is one of the central and most important of these pathways and intertwines immunological functions like inflammatory cytokine production with additional functions like cell survival, proliferation and apoptosis. It is triggered through the activation of many immune system relevant receptors, but also through different stimuli like DNA damage. Under resting conditions, the NFκB complex is bound by its inhibitor, the inhibitor of κB (IκB), and sequestered in the cytoplasm, which prevents it from performing its functions as transcription factor. After activation of the pathway, IκB is phosphorylated by the inhibitor of κB kinase (IKK) complex, which leads to its ubiquitin dependent degradation and frees the NFκB complex to translocate into the nucleus.

After this translocation, NFκB acts as transcription factor for proteins not only involved in the defense against invading pathogens, but also in cell homeostasis, proliferation and the repair of damaged DNA. It is therefore not only important during infections, but also during cancer and in the DNA damage response. As miss-regulation of this pathway can have detrimental effects, ranging from immune deficiency, autoimmunity and cell death to cancer, a fine-tuned regulation through multiple instances is of upmost importance.

Aside from the IκB and IKK proteins, many more proteins are involved in the regulation and fine-tuning of this pathway. One important protein family which is heavily involved in the regulation of the innate immune system, and also NFκB, is the family of tripartite-motif (TRIM) proteins. TRIM proteins are a family of putative E3 ubiquitin ligases and are defined by an N-terminal tripartite sequence motif, consisting of a RING domain, up to two B-Box domains and a coiled-coil domain. There are already many TRIM proteins which are known to interact with and regulate the NFκB pathway, but recently, a new possible regulator of this pathway has been found in TRIM29.

TRIM29 is a novel, but still widely unknown, member in the regulation of the innate immune system. It has been shown to be an important factor in the DNA damage response, as well as promoting cell-survival and proliferation through implications in the regulation of the Wnt and the p53 pathway. In correlation with these findings it is also a biomarker and possible oncogene in several forms of cancer. Furthermore, recent studies in lung and bladder cancer cell lines have found TRIM29 to promote proliferation and cell-cycle progression through activation of the NFκB pathway. The exact mechanism behind this activation is still unknown. However, due to the fact that both the IKK protein NEMO as well as TRIM29 perform functions in the DNA damage response, as well as being found in yeast-two-hybrid screens to be possible interaction partners, we hypothesized that TRIM29 may activate NFκB through an interaction with the IKK complex.

In this study we investigate whether TRIM29 interacts with members of the IKK complex in an attempt to discover the underlying mechanism in a TRIM29 dependent NFκB activation. We approach this topic through co-immunoprecipitation experiments to identify one of the IKKs as possible interaction partner of TRIM29. In addition we set out to determine co-localization of TRIM29 and the IKK protein NEMO through immunofluorescence. To further explore the relevance of TRIM29 in the activation of NFκB through various DNA damaging and pathogenic stimuli, we established western blot based assays to compare NFκB activation in wild-type and *Trim29* KO cells. Lastly, we also performed MTT assays to research the influence of TRIM29 on cell survival against DNA damaging and pathogenic threats.

Concluding these experiments, even though we were not able to confirm an interaction of TRIM29 with the IKKs or an NFκB activating effect, the available data on TRIM29 suggests a complex interaction with the NFκB pathway, which alternates between cellular environments.

Introduction

NFκB is a manifold factor present in many different cell types and has important implications in cellular processes like the innate immune system, proliferation and apoptosis. It was first published in 1986 by Sen and Baltimore as a regulator for the κB light chain expression in mature B-cells (Sen and Baltimore, 1986a, 1986b). Following this first discovery, more and more scientists began investigations regarding this remarkably versatile protein complex and with time, more and more aspects of NFκB have been discovered.

The knowledge of the vast network surrounding NFκB up till now includes dozens of activating or repressing stimuli, co-factors, interactors as well as hundreds of target genes which feature a binding-motif for NFκB and rely on its function as transcription factor. As one of the major components of the innate immune system, it is activated by a broad range of stimuli which reach from viral and bacterial infections to cytokines like TNF-α and IL-6. In this context, NFκB is essential for the function of the innate immune system and the survival of the infected host.

Additionally, it can also become activated by numerous non-infection stimuli to perform important functions aside from an immunological standpoint, like proliferation and cell growth. The importance of these processes is further underlined by the sheer amount of malign effects and diseases resulting from malfunctioning NFκB activation, including auto-immune diseases and cancer. This points out the importance of regulation in this matter, ensuring fast and specific activation when needed, as well as effective down-regulation when not needed anymore to prevent damage to the host. Regulation is ensured by a system of activators and repressors, concentrated around a single main-repressor, the inhibitor of κB (IκB), and on modifications of the NFκB subunits themselves.

Structure of the NFκB complex

The family of NFκB transcription factor proteins consists of five members, p65 (RelA), RelB, c-Rel, p50/p105 (NFκB1) and p52/p100 (NFκB2), which localize inside the cytoplasm in the form of either heterodimers or homodimers to form the functional complex (Chen and Ghosh, 1999; Gerlach et al., 2011) (Figure 1). Only p65, RelB and c-Rel possess a transcriptional activation domain (TAD), making them competent to perform functions as a transcription factor. p50 and p52 lack these domains and therefore homodimers of these proteins are transcriptional inactive (Hayden and Ghosh, 2008). They are both expressed in the form of larger precursor proteins, p105 and p100 respectively, which are further processed into their shorter form by ubiquitin dependent proteasomal processing (Palombella et al., 1994; Xiao et al., 2001).

Overall 15 different combinations of dimers could result out of these proteins, however not all of them have been described to exist or to be of biological relevance. Some of the described dimers are: p50/p65, p50/p50, p65/p65, p65/c-Rel, p65/p52, c-Rel/c-Rel, p52/c-Rel, p50/c-Rel, RelB/p50, and RelB/p52. While p50/p65 being clearly the most abundant combination which is present in nearly every cell, most of the other combinations are more rare, existing only in a limited amount of cell types (Hayden and Ghosh, 2004).

All of the factors in the NFκB family harbor a common 300 amino acid long amino-terminal sequence motif, the Rel-homology-domain (RHD), which is required for both dimerization and DNA binding (Ghosh et al., 1998). Crystal structures of p50 homodimers and p50/p65 heterodimers have shown, that the amino-terminal part of the RHD seems to be required for DNA binding, especially to the NFκB binding sequence on target genes, whereas the C-terminal part of the RHD seems to be important for dimerization (Chen et al., 1998; Ghosh et al., 1995). Additionally, there is another factor involved in the NFκB complex, namely ribosomal protein S3 (RPS3), which seems to be essential for the Rel dimers to get full binding and transcriptional activity (Wan et al., 2007).

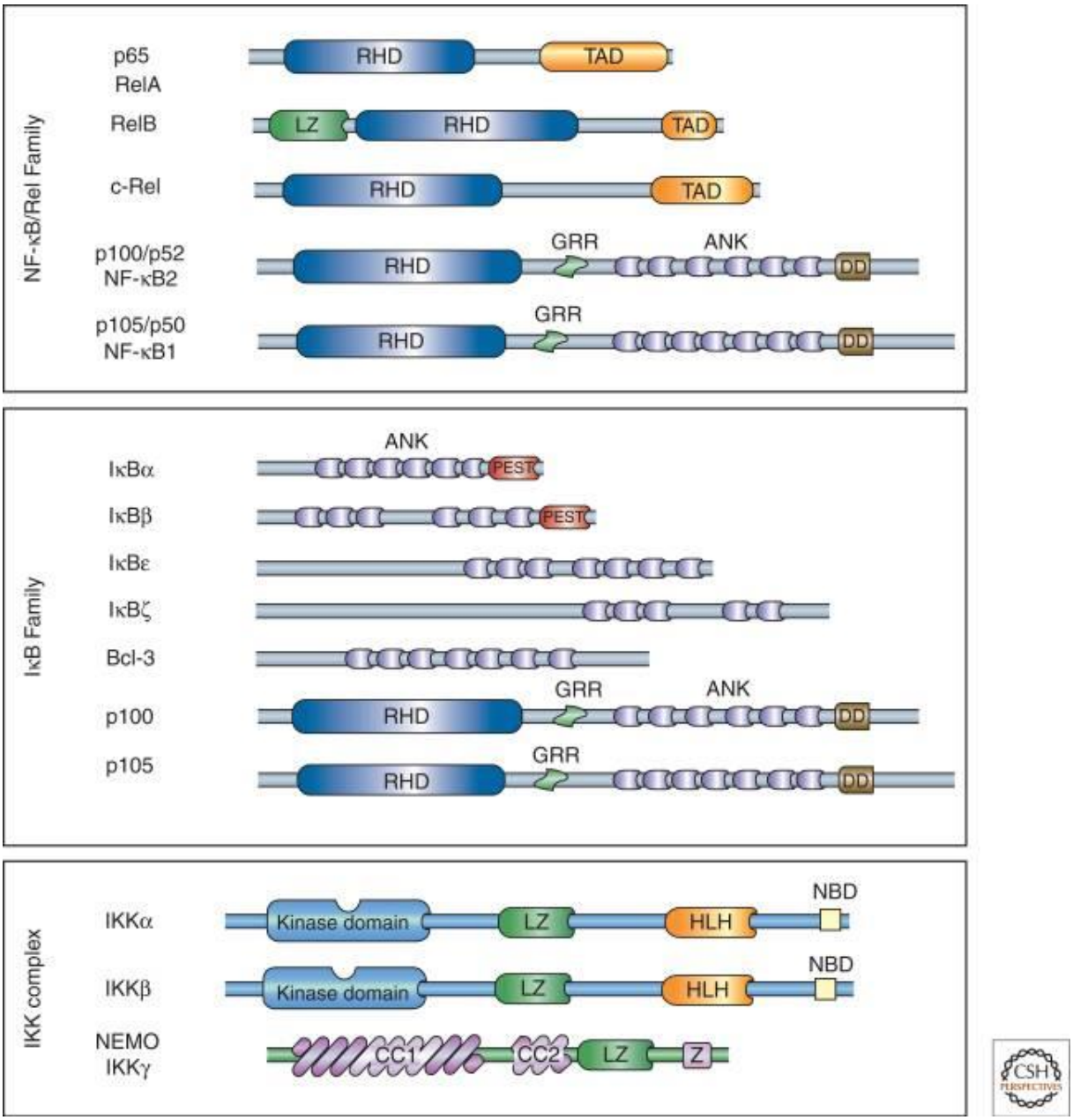


Figure 1. Members of the NF-κB, IκB, and IKK protein families. The Rel homology domain (RHD) is characteristic for the NF-κB proteins, whereas IκB proteins contain ankyrin repeats (ANK) typical for this protein family. The precursor proteins p100 and p105 can therefore be assigned to and fulfill the functions of both the NF-κB and IκB protein families. The domains that typify each protein are indicated schematically. CC, coiled-coil; DD, death domain; GRR, glycine-rich region; HLH, helix-loop-helix; IKK, IκB kinase; LZ, leucine-zipper; NBD, NEMO binding domain; PEST, proline-, glutamic acid-, serine-, and threonine-rich region; TAD, transactivation domain; ZF, zinc finger.

From: Oeckinghaus, A. & Ghosh, S. The NF-κB Family of Transcription Factors and Its Regulation. *Cold Spring Harb. Perspect. Biol.* 1, (2009).

Activated NFκB binds to the NFκB consensus sequence 5'-GGGPuNNPyPyCC-3', which is located in the regulatory elements of NFκB target genes. This consensus sequence is essential for NFκB binding, however it is not sufficient (Chen et al., 1998). There are about $1.4x10^4$ or more potential binding sites spread throughout the whole human genome, however only a small subset of relevant κB binding sites are bound at a given time and the composition of these subsets is further

defined by the stimulus which leads to the activation of the NFκB pathway. This specificity for certain binding sites is controlled by a subset of both modifications to the chromatin, which are required to allow access to the embedded binding motif itself (Natoli, 2006; Natoli et al., 2005), as well as by modifications to and interactions with the NFκB complex itself.

The actual sequence of the NFκB consensus sequence is very often highly degenerated, and yet NFκB can bind many of them with high affinity. Most of the variation in these consensus sequences happens in the 3' part of the motif, whereas the 5' part (GGGPu) seems to be more consistently conserved (Natoli et al., 2005). These variations do generally not lead to dimer-specificity for certain combinations of Rel proteins. There is no indication that certain sequences correlate with specific features of certain dimers. Also some dimers can be exchanged on the same gene over time and some genes can even bind all of the NFκB dimers without any specificity (Saccani et al., 2003). Still, it is clearly shown that some genes require binding of a specific set of NFκB proteins for transcription (Hoffmann et al., 2003). Also it seems that even slight variations in binding affinity can have a significant biological effect (Udalova et al., 2002).

In addition to that, NFκB dimers seem to be highly flexible due to a short linker region connecting the N-RHD to the C-RHD, which gives the proteins the ability to twist and rotate relative to each other. This further increases the specificity of certain dimers for DNA binding, as they are able to alter their conformation in favor of a stronger DNA-protein interaction (Chen et al., 1998).

Another layer in terms of controlling NFκB specificity is the chromatin structure. It has been shown, that some genes, despite having similarly strong binding affinity, respond very differently concerning recruitment of NFκB. Time frames from 10 minutes to 2 hours post activation have been observed in similar sites and even between identical genes, but in different cell types (Saccani et al., 2001). This is due to initial accessibility of the certain site to the transcription factor. Very often, an NFκB binding sequence is inaccessible due to surrounding chromatin structure, and often times, cooperation with additional factors is required to make certain sites accessible for the transcription factor. This is a very important step in regulation of NFκB activity, as it allows more specification on the target genes that have to be turned on as well as it allows turn-on of the genes in a timed manner. These accompanying factors can either be direct chromatin modifying enzymes or co-factors which are responsible of bending the DNA in a certain way, to make the site accessible for NFκB binding. For example, nucleosome remodeling through SWI/SNIF is a requirement for LPS-induced inflammation in macrophages (Ramirez-Carrozzi et al., 2006).

Moreover, interactions with co-factors can further enhance NFκB activity without having to modify the chromatin structure. A good example for such an interaction is the transcription factor IRF3 (Taniguchi et al., 2001). IRF3 is a transcription factor that is activated upon detection of viral infections by a protein kinase called TBK1 (TANK binding kinase 1). Activated IRF3 can bind special sequence motifs on the DNA called ISRE sites, which leads to the expression of inflammatory cytokines, one of them being interferon β (IFNβ). Interestingly, besides working by itself, it can also bind p65 (RelA) to form stable complexes (Wietek et al., 2003).

Actually, Ogawa *et al.* observed that about one third of LPS-induced NFκB target genes require the formation of a p65/IRF3 complex, which is repressed by the glucocorticoid receptor and therefore also requires glucocorticoid binding for activation (Ogawa et al., 2005). In these cases, a complex is formed which can bind both ISRE and κB sites, whereas always one factor binds its respective site and the other one does not directly bind to the DNA, but rather functions as a necessary co-factor for transcription.

The inhibitor of κB (IκB)

The main act in NFκB activation however focuses on different sets of proteins. Activation of NFκB does not require *de novo* protein synthesis, but resolves around a particular family of repressors, called the inhibitor of κB (IκB) (Beg et al., 1992). The IκB family consists of eight members: IκBα, IκBβ, IκBγ, IκBε, IκBζ, Bcl-3, p100 and p105 (Figure 1). All of them share a characteristic structural set of multiple ankyrin repeats which are required to bind the NFκB dimers at their RHD under resting conditions (Jacobs and Harrison, 1998).

In order for NFκB to be active and perform its function as transcription factor, it has to translocate into the nucleus. However, binding of IκB to the RHD of NFκB masks its nuclear localization signal, preventing it from translocating into the nucleus and therefore sequester it inside the cytoplasm. Precisely, when looking at the two most common members of the two families, IκBα and the p65/p50 dimer, IκBα only binds to p65 and masks its NLS, whereas the NLS from p50 is still exposed. This free NLS together with the nuclear export sequence (NES) from IκBα leads to a constant shuttling of NFκB between the nucleus and the cytoplasm, preventing longer retention times inside the nucleus and therefore gene induction (Ghosh and Karin, 2002).

Degradation of IκBα greatly enhances kinetics of translocation in favor for nuclear localization. Aside from the other members of the IκB family, the protein p100 is not degraded, but processed by the proteasome to form the NFκB subunit p52, which in turn again forms a free and functional complex. Degradation of the other IκBs is marked by multiple phosphorylation events caused by the inhibitor of κB kinase complex (IKK complex), followed by K48 linked ubiquitination and transport to the proteasome.

Structure and function of the IKK complex

The IKK complex is a trimeric protein complex consisting of the two highly homologous serine/threonine kinase subunits IKKα/IKK1 (CHUK) and IKKβ/IKK2 (IKBKB) as well as the regulatory subunit IKKγ/NEMO (NFκB essential modulator) (Häcker and Karin, 2006) (Figure 1). NEMO is a scaffold protein and has no catalytic functions by itself, but is essential for the canonical NFκB pathway. IKKα (85kDa) and IKKβ (87kDa) share about 50% sequence homology and both harbor an N-terminal helix-loop-helix kinase domain, a zinc-finger dimerization domain and a C-terminal NEMO binding domain (Israël, 2010). IKKα also features a predicted nuclear-localization signal (Sil et al., 2004). Kinase activity is dependent on lysine 44 and two T-loop serines, S176 and S180 for IKKα as well as S177 and S181 for IKKβ (Ling et al., 1998; Zandi et al., 1997). Mutations of these Ser to Ala residues renders the proteins inactive, whereas a phosphomimetic mutation to Glu results in an constitutively active complex.

How phosphorylation of these T-loop residues occurs is still not entirely clear. There is evidence that TAK1 acts as an IKK kinase, and it has indeed been shown that TAK1 can phosphorylate IKKβ in the activation loop. Furthermore, down-regulation of TAK1 reduces IKK activation and mutations in the TAK1 gene in Drosophila interfere with NFκB pathways (Silverman et al., 2003). However, tissue specific deletions of TAK1 seems not to impair NFκB activation in response to B-cell antigens (Sato et al., 2005).Another likely candidate for IKK phosphorylation is MEKK3, as cells which lack MEKK3 show impaired NFκB activation in response to certain stimuli (Huang et al., 2004; Yang et al., 2001).

Finally, it would also be possible that phosphorylation is achieved in an autophosphorylation type manner. This might be the case in NFκB activation through certain virus derived proteins like the HTLV protein Tax (Israël, 2010), which seems to require conformational change, rather an upstream kinase. IKKα can also perform NFκB independent functions and is known to bind the promoter regions of certain estrogen responsive genes like c-Myc and cyclin D1 to activate their transcription

through formation of a transcription complex with the estrogen receptor ErR and the co-activator AIB1/SRC-3 (Park et al., 2005).

The role of NEMO

NEMO is a small (48kDa) molecule without catalytic activity, but important regulatory functions which are required for the canonical NFκB pathway as well as several other cellular processes. Structurally, x-ray crystallography has shown that it is a long dimeric coiled-coil structure over the whole molecule aside from the C-terminus (Rushe et al., 2008). The N-terminus (aa 47-120 in human NEMO) is responsible for interaction with the other IKK subunits (Marienfeld et al., 2006), especially aa 44-111 are required for binding to aa 701-746 in IKKβ (Rushe et al., 2008). This forms an asymmetric 4-helix bundle, made of a parallel NEMO dimer, where each monomer is bent in a crescent α-helical shape. Each of these monomers binds a single, also mainly α-helical IKKβ peptide by hydrogen bonding and hydrophobic interactions (Rushe et al., 2008). Aside from that, the protein features two coiled-coils (CC1, aa 100-196 and CC2, aa 255-291), a leucine-zipper (LZ, aa 300-343) and a C-terminal zinc-finger (ZF, aa 394-419). The ZF seems to be required for efficient IκBα binding, recruiting it to the kinase subunits.

NEMO can specifically recognize poly-ubiquitin chains through its CC2-LZ region and therefore plays a central role in ubiquitin dependent IKK activation (Laplantine et al., 2009; Wagner et al., 2008). Mutations in this ubiquitin binding region (UBAN region), which impair ubiquitin binding, seem to be a cause for people to suffer on ectodermal dysplasia with immunodeficiency (EDA-ID) (Hubeau et al., 2011). The NEMO UBAN domain is able to bind different kinds of poly-ubiquitin chains, mainly being M1 linked and K63 linked chains. The actual binding properties and preferences as well as the exact biological outcome of these binding differences have not been determined yet.

NFκB activation

As mentioned before, NFκB can be activated through a broad range of both exogenous and endogenous stimuli. Two of the most prominent families of immune receptors working for the innate immune system are the toll-like receptor (TLR) and the RIG-like receptor (RLR) families.

IKK activation generally can be divided in a canonical and a non-canonical pathway (Mitchell et al., 2016) (Figure 2). The canonical pathway, the more abundant of the two, is started through recognition of a broad range of stimuli. Cytokines (like TNFα, IL-1), activation of pattern-recognition receptors (PRRs) (like TLRs, RIG-I) through bacterial or viral infections, radiation and stress signals can all lead to its activation.

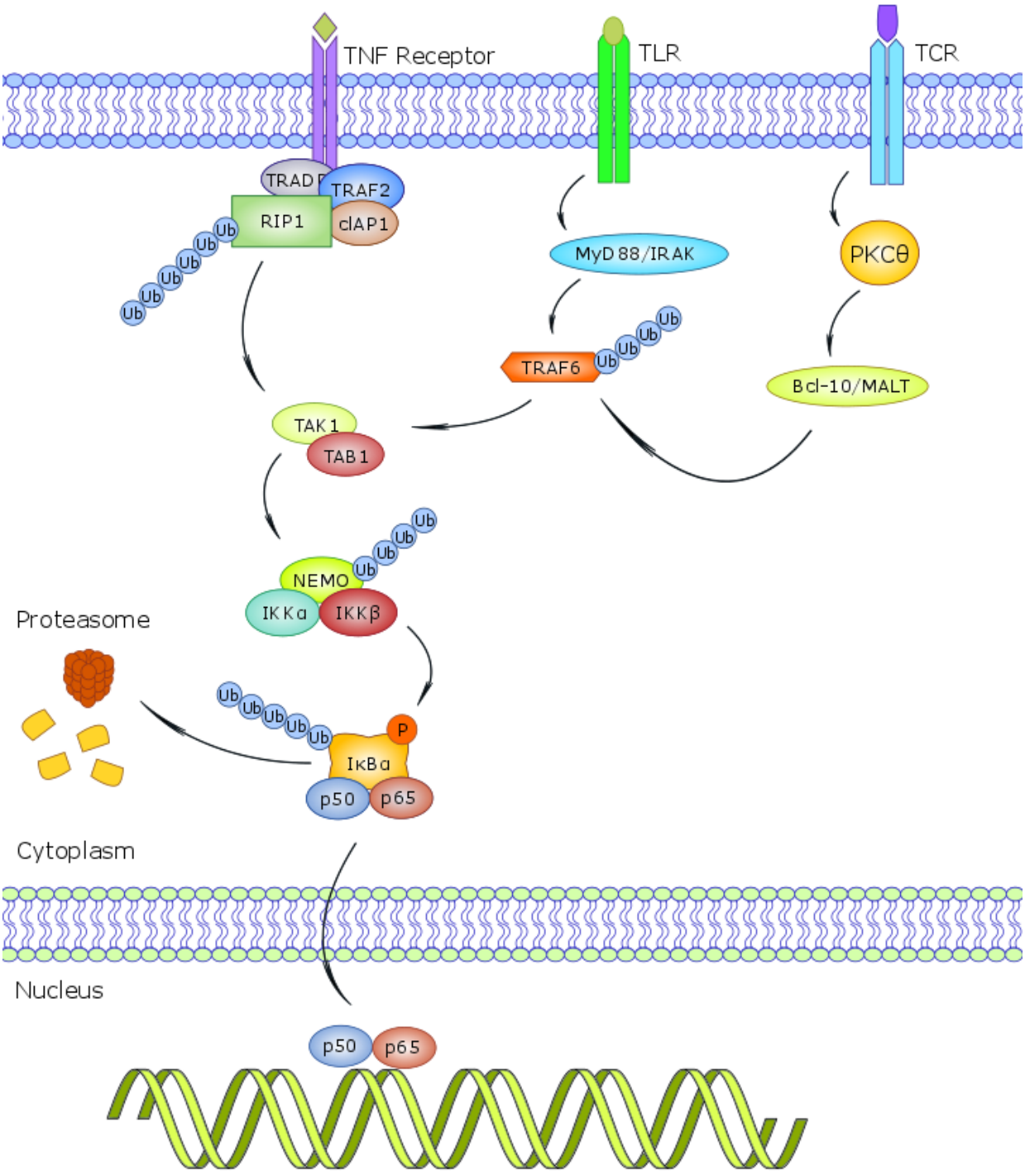


Figure 2. Depiction of the canonical NFκB pathway through TNF receptor, T-cell receptor or Toll-like receptor activation. Binding of TNF to the TNF receptor recruits TRADD, TRAF2, the E3 ligase clAP1 and/or 2 as well as RIP1. RIP1 becomes ubiquitinated and subsequently activates TAK1 which further more activates the IKK complex. Activation of TLRs leads to signaling through MyD88 and IRAK and further TRAF6 ubiquitination. Activation through the TCR leads to the ubiquitination of TRAF6 through PKC and further Bcl-10 and MALT. Ubiquitinated TRAFs can further activate the TAB/TAK1 complex. The IKK complex then promotes phosphorylation and further K48 linked ubiquitination of IκBα, which results in its degradation by the proteasome. The freed NFκB complex, depicted as p50/p65, can then translocate to the nucleus and perform its functions as transcription factor.

TLRs recognize a broad range of pattern-associated-molecular patterns (PAMPs), which are common structures exclusively expressed by pathogens and include structures like lipopolysaccharide (LPS), cell wall components (like peptidoglycan), bacterial flagellin as well as double-stranded RNA and bacterial DNA (Mahla et al., 2013; Silhavy et al., 2010). There are in total 10 TLRs in the cells of the human organism (TLR1-TLR11, no TLR10 in humans), which all have specificity for a specific type of PAMP (Medzhitov et al., 1997; Takeda and Akira, 2005). TLRs are a type of type I trans-membrane proteins and are characterized by an extracellular domain which contains leucine-rich repeats (LRRs) and a cytoplasmic part which contains a conserved region, called the Toll/IL1-receptor (TIR) domain (Botos et al., 2011; O'Neill and Bowie, 2007). They are localized in the plasma membrane, with the exceptions of TLR3, TLR7 and TLR9, which are located in endosomal compartments (Nishiya and DeFranco, 2004).

Upon binding of a TLR to a certain PAMP, it recruits a number of co-factors and adaptor proteins to its TIR domain (Figure 2). One of them, which is common to all TLRs except TLR3, is MYD88 (Adachi et al., 1998). Signaling pathways through MYD88 lead to the induction of inflammatory cytokines, whereas some TLRs also have a MYD88 independent pathway, which is important for IFNβ induction. Recruitment of MYD88 leads to binding of IRAK1 and IRAK4, after which IRAK1 is phosphorylated by IRAK4 (Kawasaki and Kawai, 2014). After that, the two proteins leave MYD88 and associate with TRAF6, which gets in turn K63 auto-ubiquitinated through additional input by the adaptor proteins BCL10 and MALT1 (Sun et al., 2004). Subsequently, TRAF6 then forms a complex with the TAB1/TAB2/TAK1 complex and further induces TAK1 activation (Kanayama et al., 2004). Activated TAK1 is in turn one of the most prominent candidates for IKK activation through NEMO recruitment.

In contrast, activation trough the TNF-receptor pathway works in a different manner. Binding of the substrate to the receptor leads to the formation of complex I at the membrane bound receptor, and comprised of TRADD, RIP1 and TRAF2 (Figure 2). This complex promotes the activation of the ubiquitin ligases clAP1 and clAP2 as well as its kinase RIP1, which are further implicated in the activation of the NFκB pathway. In this process RIP1 gets K63 ubiquitinated which is thought to recruit TAK1 as well as IKKs (Oeckinghaus et al., 2011). It resolves around a signaling cascade and ubiquitination events that result in the activation of the IKK complex with IKKα and IKKβ as the catalytic subunit responsible for IκBα phosphorylation and NEMO as crucial adaptor. Though IKKβ is the more prominent subunit in this pathway, the two catalytic subunits seem to be somewhat redundant. Phosphorylated IκBα is then further K48 ubiquitinated by the E3 ubiquitin ligase SCF-β-TRCP and degraded by the proteasome.

In another step within one hour after activation, TRADD, RIP1 and TRAF2 dissociate from the TNF-receptor and bind FADD (fas-activated death domain), caspase-8 and caspase-10, leading to the formation of complex II. Opposed to complex I, complex II is pro-apoptotic and is thought to perform its apoptosis inducing functions only upon defective NFκB signaling or insufficient amounts of anti-apoptotic proteins such as XIAP and FLIP. The transformation of complex I into complex II thus represents a checkpoint to sort out cells with defective NFκB signaling.

Another TNF-receptor dependent signaling cascade, the non-canonical pathway is much more strict concerning its activation and is only triggered by a few members of the TNF cytokine family, including the CD40 ligand, BAFF and lymphotoxin β (Hayden and Ghosh, 2012; Sun, 2011). This pathway leads to the posttranslational stabilization of NFκB inducible kinase (NIK) which features an intrinsically active kinase domain (de Leon-Boenig et al., 2012).

Under resting conditions, NIK is K48 ubiquitinated by a TRAF-cIAP complex and degraded. Upon activation of the non-canonical pathway, the complex is recruited to the TNF receptor. K63 ubiquitination of cIAP 1/2 by TRAF2 leads to a shift from K48 ubiquitination of NIK to ubiquitination of TRAF3 (Vallabhapurapu et al., 2008; Zarnegar et al., 2008). NIK is then thought to phosphorylate IKKα, which is therefore activated and able to phosphorylate the IκB family member p100 to mark it

for C-terminal processing to generate p52 containing complexes. This phosphorylation event is completely independent of IKKβ or NEMO (Sun, 2011). Attenuation of the pathway is caused by the deubiquitinase (DUB) OTUD7B, which removes the K48 ubiquitination on TRAF3.

RLRs are a family of cytoplasmic PRR which are in charge of recognizing foreign dsRNA and DNA (Onoguchi et al., 2010). The two most prominent members of the family being RIG-I (retinoic-acid-inducible protein 1), which is specific for 5'-ppp RNA, and MDA5 (melanoma differentiation-associated gene 1), which recognizes dsRNA fragments (Yoneyama and Fujita, 2007).

RIG-I and MDA5 contain a DExD/H box RNA helicase and two caspase recruiting domains (CARD) (Leung and Amarasinghe, 2012; Yoneyama et al., 2005). The C-terminal domain binds to their RNA targets and the CARDs are in charge of relaying the out-coming signal to their downstream targets. Despite their overall similarity, the two proteins bind different kinds of RNA, with RIG-I having a preference for both shorter single-stranded and double-stranded 5'-ppp RNAs and MDA5 binding to longer forms of dsRNA (Kato et al., 2008; Pichlmair et al., 2006). Upon binding of RNA, both RIG-I and MDA5 translocate and bind to MAVS (also called IPS-1), located at the mitochondrial membrane (Kawai et al., 2005). This triggers further signal to activate several transcription factors which includes, alongside TBK1/IKKε dependent IRF3 and IRF7 activation, also NFκB activation in a RIP1 and TRAF6 dependent manner (Loo and Gale, 2011).

TRAF6 was identified as the first ligase, that together with Ubc13 and Uev1A, could trigger K63 linked auto-ubiquitination on target proteins and subsequently trigger IKK activation (Chen, 2012). Aside from the TLR and RLR pathways, it plays also an important role in the IL-1R and TNFR pathways. However, opposed to the other pathways, IKK activation in the TNFR pathways is not entirely dependent on K63 ubiquitination (Xu et al., 2009). Precisely, linear M1 linked ubiquitination of NEMO and RIP1, mediated by the linear ubiquitination assembly complex (LUBAC), seems to be the main activator in this pathway (Gerlach et al., 2011; Tokunaga et al., 2009). LUBAC is a trimeric E3 ubiquitinase complex consisting of the proteins HOIL1, HOIP and SHARPIN and is recruited to the TNFR in a TRADD, TRAF2 and cIAP 1/2 dependent manner. Besides TNFα, LUBAC mediated M1 ubiquitination seems play a role in IL-1, CD40, parkin, TACI and DNA damage induced NFκB activation (Iwai, 2012).

NFκB in DNA damage response

Cells are continuously exposed to foreign influences which pose a threat to their genomic integrity. This can be DNA damaging reagents, radicals or radiations, which lead to breaks in the DNA chain or to mutations. The DNA damage response (DDR) is a set of mechanisms to constantly check the DNA for such damages, repair them and keep the genome intact. Alternatively, depending on the type of damage, it can also lead to cellular senescence and apoptosis.

The main players in initiating DDR are a family of phosphoinositol-2-kinase like proteins: ATM, ATR and DNA-PK, whose actions coordinate downstream effects, often through phosphorylation events (Maréchal and Zou, 2013; Yang et al., 2003). Recruitment of these factors to break sites is mediated by special phosphopeptide binding motifs (FHA), which are present in many of the accompanying co-factors important for the DDR (Durocher et al., 1999). These co-factors often incorporate enzymatic activities like ubiquitination, SUMOylation and (poly) ADP-ribosylation.

One of the most severe forms of DNA damage are DNA double-strand breaks (DSBs). DSBs are sensed by the DDR factor ATM, which has a major role in the activation of downstream transcription factors, like p53, to activate counter-measures. Alongside many others, it is shown that also NFκB is activated in an ATM dependent manner upon DSBs (Huang et al., 2003; Piret et al., 1999).

NFκB activation through DNA damage depends on posttranslational modifications of NEMO and relies on its zinc-finger region, which is not an essential requirement in the canonical NFκB

pathway, but plays a crucial role in response to DNA damage. The most striking modifications seem to be, aside from phosphorylation and ubiquitination, SUMOylation of NEMO on its lysine residues 277 and 309, which is mediated by the SUMO E3 ligase PIASy (protein inhibitor of activated STATy; PIAS4) (Mabb et al., 2006) (Figure 3). Mutations of these residues completely abrogates NFκB activation through DNA damage. However, it seems to have no major effect on canonical NFκB activation. Genomic data also suggest that the ZF domain of NEMO seems to be required for SUMOylation, but it is not exactly clear which role the ZF domain plays in this context. Binding of NEMO to PIASy does not require the ZF region, but is dependent on the N-terminal CC1 domain. Interestingly, binding of PIASy and IKKβ to NEMO are mutually exclusive, as the binding locations of NEMO:PIASy and NEMO:IKKβ overlap (Mabb et al., 2006). This further underscores NEMOs exclusive role as a singular factor, being independent of the IKK complex during activation.

Shown by several reports, NEMO SUMOylation seems to alter its cellular localization, preferring a nuclear state. NEMO SUMOylation is also mediated by additional factors, the p53 inducible death domain-containing protein (PIDD), which is known for its function in caspase-2 activation and together with RIP1 sits in the nucleus forming a signaling complex called the PIDDosome to promote SUMOylation (Janssens et al., 2005). The relevance of these observation is further bolstered, by RIP1 being required of NFκB activation through genotoxic agents (Hur et al., 2003). However, RIP1 also has to perform an essential cytoplasmic role in NFκB activation (Yang et al., 2011). Furthermore, apoptotic responses to genotoxic reagents in *Pidd* $^{-/-}$ MEFs were largely undisturbed, indicating PIDD being non-essential in this context (Manzl et al., 2009).

Another important protein for genotoxic NEMO activation is poly (ADP-ribose) polymerase 1 (PARP-1). PARP-1 has a role in forming a nuclear complex consisting of PARP-1, NEMO, ATM and PIASy. Upon recruitment to DNA damage, PARP-1 causes several PAR modifications to different subunits, as well as auto-modifications (Krishnakumar and Kraus, 2010). NEMO is getting recruited to the complex by a binding with PARP-1 and together with the NEMO: PIASy binding, maps to the same N-terminal region that excludes IKK-binding. PIASy recruitment is mediated by binding to a PAR domain on modified PARP-1 via two PAR binding domains and ultimately promoting SUMOylation of NEMO.

Additionally there are further studies concerning the role of PARP-1 in DNA damage induced NFκB activation. Publications show that PARP-1 is an important co-factor for p65 in NFκB transcriptional activity upon DNA damage (Hassa and Hottiger, 1999). This function is independent of its DNA binding capacity or its catalytic activity. Furthermore, it has been shown that *Parp-1*$^{-/-}$ MEFs have no impairment in IκB degradation or p65 translocation to the nucleus upon ionizing radiation, but binding to κB sites is impaired (Veuger et al., 2009).

Besides SUMOylation, also phosphorylation and ubiquitination play an important role in DNA damage induced NEMO activation. Following DSBs, NEMO is phosphorylated on S85 by ATM itself (Wu et al., 2006) (Figure 3). Mutation of this serine residue to alanine does not impair SUMOylation, but inhibits mono-ubiquitination of NEMO. The E3 ligase responsible for NEMO ubiquitination on position K277/K309 during genotoxic stress is cIAP1 (Jin et al., 2009).

Ubiquitination of NEMO by clAP1 requires both ATM kinase activity as well as an intact S85 site. This ubiquitination is deemed necessary for NEMO activation and export to the cytoplasm to coordinate with the rest of the IKK complex, following exposure to VP16 (Etoposide). 1.3E2 cells expressing K277A and K309A mutant forms of NEMO fail to activate IKK and NFκB following diverse genotoxic stimuli and have increases susceptibility against ionizing radiation. Surprisingly, a fusion protein with an ubiquitin moiety fused in frame onto the N-terminus of S85A mutated NEMO restores IKK and NFκB activation, but is still dependent on ATM activity (Wu et al., 2006). This implicates further ATM involvement in this pathway downstream of NEMO ubiquitination.

The relation between SUMOylation and ubiquitination is not entirely clear as both use the same lysines as acceptor sites. However, kinetic analysis implicates that NEMO undergoes transient

SUMOylation events before being subsequently phosphorylated and ubiquitinated (Huang et al., 2003). It seems, that both have a function in subcellular localization, in with SUMOylation is responsible for nuclear retention, whereas ubiquitination seems to promote a cytoplasmic localization.

Moreover, ubiquitinated NEMO can transfer a small portion of ATM to the cytoplasm and correlates with the emergence of a NEMO:ATM:IKK complex which further facilitates NFκB activation (Wu et al., 2006). A first hint for the cytoplasmic activity of ATM came from Jin *et al.* who show, that TAK1, which is important for IKK activation in the canonical pathway, is also important for DNA damage induced IKK activation (Jin et al., 2009).

TAK1 is part of a multi-subunit complex together with TAB1, TAB2 or the related protein TAB3. It is activated by binding of the TAB subunits to K63 linked ubiquitin chains and in the canonical NFκB pathway facilitates phosphorylation of the T-loop serines 177 and 181 of Ikkβ. Several groups have now demonstrated, that ATM dependent TAK1 activation is necessary for DNA damage induced IKK activation (Hinz et al., 2010; Wu et al., 2010; Yang et al., 2011). Although the exact matter of TAK1 activation is not entirely clear, several models have been proposed for mechanisms involving this process.

The research of Jin *et al.* demonstrates that TAB/TAK activation is dependent on the E3 ligase XIAP (Jin et al., 2009). In detail, it specifically requires the intact BIR and RING domain of XIAP, showing a dependence on its catalytic activity. In addition, TAK1 activation requires polyubiquitination of ELKS, which has been shown to be required for DNA damage induced NFκB activation before (Ducut Sigala et al., 2004) (Figure 3). Investigations in cells derived from $Elks^{-/-}$ mice have shown that TAK1 activation is defective in the absence of ELKS (Wu et al., 2010). The interaction between ELKS and TAK1 is dependent on ATM and NEMO whereas the polyubiquitination of ELKS seems to be dependent on the activity of the ubiquitin ligase XIAP in combination with the K63-specific E2 ligase Ubc13. Polyubiquitination of ELKS results in the formation of a NEMO, ATM, ELKS and TAK1 containing complex in the cytoplasm, whereas TAK1 binding to ELKS through the ubiquitin binding ability of TAB2. Even though TAK1 recruitment ultimately requires NEMO, ELKS and ATM, the E3 ligase activity of XIAP is required for full TAK1 activation (Figure 3).

A second model in ATM dependent TAK1 activation includes the interaction of ATM with TRAF6 (Hinz et al., 2010). Hereby, ATM is exported to the cytoplasm and interacts with TRAF6 through a TRAF6 interaction motif on ATM. This then activates TAK1 in a TRAF6 dependent manner, however the mechanism behind this process is not entirely known.

Another mechanism in TAK1 activation involves K63 polyubiquitination of RIP1 (Yang et al., 2011). Hereby, RIP1 is first SUMOylated by PIASy in a DNA damage dependent matter. This is required for further ubiquitination in the nucleus. Afterwards, a cytoplasmic complex is formed containing at least RIP1, NEMO, ATM, TAK1 and IKKβ. RIP1 mutants which cannot be SUMOylated fail to interact with TAK1, whereas an in frame fusion of mutated RIP1 with polyubiquitin restores TAK1 activation.

Which one and if any of these models is in function under certain conditions is however still a matter of investigations.

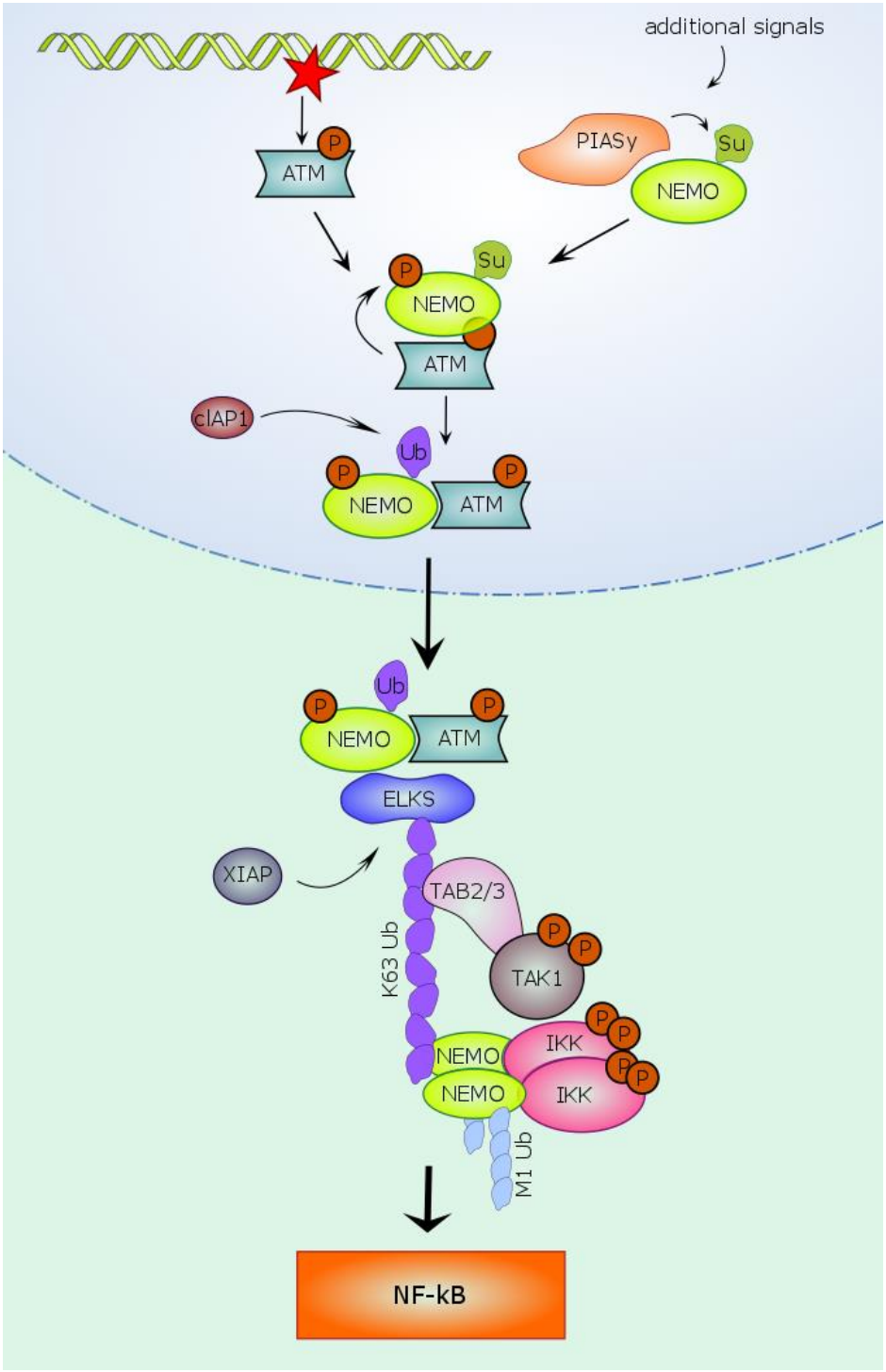


Figure 3. One of the possible pathways by which DNA damage can activate NFκB. Genotoxic stress leads to the phosphorylation of ATM and at the same time NEMO is SUMOylated by the E3 likase PIASy. Afterwards NEMO becomes phosphorylated by ATM, which is a requirement for further ubiquitination by clAP1. Ubiquitinated NEMO translocates into the cytoplasm together with ATM to recruit ELKS and the TAB/TAK1 complex. ELKS is K63 ubiquitinated by XIAP, which is required for TAB2 binding and further TAK1 activation. TAK1 then phosphorylates IKKβ, resulting in IKK-complex and further NFκB activation. In other models, depending on the genotoxic stress and additional signals, TAK1 can also be activated in a RIP1 or TRAF6 dependent manner. Also activation of NEMO in the nucleus through a complex called the PIDDosome is possible.

Regulation of NFκB activity

Errors in the correct execution of NFκB activity have often times quite substantial effects. In most of the cases the symptoms of such malignancies are chronic inflammatory diseases, immunodeficiencies and cancer (Courtois and Gilmore, 2006). An example for one of the first inheritable genetic diseases which has been discovered and is based on a defective NEMO subunit is Incontionentia Pigmenti (Berlin et al., 2002). It is characterized by strong inflammation in the skin, followed by hyperproliferation of keratinocytes and accumulation of melanocytes. At the final step, the cutaneous areas regress and result in hypopigmented areas. It is embryonically lethal in males and therefore only occurs in females, which is caused by the fact that NEMO is encoded at the X-chromosome (Xq28). The cause of the disease is shared by 80% of the affected individuals and is a deletion of exon 4-10 of the NEMO gene (Smahi et al., 2000). The resulting truncated NEMO form is still able to bind IKKα and β, but is unable to activate the complex.

Another interesting example of NFκB dysregulation is Hodgekins Lymphoma (HL), a common B-cell lymphoma. HL can have various causes, but approximately 10% are caused due to IκBα loss-of-function mutations. These are marked by small insertions, deletions or point mutations in an IKBA allele and a deletion or inactivation of the second allele (Wood et al., 1998). As a consequence of this constitutively active NFκB, many of its target genes are overexpressed, including the proliferative factors cycline-D1, CD86 and CD40 (Hinz et al., 2001). Interestingly, reactivation of IκBα can lead to apoptosis of these cells.

Aside from that, about 50% of HL and about 10-20% of non-Hodgkin B-cell lymphomas show a significant abundance of REL proteins. This is caused by gene duplications which leads to 4-75 copies of the corresponding REL gene in the cancer cells genome (Gilmore et al., 2004). Taken together, there are many well described diseases caused by dysfunctional NFκB, not limited to NEMO, which underlines the importance of the regulation of this pathway.

Regulation through IκB negative feedback

The main regulatory mechanism of NFκB is through is inhibitor IκB. Under native conditions, it keeps NFκB sequestered and inactive in the cytoplasm. In order to free NFκB, IκB has to be degraded. In the case of IκBα, degradation is a rapid process, taking only minutes after incoming TNF or LPS stimulus until it is fully degraded. The exact amount of time however strongly varies dependent on the stimulus. Following activation, NFκB has approximately up to one hour of time until activity is efficiently shut off by a negative feedback mechanism (Werner et al., 2008).

This negative feedback mechanism is strongly dependent on IκBα, which is required, but not sufficient in this matter and requires the cooperation of IκBβ, which both have to be transcriptionally induced under NFκB dependent promoters. Re-accumulation of IκBs results again in the binding of the NFκB dimers and their export and sequestering in the cytoplasm. IκBβ seems not to be able to induce this negative feedback mechanism, even when under a NFκB promoter, and requires IκBα to perform this function (Fagerlund et al., 2015). In addition, IκBα has been shown to be able to directly strip NFκB from the DNA, which IκBβ seems not be able to do (Bergqvist et al., 2009).

The combination of effective IκBα feedback together with stimulation conditions that allow for prolonged IKK activation can lead to repeated NFκB activation cycles and oscillatory NFκB activity in these cells (Nelson et al., 2004). This is marked by shuttling of Rel subunits in and out of the nucleus, whereas kinetics is majorly dependent on the transcription of IκBα. It is however unclear what the biological function of this oscillatory mechanism is and how certain downstream networks react to these oscillations (Behar and Hoffmann, 2010).

Just like IκBα and β, IκBδ is an NFκB repressor which is under the control of an NFκB dependent promoter and is therefore part of a negative feedback loop. However, opposed to IκBα, both

transcription and translation occur in a much slower way (Shih et al., 2009). This is partly caused by the sheer length of the protein, and hence the ORF, which has a negative impact on mRNA stability and the subsequent oligomerization steps also take several hours (Savinova et al., 2009). This gives IκBδ a role in attenuating persistent signal, in contrast to IκBα, which more rapidly stops NFκB activity. Additionally, due to it being target of the non-canonical pathway, it is not targeted by attenuated canonical signal and therefore is not a subject of oscillatory activity. Further, due to its longer half-life than other IκBs, it contributes to a signaling memory in which subsequent activations of NFκB are dampened (Shih et al., 2009).

Regulation and posttranslational modifications of NFκB

There are quite a lot of additional feedback mechanisms in the NFκB pathway, both repressing and activating in nature. The de-ubiquitinase A20 is part of the NFκB targets in the TNF pathway and has a role in repressing the pathway by removing activating ubiquitin chains. However, due to its long half-time, its role is less in an acute stop to the NFκB signaling than to dampen sequential activating signals after prior exposure events (Werner et al., 2008).

Also, TNF itself is transcribed in an NFκB dependent manner, but additional co-factors for splicing, mRNA stability, pro-TNF processing and secretion are required. This makes TNF expression more a fast-forward loop upon PAMP detection than a pure positive feedback loop (Caldwell et al., 2014). In conclusion, feed-forward mechanisms are a vital part in organized NFκB function, but they are by themself not sufficient and need further regulations through different means.

Given the wide range and diversity of NFκB functions it is clear that a broad range of regulators have to be in place to control the specific outcomes. We already mentioned IκBs as the prime repressors and the IKKs as the activators of NFκB, but turning the system on and off alone is not sufficient, therefore another layer of regulations has to be in place. Posttranslational modifications represent this layer that is necessary to fine-tune the response. As already mentioned above, many components in the signaling chain of the NFκB pathway are subject to posttranslational modifications like phosphorylation, ubiquitination, SUMOylation and acetylation. All of these modifications play some role in activating or repressing certain factors or are important for binding capabilities. The modifications themselves are also controlled and mostly reversible, if they don't lead to protein degradation.

Besides all the modifications to the subunits of the signaling chain, also the Rel subunits themselves are subject to posttranslational modifications, including phosphorylation, acetylation and ubiquitination (Perkins, 2006). Some of these modifications also represent important means of cross-talk between various other pathways (Perkins, 2007). Degradation of IκB and translocation of NFκB to the nucleus alone is not sufficient for effective gene expression. Modifications to the subunits are necessary for this matter and the best studied subunit for posttranslational modifications is p65.

The first discovered modifier for p65 was protein kinase A (PKA) and it has been shown to be critical for the expression of many, but not all, of the p65 targets (Naumann and Scheidereit, 1994; Neumann et al., 1995). PKA resides in a complex together with IκBα:NFκB in the cytosol and is activated upon IκBα degradation, after which it phosphorylates p65 at position S276. p50 and c-Rel are also modified by PKA at equivalent sites (Perkins, 2006). It is thought that the phosphorylation at S276 on p65 breaks an intramolecular bond between the carboxy-terminal part and the RHD, thereby facilitating DNA binding and interactions with the accessory proteins p300 and CBP (CREB binding protein) (Zhong et al., 1997, 2002).

Not only is S276 phosphorylated by PKA, but also by MSK1 and MSK2 (mitogen – and stress-activated protein kinase). This phosphorylation is thought to possible mediate crosstalk between the NFκB and the ERK and p38 MAPK pathway (Perkins, 2006; Vermeulen et al., 2003).

There are quite a few additional phosphorylation sites in the human p65 protein. S536 in the TAD of p65 seems to be targeted by several kinases, including IKKβ. The main effect of this modification seems to be the control of nuclear localization and interactions with CBP and p300 (Chen et al., 2005). S529 has been shown to be inducibly modified by CK2, but the effect on transcriptional activity is not so clear (Wang et al., 2000). The protein kinase PKCζ has been shown to modify S311 in response to TNF signaling and seems to have a positive effect on p65:CBP interaction (Duran et al., 2003). The purpose of all these modifications seems to be to fine-tune transcriptional activity and not to just turn the activity on or off.

Also acetylation events play an important role in p65 activity. p65 can be acetylated on different sites, most of them by the actions of CBP / p300 and accompanying HATs. Acetylation of K310, which requires prior phosphorylation of S276, has been shown to enhance transcriptional activity without changing DNA binding or IκB binding abilities, probably by creating a binding site for certain co-activators (Chen et al., 2002). Acetylation of K221, in contrast, impedes IκB interactions, thereby enhancing DNA binding. Also, acetylation on lysine 218 has been shown to promote p65 transactivation activity, while acetylation on K122 and K123 has an inhibitory effect. These modifications on p65 have been shown to be counteracted by different histone deacetylases like HDAC3 and SIRT1 (Chen and Greene, 2004; Oeckinghaus and Ghosh, 2009).

Shutting off NFκB activity

Much less is known about the termination of NFκB activity compared to the activation. One of the main shut-off mechanisms involves the synthesis of new IκB subunits, which can be expressed under an NFκB activated promoter. They can bind, inhibit and sequester NFκB as well as directly remove it from the DNA. Combined with deactivation of the inducing stimuli, through dephosphorylation or deubiquitination events, this can effectively turn off NFκB. In addition, there are inhibitory modifications, like acetylation, which can inhibit DNA binding or binding to required co-factors. Because acetylation has been shown to negatively impact DNA binding of p65, interactions with HATs and HDACs can have an important function in NFκB inhibition (Kiernan et al., 2003).

Ubiquitination also plays an important role in the inhibition of NFκB. SOCS-1 can directly ubiquitinate promoter-bound p65, leading to its proteasomal degradation (Ryo et al., 2003; Saccani et al., 2004). Also, the nuclear ubiquitin ligase PDLIM2 ubiquitinates p65 for proteasomal degradation, as well as promoting its translocation to promyelocytic leukemia (PML) nuclear bodies, which are also places for protein degradation, and therefore contributing to NFκB transcriptional silencing (Tanaka et al., 2007). Interestingly, even IKKα has been shown to induce p65 and c-Rel turnover and removal from pro-inflammatory promoter regions in macrophages (Lawrence et al., 2005).

These examples represent just the tip of the iceberg of possible modifiers which are part of the vast network to control this large and diverse pathway. There are still numerous protein families, both investigated and still unknown, which play major to minor roles in this concern. One very interesting protein family in this matter is the family of tripartite-motif proteins (TRIMs).

The family of TRIM proteins

TRIM proteins are unique members of a family of putative E3 ligases with the first member being discovered in 1991 as the Xenopus nuclear factor 7 (Xnf7) (Reddy et al., 1991). Since then, TRIM proteins received a steadily increasing amount of attention and many new members of the family have been discovered up till now. The family of TRIM proteins is characterized by a unique N-terminal tripartite sequence motif, consisting of a RING domain, up to two B-Box domains and a coiled-coil domain (RBCC) (Micale et al., 2012) and a C-terminus which can contain various additional domains. Even though some of the members do not feature the full set of the tripartite-motif domains, they are still highly conserved in order and spacing, making them part of the TRIM family. The order and

function of this RBCC motif is also conserved through various species which clearly marks it as the signature structure of this protein superfamily.

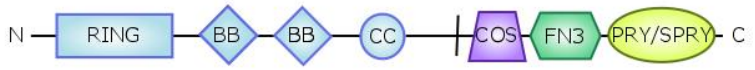
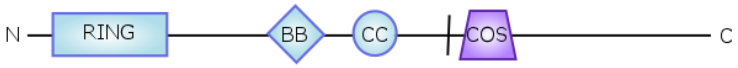
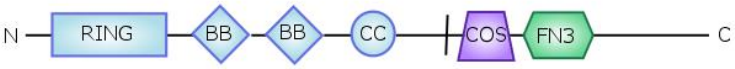
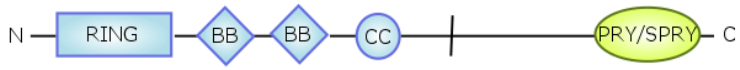
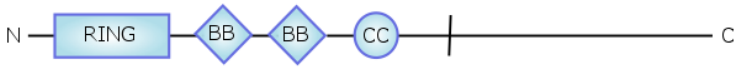
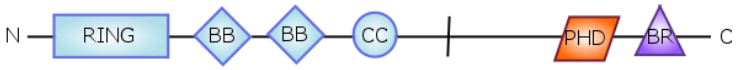
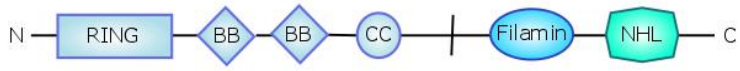
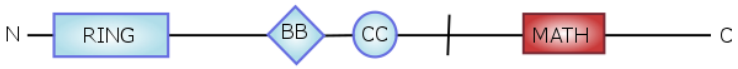
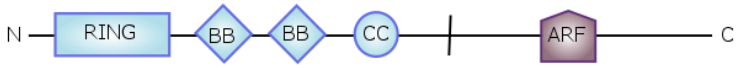
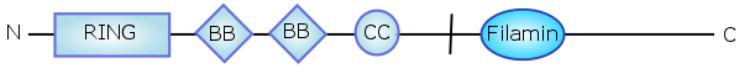
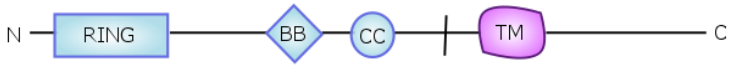
Subgroup	TRIM proteins	Domain Organization
I	1, 9, 18, 36, 46, 67	
II	54, 55, 63	
III	42	
IV	4, 5, 6, 7, 10, 11, 14(ΔRING), 15, 16(ΔRING), 17, 20 (ΔRING), 21, 22, 25, 26, 27, 34, 35, 38, 39, 41, 42, 47, 48, 49, 50, 51(ΔBB, ΔPRY), 53, 58, 60, 62, 64, 65, 68, 69, 70(ΔRING, ΔBB), 72, 75, 1L(ΔBB)	
V	8, 19, 29(ΔRING), 31, 40, 44(ΔRING), 52, 56, 61, 73, 74	
VI	24, 28, 33, 66(ΔRING)	
VII	2, 3, 32, 71	
VIII	37	
IX	23	
X	45	
XI	13, 59	

Table 1. TRIM proteins can be classified into 11 subgroups which differ in terms of their RBCC domain and their C-terminal domains. The TRIM proteins are further differentiated in true TRIM proteins of a certain class and TRIM-like proteins, which lack certain domains otherwise significant for their subgroup.

Up till now, over 70 members of TRIM proteins have been discovered in the human genome and many of them have orthologs in other species as well. However, the function of many of them remains unknown. Structurally, they mainly differ in the composition of their C-terminal domain, therefore which motifs they contain and in which combination. Additionally, some of them also differ in their RBCC motif, as there are TRIMs which lack one of the B-Boxes or even their RING domain (Table 1).

The RING domain is a 40-60 bp long C_3-H-C_4 zinc-finger which binds two zinc-ions and is localized about 10-20 bases from the C-terminus in most of the TRIMs. Its main function is predicted to perform E3 ubiquitin ligase activity, therefore working as an adaptor to transfer single ubiquitin molecules from E2 proteins onto their corresponding target. The structure of the resulting poly-ubiquitin chain is hereby mainly determined by the E2 protein (Joazeiro and Weissman, 2000). However, despite the possibility to be a common feature of TRIM proteins, real E3 ligase activity has not been shown for all RING containing TRIMs and is still a matter of investigations. Additionally, the RING domains of some TRIMs are capable to perform E3 ligase activity for other ubiquitin like moieties, like SUMO and ISG15 (Chu and Yang, 2011).

Following the RING domain are up to two B-Box domains, B1 and B2, which are characterized by 40 bp long C-H-C-(C/D)-C_2-H_2 zinc-finger motifs, a quite similar motif as in the RING finger domains (Massiah et al., 2007). The two B-Box domains can be discriminated by their amount and distribution of conserved histidine and cysteine residues (Borden et al., 1996). Also, the B2 domain is able to be present alone, whereas the B1 domain is always in combination with the B2 domain. The B-box is a very rare domain that is only found in TRIM proteins and some proteins in plants, which do not have TRIM proteins (Khanna et al., 2009). They are implicated in higher-order protein structure formation in some proteins (Wagner et al., 2016), however, aside from possible autoimmunity upon deletion of the domain, not much more is known about the cellular functions they perform. It is however, due to their similarity with RING finger motifs, suggested that they may also convey protein binding and E3 ligase activity, which is further underlined as some RING-less TRIMs are still able to perform E3 ligase activity *in vitro* (Bell et al., 2012).

The third signature domain of the RBCC motif is the coiled-coil domain, which features a secondary structure consisting of intertwining α-helices. The coiled-coil domain can act as an interaction feature with other CC domains and therefore enables TRIM proteins to form homo- or hetero-dimers with other TRIMs (Li et al., 2011b). It also acts as interaction domain with other proteins and is even capable of forming higher order stable protein complexes.

The best known example among one of these complexes are PML nuclear bodies (Lallemand-Breitenbach and de Thé, 2010). These are highly organized compartments in the nucleus consisting of proteins and DNA which are held together by the SUMOylated scaffold protein PML (Promyelocytic Leukemia Protein; TRIM19). The complex serves several critical functions, being oncogenesis or resistance against viral infections. It also seems to serve a function in enhancing the expression of nearby genes, as increased RND expression is observed in its vicinity (Bernardi and Pandolfi, 2007). Lastly, the CC domain also plays a role in cellular localization. Hereby, the CC domain is both required and sufficient for the formation of multimers, which then can compartmentalize. The RING and B-Box domains can hereby also further determine the type of subcellular compartment (Reymond et al., 2001).

TRIM proteins can be divided into 11 sub-groups, which are based on the C-terminal domains they possess. The motifs on the C-termini can be quite diverse and currently, 11 different combinations of domains are known (Kawai and Akira, 2011) (Table 1). The most common of these motifs is the PRY/SPRY domain (Grütter et al., 2006). Numerous reports describe various functions for this domain, however its predominant function is protein-protein interaction (Nisole et al., 2005). This interaction mainly entails E3 target binding to bring the protein in close proximity to the RING domain and the accompanying Ub-loaded E2 protein. One well known TRIM with a PRY/SPRY domain is TRIM21, which can bind the Fc region of cytosolic virus-antibody complexes through its PRY/SPRY domain and is thought to target them for proteasomal degradation (D'Cruz et al., 2013).

Other common domains of TRIM proteins include COS domains (Short and Cox, 2006), which appear most common in combination with FN3 and (PRY)/SPRY domains and facilitate interactions with microtubule whereas the FN3 domain mainly interacts with DNA and heparin. PHD domains are involved in chromatin-mediated gene regulation and bromodomains, which are always located

downstream of PHD domains in TRIM proteins, recognize acetylated lysine residues (Ivanov et al., 2007). Together they function as transcriptional repressors. NHL and MATH domains are both able to mediate protein-protein interactions. FIL domains can perform actin crosslinking and lastly ARF domains are responsible for intracellular trafficking (Kawai and Akira, 2011). This high variety of functions in this protein family makes it extremely versatile and lets them play a role in many cellular processes, including virtually every step in the innate immune system, from detection to signaling and execution.

TRIM proteins and the immune system

TRIMs are critical molecules for the regulation of the innate immune response, especially in the defense against viral infections. There are various reports showing the antiviral activity of TRIM proteins against viruses like human immunodeficiency virus type-1 (HIV-1), influenza virus, N-tropic murine leukemia virus and cytomegalovirus (Uchil et al., 2008). One of the most notable and best studied antiviral TRIM proteins is TRIM5α (Pertel et al., 2011). Both infection experiments and *in vitro* assays show, that TRIM5α can recognize the oligomeric retroviral capsid lattice of certain viruses, predominantly observed with HIV-1, and trigger the synthesis of K63 linked unanchored ubiquitin chains. These ubiquitin chains can in turn non-covalently interact with the TAB/TAK kinase complex which then activates NFκB and proinflammatory cytokine production. Therefore, TRIM5α works both as an antiviral PRR as well as an activator for NFκB (Pertel et al., 2011).

As another example for TRIM based receptor functions, TRIM21 functions as a cytoplasmic antibody by binding the Fc region of antibody-opsonized bacteria and non-enveloped viruses with its SPRY domain. The exact mechanism behind this function is still unknown, however, adenoviral infections of *Trim21*$^{-/-}$ mice and MEFs show that it is indeed critical for the correct defense against already opsonized pathogens (McEwan et al., 2013; Vaysburd et al., 2013).

TRIM functions in the innate immune system are versatile, and many play important roles as co-factors in nearly every stage of the immune signaling pathways, highly concentrated on the IFN and inflammasome pathways, but also in NFκB activation. RIG-I and MDA-5 recognize viral RNAs in the cytoplasm, however this interaction alone is not sufficient for hosting an antiviral defense. Additional interactions with co-factors and the resulting modifications to the receptors are mandatory in this context.

For example, RIG-I oligomerization is dependent on the formation of K63 linked ubiquitin chains in addition to viral RNA binding. One important activator in this matter has been found in the form of TRIM25 (Gack et al., 2007). Experiments in *Trim25*$^{-/-}$ mice and MEFs have shown that TRIM25 binds RIG-I and mediates the synthesis of K63 linked Ubiquitin chains. The fact if these chains are unanchored or anchored in nature is however still a matter of controversy. Recent crystal structures of RIG-I indicated that both forms would be feasible for its activation (Peisley et al., 2014). Independent of this discussion, the results identified TRIM25 as a critical regulator for the RIG-I pathway.

Another TRIM that is of interest for both the IRF3 and the NFκB pathway is TRIM14 (Zhou et al., 2014). It acts further downstream of the RIG-I / MDA-5 pathways by binding the adaptor protein MAVS. It has been shown that TRIM14 co-localizes with MAVS along microtubules after SeV infection induced IFNβ stimulation. This localization as well as the IRF3 interaction is mediated by the B-Box domains, as it has been shown by three point mutations in expression studies of this protein. TRIM14 is one of the RING-less TRIMs and no E3-ligase activity has been indicated, however it seems its own K63 ubiquitination is able to activate NEMO after recruitment to MAVS.

Many other TRIMs are implicated in the regulation of the NFκB pathway (Table 2). TRIM8 activates the NFκB pathway by K63 ubiquitination of TAK1 after stimulation with TNF-α or IL-1β (Li et al., 2011a). This leads to the activation of the TAK1/TAB1 complex and phosphorylation of IKKβ,

thereby activating the IKK complex. TRIM20, also known as MEFV (Mediterranean Fever Protein, Pyrin), positively regulates NFκB by interacting with p65 as well as promoting the turnover of IκBα. It is targeted by caspase-1 which cleaves off an N-terminal fragment, which includes the so-called pyrin domain. This fragment can bind to p65 to further facilitate its translocation into the nucleus. Additionally, the fragment can also bind to IκBα and mediate its degradation in a calpain-dependent manner (Chae et al., 2008).

TRIM	Effect on NFκB	Mechanism
TRIM4	positive	K63 linked RIG-I poly-ubiquitination (Yan et al., 2014)
TRIM8	positive	K63 linked TAK1 poly-ubiquitination (Li et al., 2011a)
	positive	Promoting PIAS3 cytoplasmic localization and turnover (Tomar et al., 2012)
TRIM9	negative	Binding and repression of β-TrCP, blocking IκB degradation (Shi et al., 2014)
TRIM13	negative	Ubiquitin mediated NEMO degradation (Tomar and Singh, 2014)
TRIM19	negative	Recruitment of NFκB to PML nuclear-bodies (Wu et al., 2003)
TRIM20	positive	Enhancing nuclear translocation of p65 and calpain-mediated degradation of IκBα (Chae et al., 2008)
TRIM21	negative	Mediates autophagic degradation of IKKβ (Niida et al., 2010)
TRIM22	negative	Inhibition of TRAF6 activation (Qiu et al., 2013)
	positive	Activation of the non-canonical pathway by interaction with IKKα and p100/p52 processing (Qiu et al., 2015)
TRIM23	positive	Activates NFκB through interaction with the human cytomegalovirus protein UL144 (Poole et al., 2009)
	positive	K27 linked NEMO poly-ubiquitination (Arimoto et al., 2010)
TRIM25	positive	K63 linked RIG-I poly-ubiquitination (Gack et al., 2007)
TRIM27	negative	Inhibits IKKs (Zha et al., 2006)
TRIM30α	negative	Promotes degradation of TAB2/3 (Shi et al., 2008)
TRIM38	negative	Proteasomal degradation of TRAF6 (Zhao et al., 2012)
	negative	Lysosome dependent degradation of TAB2/3 (Hu et al., 2014)
TRIM40	negative	Neddylation of NEMO (Noguchi et al., 2011)
TRIM45	negative	Negative regulation of NFκB (Shibata et al., 2012), possibly through inhibition of RACK1 (Sato et al., 2015)
TRIM59	negative	Interacts with ECSIT, Overexpression inhibits NFκB (Kondo et al., 2012)

Table 2. TRIM proteins interacting with the NFκB pathway.

There are also many negative regulators of NFκB in the TRIM family, which are of equal importance as the positive regulators. As an example for such negative regulation of NFκB, TRIM21, a known interferon regulator and auto-antigen, should be mentioned. Patients with lupus erythematosus or Sjogrens syndrome target endogenous TRIM21 (Ro52), inhibiting their E3 ligase

ability by blocking the interaction with the cognate E2 ligase (Espinosa et al., 2011). The prime function of TRIM21 is the repression of the constitutive activation of IKKβ, which is caused by phosphorylation through the tax oncoprotein during HTLV-1 infections. It facilitates mono-ubiquitination of IKKβ, promoting its autophagy mediated degradation. This degradation of IKKβ can efficiently repress activation of NFκB (Niida et al., 2010).

Furthermore, TRIM21 seems to be implicated in the regulation of IRF3 and IRF7. However, these reports seem to be somewhat divergent, as they show both a negative regulation through K48 linked ubiquitination of IRF3 (Higgs et al., 2008) and IRF7 (Higgs et al., 2010), as well as a positive regulation of the former one through a steric inhibition of another IRF3 repressor, Pin1 (Yang et al., 2009).

These are just a few examples of the many implications where TRIM proteins play a role in the immune system. The knowledge about the regulatory capabilities of TRIM proteins increased quite substantially over the last decade. In fact, it has been shown that over half of all the 70+ TRIMs in the human genome have implications in the regulation of immune pathways (Versteeg et al., 2013). However, up till now, many of these regulatory functions remain a matter of investigations. One of the most promising TRIMs to be potentially implicated in immune system, and especially NFκB, regulation is TRIM29.

TRIM29

TRIM29 (Ataxia Telangiectasia group D Complementing gene; ATDC) is a novel and quite interesting candidate for innate immune system regulation. It was originally found 1992 in search for the responsible gene locus for the Ataxia Telangiectasia disease (AT) (Kapp et al., 1992).

AT is an autosomal recessive inheritable disease and is caused by a mutation in the ATM gene (Savitsky et al., 1995). Symptoms include Ataxia (Difficulties with the control of movement, including limb, head and eye movement as well as spontaneous, unintended movements), Telangiectasia (dilated blood vessels, especially visible in the eye), cancer, a weakened immune system including preposition against many infections as well as premature aging of the skin and hair. Many of these symptoms are caused by lack of the DNA damage repair functions of ATM, like DNA DSB repair, as well as control of the cell cycle upon DSBs, including cell cycle arrest and apoptosis (Derheimer and Kastan, 2010). It is both necessary for the repair of external caused damages through means like radiation or reactive oxygen species, as well as for endogenous causes, playing an important role in V-(D)-J recombination in the development of mature lymphocytes (Bredemeyer et al., 2006). Up until the discovery of ATM being the sole reason for AT, several potential loci for containing the possible cause for AT were observed, including TRIM29, hence the name.

The full human TRIM29 is a 588 aa long (~66kDa) protein encoded on chromosome 11 q23.3 (Leonhardt et al., 1994). It is quite a unique member of the TRIM family as it is part of the TRIM subgroup V, but completely lacks a RING domain. Besides that, it does not feature any specific domain on its C-terminus. This domain composition of only possessing B-Boxes and a CC domain makes it quite special among TRIM proteins, only resembling TRIM44 in term of domain composition (Kawai and Akira, 2011).

TRIM29 shows highly elevated expression levels in the skin, specifically keratinocytes, as well as marginal elevated expression levels in the tongue and the eye, based on data from BioGPS as well as our own data from qPCR experiments. Additionally TRIM29 is highly overexpressed in many types of cancer cells and is considered a biomarker for several forms of cancer including pancreatic, gastric, bladder, colorectal, ovarian and endometrial cancer, as well as in plasma cell myeloma (Dyrskjøt et al., 2004; Glebov et al., 2006; Hawthorn et al., 2006; Kosaka et al., 2007; Mutter et al., 2001; Ohmachi et al., 2006; Santin et al., 2004; Wang et al., 2009; Zhan et al., 2002).

Inside the cells, TRIM29 is mainly located in the cytoplasm, even though it is known to localize to the nucleus under some conditions (see further below). In the cytoplasm, it can be observed associated with the intermediate filaments, as it is a known interactor of the intermediate filament III protein vimentin (Brzoska et al., 1995). However, there are publications that show a nearly exclusively nuclear localization of TRIM29 in certain cancer types, especially HeLa cells (Masuda et al., 2015), which proposes a shift to a more nuclear function under certain conditions.

TRIM29 in the DNA damage response

Studies concerning TRIM29 indicate implications in multiple different cellular processes including cell homeostasis and the immune system. The best studied function of TRIM29 however is its implication in the DNA damage response. This ability is also the most probable reason for the high expression of TRIM29 in cells which are more constantly exposed to DNA damaging threats like UV radiation.

Overexpression of TRIM29 in HEK-293 cells, which do not express TRIM29 in detectable amounts, leads to increased radio-resistance in these cells against ionizing radiation (Wang et al., 2014). Based on this same hypothesis, pancreatic cancer cells were investigated for a possible TRIM29 mediated radio-resistance. Pancreatic cancer cells are a type of cancer cells which happen to express high amounts of TRIM29, as well as being highly resistant against radio-therapy. Indeed, knockdown of TRIM29 in these cells through shRNAs lead to reduced proliferation of these cells and in combination with ionizing radiation to a complete proliferation stop (Wang et al., 2014). This indeed indicates TRIM29 as a growth and pro-survival factor in many cancer cells.

Besides cancer, TRIM29 has been shown to exhibit its capabilities for radio-resistance also in more natural cellular contexts. Human hTERT keratinocytes, an immortalized keratinocyte cell line which expresses high amounts of TRIM29, were UVB irradiated and their survival measured in time. siRNAs against TRIM29 significantly reduced the survival of these cell against UVB radiation (Bertrand-Vallery et al., 2010). In addition, this group showed that the expression of TRIM29 itself is regulated by DNA damage as the protein levels were significantly elevated based on western blot analysis several hours post UVB treatment.

Also, quantitative real-time PCR analysis showed increased levels of TRIM29 mRNA after treatment with several DNA-damaging reagents, including UVB, H2O2, Taxol and Etoposide (VP-16; a topoisomerase II inhibitor) as well as PMA (Bertrand-Vallery et al., 2010). PMA is not directly involved in the DNA damage response, but is a known activator for the Protein Kinase C (PKC), whose activation also involves TRIM29, as it interacts with one of its inhibitors and with its substrate (Brzoska et al., 1995).

Similar to many other DNA damage response pathways, the activation of TRIM29 starts with the induction of ATM caused by various means of DNA damage, especially DNA DSBs. Activated ATM can activate p38/MAPK, which further facilitates the activation of MK2 (MAPKAP kinase 2) (Figure 4). MK2 in terms is able to bind to TRIM29 and induce its phosphorylation on Ser550, an evolutionally conserved site which is crucial for TRIM29s DDR activity (Wang et al., 2014).

Similar to a TRIM29 knockdown, causing a S550A mutation leads to a strongly increased susceptibility of pancreatic cancer cells to ionizing radiation. Aside from activating it, it has also been shown that the S550 phosphorylation frees TRIM29 from its interaction with DVL2 (Wang et al., 2014). Phosphorylated TRIM29 can interact with the E3 ligase RNF8, which is a well known factor in the DDR (Yang et al., 2015). Aside from S550 phosphorylation, this interaction is dependent on the TRIM29 C-terminus and the RING domain of RNF8 however it does not require any E3 ubiquitin ligase activity.

Inside the nucleus, TRIM29 does not translocate to the DNA damage repair foci themself, but instead binds to the nearby chromatin. This histone binding is dependent on the histone modifications

H3K36me2/3, H4K16Ac and H4K20me2 (Masuda et al., 2015). During the DDR RNF8 binds, together with its partner molecule RNF168, to H2A and H2AX histones and facilitates their ubiquitination through their RING domain. This ubiquitination promotes the recruitment of 53BP1 and BRCA1 ionizing-radiation induced foci (IRIF), facilitating DNA repair (Figure 4).

TRIM29 is not an absolute requirement for this function, but significantly enhances the kinetics of the process as it works as a scaffold protein for different components of the DDR (Yang et al., 2015). Hereby is it involved in the formation of a protein complex involving Tip60, BRCA1 and BASC (Masuda et al., 2015) (Figure 4). TRIM29 has been known before to bind Tip60 (Sho et al., 2011), an acetyltransferase and well known chromatin remodeling enzyme, which promotes the ubiquitin ligase activity of RNF8 and DNA repair.

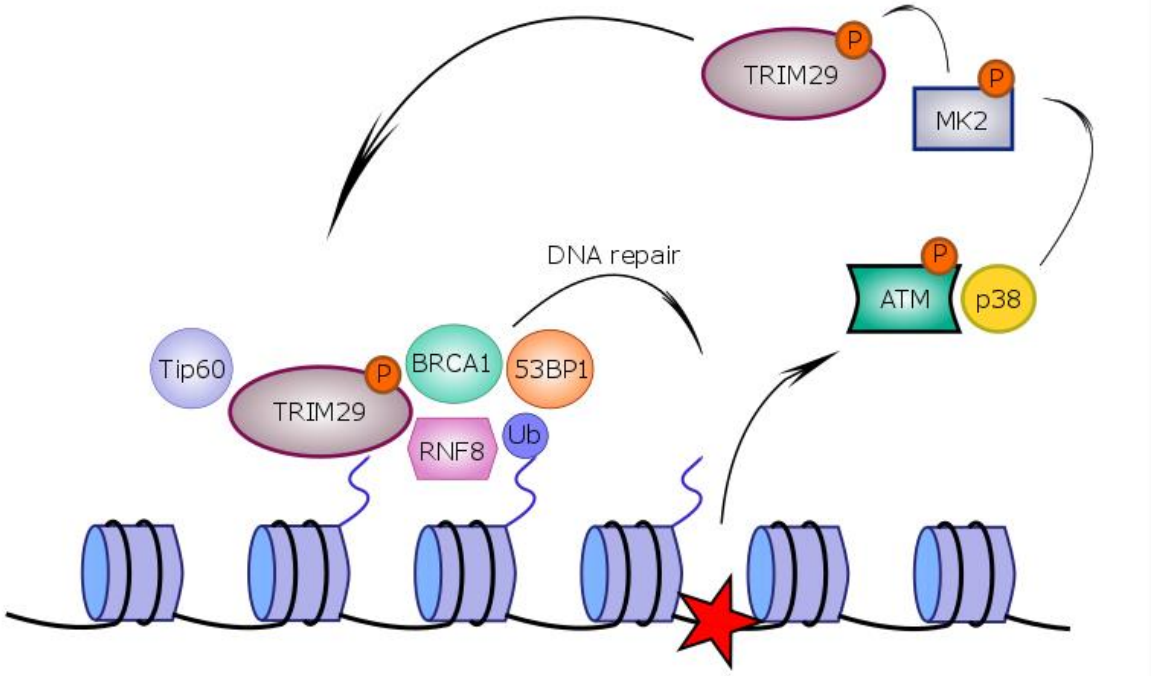


Figure 4. TRIM29 in the DNA damage response. DNA damage triggers activation of ATM, which further leads to phosphorylation of MK2 through p38. Activated MK2 can further phosphorylate TRIM29 on S550. This phosphorylation enables the TRIM29 C-terminus to interact with the RING domain of the E3-ligase RNF8, which is also prior activated in a ATM dependent manner. TRIM29 translocates to the chromatin, which is dependent on certain modifications on the histone tails, enhancing RNF8s ability in histone binding. Furthermore, TRIM29 interacts with the acetyltransferase Tip60, a known chromatin remodeling enzyme. RNF8 can now bind to H2A and H2AX histones and facilitate the ubiquitination of their histone tails. This further recruits DNA damage repair factors like BRCA1 and 53BP1 to the complex, enabling DNA damage repair.

TRIM29 and p53

The transcription factor p53 is an important tumor suppressor and considered the "guardian of the genome". It is activated upon DNA damage as well as upon expression of misregulated oncogenes and drives the expression of several downstream genes to trigger a protective response (Vousden and Prives, 2009). Two of the main outcomes of this activation are the arrest of cell growth by holding the cells in the G1/S phase of the cell cycle, as well as promoting apoptosis (Fridman and Lowe, 2003). It is also responsible for the correct execution of several parts of the DNA damage repair process (Williams and Schumacher, 2016).

TRIM29 has been shown to function as an effective repressor of p53. The interaction between TRIM29 and p53 translocates p53 to the cytoskeleton of the cytoplasm. This excludes p53 from the nucleus and prevents it from performing its nuclear functions, resulting in decreased levels of the downstream proteins p21 and NOXA (Yuan et al., 2010a). Mutation studies have shown that the interaction between TRIM29 and p53 is dependent on the acetylation of lysine 116 in the TRIM29 N-terminus. HDAC9 has been found as a negative regulator for this TRIM29 – p53 interaction by removing the acetylation from lysine 116, increasing p53s transcriptional activity (Yuan et al., 2010b).

In addition, TRIM29 has been shown to function as an inhibitor for the p53 activator Tip60 (Sho et al., 2011). Binding of TRIM29 to Tip60 leads to its degradation through the proteasome. How this mechanism is performed is not yet entirely clear, as no ubiquitination or similar modifications were observed. One of the main functions of Tip60 is activation of p53 through acetylation of its lysine K120 (Reed and Quelle, 2014). Degradation of Tip60 and therefore its absence renders p53 unable to perform its apoptotic functions and also leads to a shift from the G1 into the S phase of the cell cycle.

In this context, TRIM29 functions as a pro-survival factor in cells, by inducing proliferation and inhibiting apoptosis (Figure 5). Its ability to stop cell cycle arrest and apoptosis further contributes to its function as DNA damage survival factor. The enhanced cell proliferation in combination with the repression of the tumor suppressive activity of p53 is also one of the many reasons why certain cancer cell types have such an unusually high expression of TRIM29.

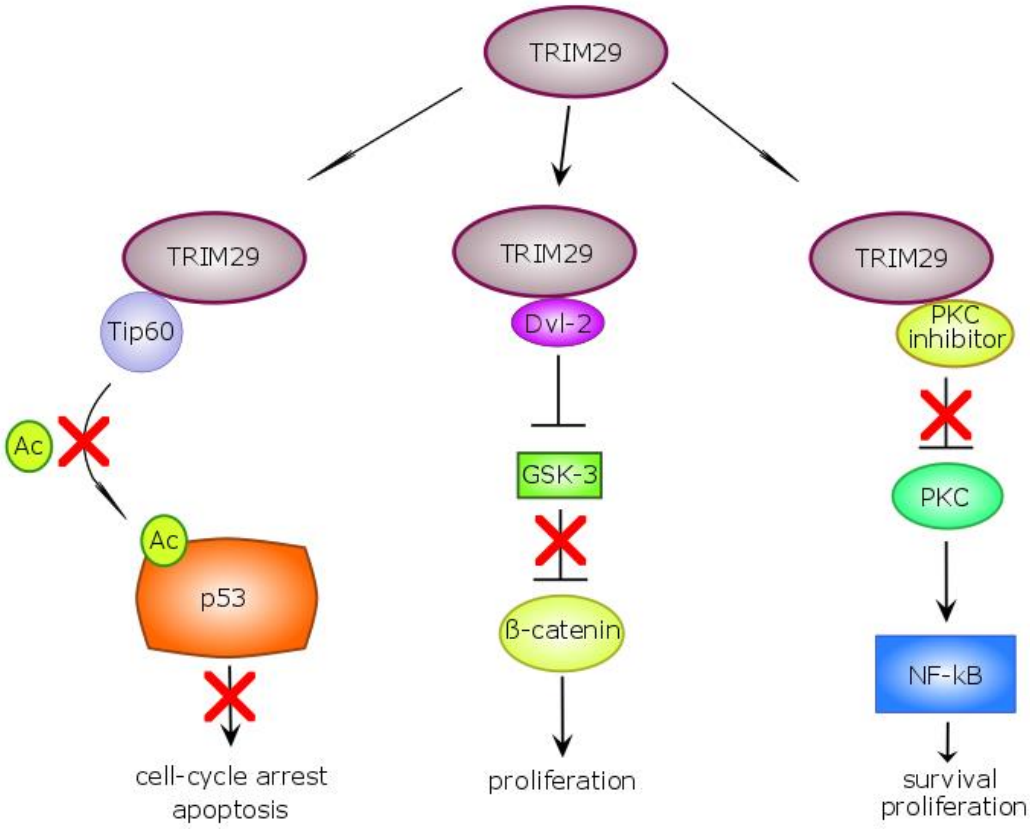


Figure 5. TRIM29 can contribute to cell survival and proliferation in several different ways. On the one hand it can interact with Tip60, preventing acetylation of p53, which is needed for p53 mediated cell-cycle arrest and apoptosis. In addition, TRIM29 can also inhibit p53 through a direct interaction, leading to their translocation to the cytoplasm. TRIM29 can also bind and stabilize Dvl-2, which would be degraded under resting conditions, which further inhibits GSK-3, a β-catenin repressor. This leads to β-catenin accumulation and transcription of pro-proliferative genes. In a third pathway, TRIM29 can lead to the activation of NFκB and to onset of proliferation. This has been shown to be due to binding and repressing the PKC inhibitor, leading to NFκB activation through PKC. If this is the sole reason for NFκB activation is however still a matter of investigations.

TRIM29 and the Wnt pathway

Another feature of TRIM29 which greatly contributes to its role as an oncogene in cancer is its ability to interact with the Wnt pathway. The canonical Wnt signaling pathway resolves around the accumulation of β-catenin in the cytoplasm and its translocation to the nucleus, where it functions as transcriptional co-activator or transcription-factor.

Under resting conditions, β-catenin is readily degraded by the proteasome caused by the inhibitors glycogen synthase kinase 3 (GSK3) and casein kinase 1α (CK1α) which phosphorylate β-catenin and prime it for further ubiquitination by the E3 ligase β-Trcp. Binding of Wnt to the Frizzled (Fz) rezeptor and the co-receptor LRP5/6 activates a signaling cascade in which the protein disheveled (dsv) is phosphorylated and can repress GSK3 activity (MacDonald et al., 2009; Nusse, 2005). Among the targets of β-catenin are several genes for the expression of pro-proliferative proteins (Kaldis and Pagano, 2009), giving it a significant role both in normal cell proliferation as well as in tumorigenesis.

Studies in pancreatic cancer cells have shown correlation of high overexpression of TRIM29 with increased pools of free intracellular β-catenin as well as increased proliferation (Wang et al., 2009). Knockdown of TRIM29 lead also to reduced amounts of β-catenin. The cause for this

correlation has been found in a direct interaction between TRIM29 and the Wnt pathway protein Dvl-2, a member of the dsv protein family which consists of Dvl-1, Dvl-2 and Dvl-3 (Lee et al., 2008). Under resting conditions, these proteins are ubiquitinated and degraded by the proteasome. Binding of TRIM29 to Dvl-2 stabilizes the protein in a similar fashion to the canonical Wnt pathway activation, enabling it to repress GSK3 (Wang et al., 2009) (Figure 5).

As knockdown of TRIM29 in pancreatic cancer cells showed a nearly identical reduction on tumor growth as knockdown of β-catenin, this interaction can be seen as critical for pancreatic tumor formation (Wang et al., 2009). The importance if this finding is further underlined by the presence of high amounts of TRIM29 in several types of cancer, including non-small-cell lung cancer, where it has been shown to also correlate with high β-catenin levels (Zhou et al., 2012).

Involvement of TRIM29 in the ERK and JNK pathways

Overexpression and knockdown studies in the lung cancer cell lines HBE, A549 and H1299 showed that TRIM29 plays a significant role in cell migration and invasion (Tang et al., 2013a). Invasion and metastasis is a complex phenomenon and requires several preliminary steps like decreased adhesion, increased motility and proteolysis (Johnsen et al., 1998; Liotta and Stetler-Stevenson, 1991). One important class of proteins implicated in these processes are matrix metalloproteinases (MMPs), which are zinc-containing proteolytic enzymes who break down extracellular matrix proteins and promote angiogenesis, tumor invasion and tumor metastasis (Egeblad and Werb, 2002; Kessenbrock et al., 2010).

TRIM29 contributes to these functions by upregulating one of the key players of this family, MMP-9. This upregulation is caused by increased activity of the transcriptionfactor AP-1, as shown by AP-1 luciferase assays and the fact, that MMP-9 has an AP-1 binding site (Westermarck and Kähäri, 1999). Also, increased levels of c-Jun and c-Fos protein and mRNA were detected with increased TRIM29 as well as their accumulation at MMP-9 promoter sites. The cause for these findings has been detected in a TRIM29 mediated increase in ERK and JNK activity. Taken together, these findings show a role of TRIM29 in cancer invasion and metastasis by activating MMP-9 through the ERK/JNK pathway (Tang et al., 2013a).

TRIM29 and the NFκB pathway

Interestingly, another involvement of TRIM29 in cell proliferation and oncogenesis has been found in non-small-cell lung cancer, which is not related to either p53 or β-catenin function (Tang et al., 2013b).

TRIM29 is highly expressed in many, but not all forms of non-small-cell lung cancer which is another indication that supports varying functions of TRIM29 in different cancers. Investigations on HBE cells, which express low amounts of TRIM29 as well as A549 and H1299 cells, which are both high TRIM29 expressers showed, that the abundance of TRIM29 again correlated with cell proliferation in all three cell lines. Hereby, overexpression of TRIM29 in HBE cells lead to increased proliferation as well as siRNA mediated knockdown of TRIM29 in A549 and H1299 lead to significant decrease of proliferation rates. Quantitative real-time PCR analysis of several genes involved in cell-cycle progression and proliferation has shown that TRIM29 levels correlate with increased levels of cyclin-D1 and c-Myc. In addition, a shift in the cell-cycle from G1 to S phase has been observed.

To investigate if p53 or β-catenin was involved in this process, β-catenin accumulation as well as p21 expression have been observed after TRIM29 knockdown or overexpression. There was no effect detectable in neither p21 nor β-catenin in HBO, A549 or H1299 cells, indicating that another process is involved in the increased expression of cyclin-D1 and c-Myc. A luciferase reporter assay with an NFκB dependent promoter showed increased NFκB activation with enhanced amounts of

TRIM29. This also correlates with an increase in p-IκBα levels, which indicate NFκB activation. The addition of Bay 11-7082, an NFκB inhibitor which blocks IκBα phosphorylation, completely abrogated luciferase reporter activation, increases in cyclin-D1 and c-Myc levels as well as enhanced cell proliferation with high amounts of TRIM29. These findings show a function for TRIM29 in cell proliferation and oncogenesis by activating cyclin-D1 and c-Myc expression through the NFκB pathway.

Similar results have been found more recently by another group in bladder cancer cells (Tan et al., 2016). Also here a TRIM29 caused expression of c-Myc and cyclin-D1 through the NFκB pathway is claimed to be the cause for cancer proliferation. In addition to the previous finding, this group investigated the mechanism behind this NFκB activation and found increased PKC phosphorylation alongside increased TRIM29 expression.

This implication has been shown before (Brzoska et al., 1995) and PKC is known to interact with the NFκB pathway in cells like B-cells, T-cells and cancer (Saijo et al., 2002; Sun et al., 2000; La Porta and Comolli, 1998). Treatment with the PKC inhibitor staurosporine also blocked IκBα phosphorylation caused by increased TRIM29 expression (Tan et al., 2016). Based on these observations it seems that TRIM29 causes NFκB dependent c-Myc and cyclin-D1 expression through repression of the PKC inhibitor (Figure 5).

Another interesting publication, which tackles TRIM29s involvement in the regulation of the NFκB pathway, has been recently released, quite some time after we started working on this current project. In it, the role of TRIM29 in alveolar macrophages (AM) is being addressed, which seems to be the only non-cancer immune cell-type which expresses high levels of TRIM29 (Xing et al., 2016).

AMs are the highest abundant immune cells in the alveoli and their abundance together with their functions mark them somewhat as the "guardian" of the respiratory tract (Hussell and Bell, 2014; Kopf et al., 2015). Indeed, AMs are the main effector cells in the lung and also perform various roles in cell differentiation, immunosurveillance and surfactant homeostasis (Westphalen et al., 2014). They have an important function in interacting with alveolar epithelial cells, dendritic cells and T-cells through receptor interactions, chemokines and cytokines, to modulate and fine-tune a cell specific response against viral, bacterial and fungal infections in the lung (Hussell and Bell, 2014; Westphalen et al., 2014).

Knockout of TRIM29 in these cells increases type I interferon and IL-6 production upon treatment with the RIG-I ligands 5'ppp-RNA and Poly:IC (Xing et al., 2016). In addition, treatment with 5'ppp-RNA as well as infections with influenza virus showed up to five times increased amounts of TNF-α and the chemokines MIP-1α, CCL-2, CCL-5 and CXCL-2. Furthermore, infection studies in *Trim29*$^{-/-}$ mice showed them to be much more resistance against infection by influenza virus, having much higher survival rates and regaining weight much earlier than their wild-type counterparts. In addition, treatment of *Trim29*$^{-/-}$ mice with LPS as well as bacterial infections showed severely increased death rates caused by septic shock.

In an attempt to find the process behind these effects, increased phosphorylations of the NFκB subunit p65 and the transcription factor IRF3 have been discovered when stimulating *Trim29* KO cells with LPS or influenza virus compared to WT cells. Immunoprecipitation of TRIM29 followed by mass spectroscopy analysis revealed NEMO as a potential binding partner of TRIM29. Deletion mutations in NEMO and TRIM29 together with immunoprecipitation experiments performed with pre-purified proteins revealed a TRIM29-NEMO interaction through their outer membrane protein H (OmpH) domain. This interaction leads to the degradation of NEMO through the proteasome.

Co-transfection of TRIM29 and NEMO in HEK-293T cells followed by western blotting showed that TRIM29 causes K48 linked ubiquitination of NEMO. This ubiquitination process is caused by the TRIM29 B-Box domain and mutation studies in NEMO revealed lysine 183 as the target site for this ubiquitination. At last, immunofluorescence experiments with tagged TRIM29 and NEMO

in HEK-293T cells showed that the two proteins co-localize with the lysosomal marker LAMP1, indicating that TRIM29 binds NEMO and then translocates to the lysosomes.

Taken together, these results show a new and interesting role of TRIM29 as a negative regulator of the NFκB and IRF3 pathway by priming the degradation of NEMO. This function is seemingly unique until now and in strong contrast to the previous findings, where TRIM29 always performed activating functions in the diverse pathways, including the NFκB pathway. Even though these discoveries are not per se mutually exclusive, it will be important to dig further in the activation and possible cell specificity of these functions to draw a clear picture of TRIM29 in this matter.

Insights we got from these publications show quite clearly that TRIM29 activates the NFκB pathway, but also seems to have the ability to repress the pathway under certain conditions. However, the question of how this activation mechanism is performed is not entirely clear. It has been claimed that the TRIM29 caused activation of NFκB is due to activation of PKC (Tan et al., 2016), but we are not convinced that this is the sole reason for this activation, even more as it has been shown that PKC dependent NFκB activation seems to have a function more tuned towards survival than cell-cycle progression (Su et al., 2002).

In Addition, as both TRIM29 and NEMO are translocated into the nucleus upon similar stimuli like DNA damage, we suspected a closer interaction between those two, aside from the negative regulation of NEMO which was not known to us by that time point. This theory was further backed up by yeast-two-hybrid screens which indicate a direct interaction between TRIM29 and NEMO (Ravasi et al., 2010).

Hypothesis

Previous publications have shown that TRIM29 has an activating effect on NFκB in various cancer cell types (Tan et al., 2016; Tang et al., 2013b). In addition, yeast-two-hybrid screens as well as mass-spectroscopy screens of the IKK proteins have indicated TRIM29 as a possible interactor of the IKK family (Ravasi et al., 2010). From this we formulated our working-hypothesis that TRIM29 is an interactor and activator of the IKK complex, thereby regulating activation of the NFκB complex.

Results

TRIM29 is highly expressed in the epidermis of the skin

As our first step in this project, we wanted to determine the expression levels of TRIM29 in different mouse organs to find a suitable cell type with high endogenous TRIM29 expression for further experiments. For this, we extracted the RNA from parts of the body skin, the eye, the bladder, the stomach, the liver, the spleen, the heart, the tongue, as well as the epidermis and purified epidermal keratinocytes of SV/129 mice. After RNA extraction, we created cDNA through reverse transcription and measured the *Trim29* mRNA levels through qPCR, normalizing the mRNA amounts to the 18S rRNA.

These qPCR experiments have shown the keratinocytes to have the highest relative *Trim29* expression with a 200,000 fold increase over background level, closely followed by epidermal mRNA (Figure 6). The whole body skin, the eye and the tongue still showed relatively high *Trim29* expression, but still about five times less than in the keratinocyte sample. We could also detect slight expression in the bladder samples, but no substantial expression in stomach, liver, spleen or heart (Figure 6).

These results show keratinocytes, which are the main cell type in the composition of the epidermis, to have the highest expression of *Trim29* mRNA, alongside some cells in the tongue and the eye. This fits well with the previously reported role of TRIM29 in DNA damage repair as it is localized in cells which are regularly exposed to DNA damaging threats like UV radiation. For this reason, further experiments to investigate the interaction of TRIM29 with the IKKs were performed in these cells.

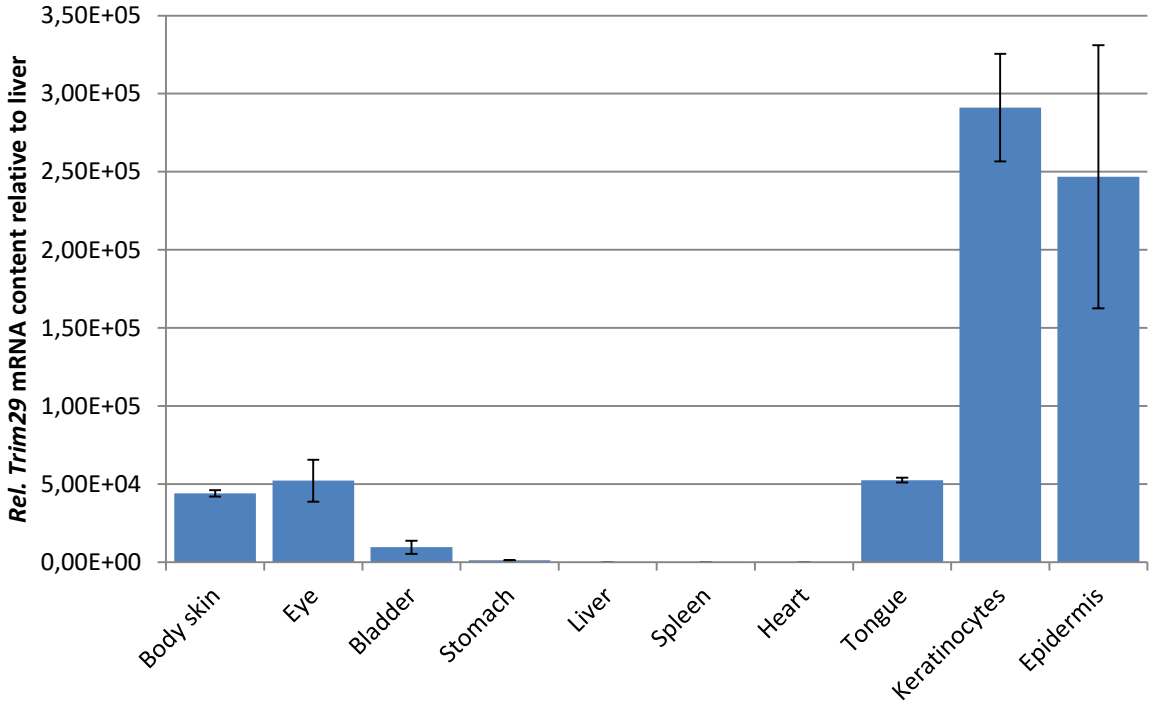


Figure 6. Trim29 mRNA is highly expressed in epidermis and keratinocytes. RNA was extracted from various tissues of a 6-weeks old SV/129 mouse. cDNA was created by reverse transcriptase reaction. Relative amounts of *Trim29* mRNA, normalized to the 18S rRNA, were measured by qPCR.

TRIM29 and IKKα/β or NEMO do not interact upon overexpression

To determine if the NFκB activating effect of TRIM29 is due to a possible activating effect on the IKKs, we wanted to investigate if TRIM29 and the IKKs are interacting with each other. To do this, we performed immunoprecipitation experiments with either TRIM29 or one of the IKKs as bait und determined co-precipitation of the other protein. For this, we transfected HEK-293T cells with plasmids expressing HA-tagged TRIM29 together with Myc-tagged NEMO (or IKKα/β in similar experiments) (Figure 7). HA-tagged GST was used as negative control to determine unspecific binding of the IKKs to the beads. HA-tagged p65 in combination with Myc-tagged IκBα was used as positive control to test if the IP settings worked in general.

All proteins were expressed at their predicted sizes in the whole cell extract (Figure 7). All HA-tagged proteins were efficiently immunoprecipitated with anti-HA beads (Figure 7). In addition, a signal of the predicted size for Myc-IκBα could be detected when blotting with an anti-Myc antibody, indicating that it indeed was co-precipitated alongside HA-p65, as the signal was absent when HA-p65 was missing. However, NEMO did not co-precipitate with our bait proteins (Figure 7).

This let us conclude that our approach did in principal work, as seen in our positive control, but that TRIM29 and NEMO did not interact under these conditions. We therefore decided to alter the stringency of our experiments to allow for weaker interactions.

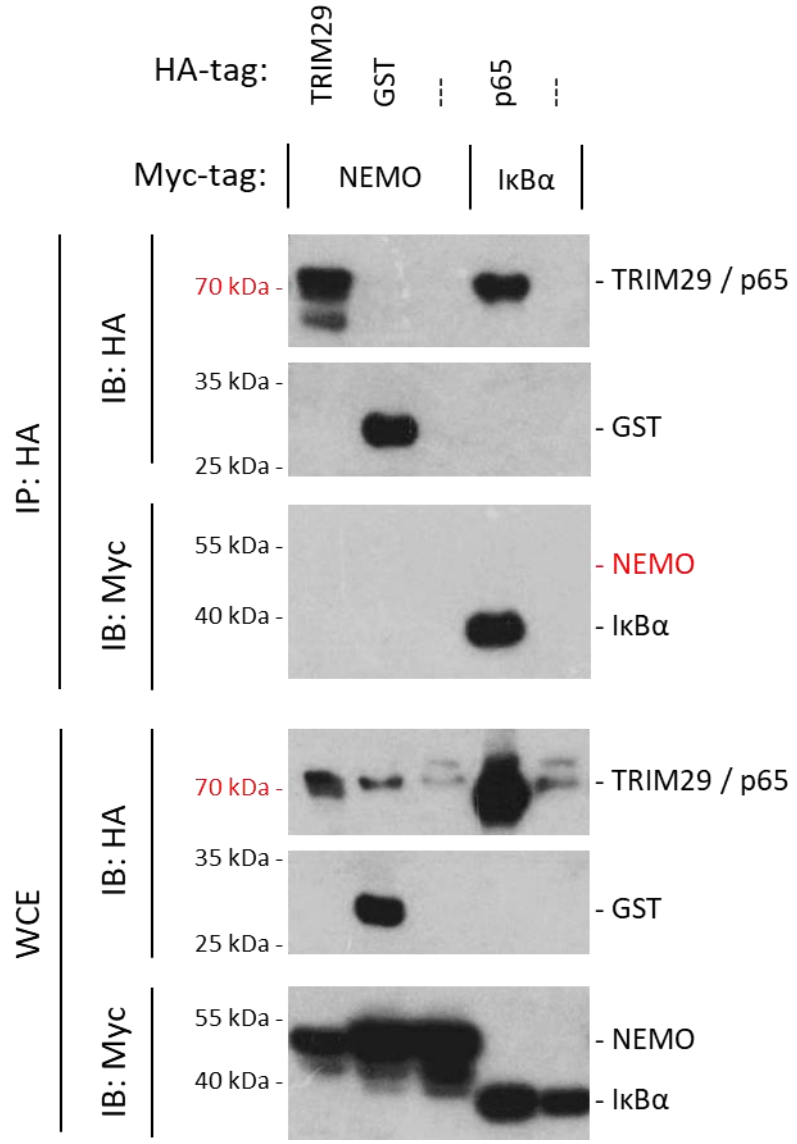


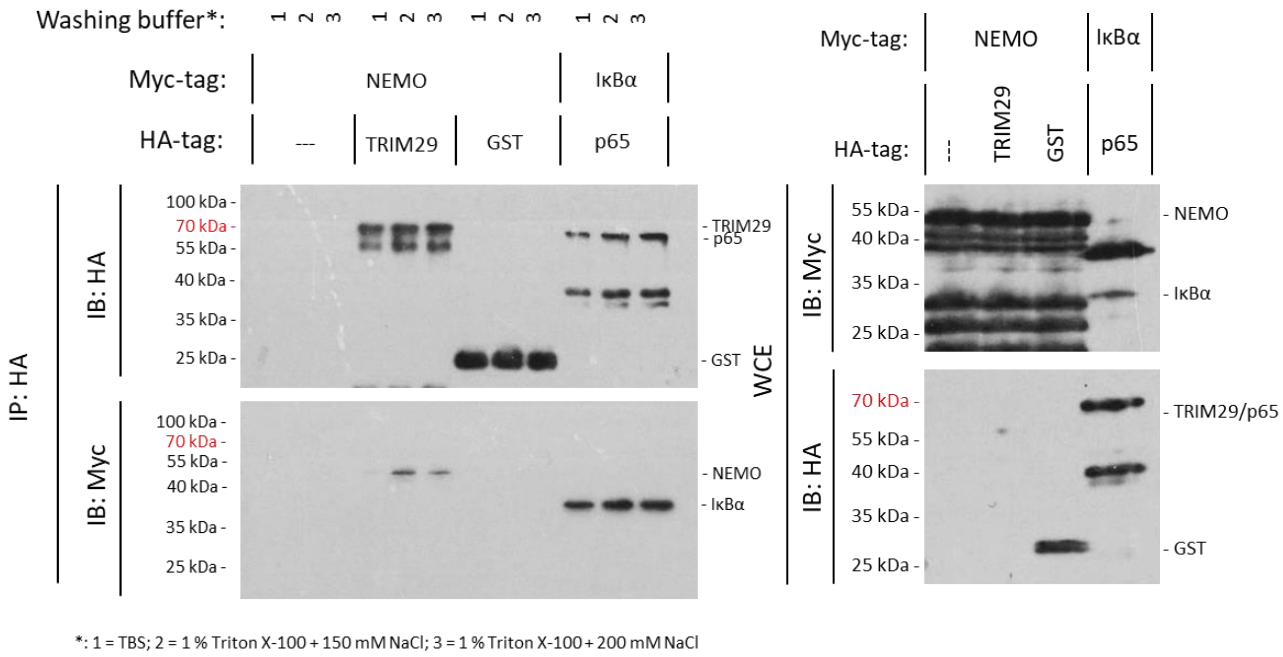
Figure 7. TRIM29 does not interact with NEMO when overexpressed in HEK-293T cells. HA-TRIM29 and Myc-NEMO expressing plasmids were transfected into HEK-293T cells. HA-GST was included as a negative control. HA-p65 and Myc-IκBα were included as positive controls. Cells were lysed 48 h after transfection in a buffer containing 1 % Triton X-100 and 250mM NaCl. IP was performed with anti-HA antibodies crosslinked to agarose beads. Proteins were eluted with 0.1 M glycin pH 2 and run on a 10 % SDS-PAGE gel for further western blot analysis.

High protein concentrations and low stringency of tested washing buffers led to co-precipitation of NEMO

We speculated that the interaction between TRIM29 and the IKKs could be very weak and therefore be disrupted by our buffer conditions, while the strong p65-IκBα interaction would stay intact. We therefore decided to alter the stringency of our washing buffer to prevent the disruption of the interaction during this experimental step. We transfected HEK-293T cells with a vector coding for Myc-tagged NEMO in combination with either an empty vector, a vector coding for HA-tagged TRIM29 or HA-tagged GST. HA-p65 in combination with Myc-IκBα was used as positive control. After lysis of the cells, identical samples were pooled, then divided in triplicates and IP was performed with anti-HA magnetic beads. Lastly, the beads in all the triplicates were washed six times in different buffers. One sample was washed in TBS, one in lysis buffer containing 150 mM NaCl and one in lysis buffer containing 200 mM NaCl. These reduced amounts of NaCl make the buffers less stringent than the former 250 mM NaCl, as higher concentrations of salt can disrupt protein bindings.

All proteins were expressed at their predicted size as seen in the whole cell extract and the amount of expressed NEMO was equal in all transfected samples (Figure 8). In the IP fraction, the signal we detected for our HA-tagged proteins TRIM29, GST and p65 were of equal strength after washing with the two lysis buffers, but slightly weaker with TBS (Figure 8). IκBα was co-immunoprecipitated alongside p65 in all samples without significant differences between the three buffers. NEMO was co-precipitated with TRIM29, but not with the empty vector sample and also not with GST. In addition, the signal for co-precipitated of NEMO was weaker with TBS as washing buffer than with the two lysis buffers (Figure 8).

We concluded from this experiment, that the lysis buffers work better than TBS for washing the beads, which is probably due to better pellet formation of the magnetic beads when a detergent is present. Furthermore, we were able to detect an interaction between TRIM29 and NEMO. To further validate these results, we set out to test whether this interaction is specific or due to a possible unspecific interaction with certain proteins features, like the coiled-coil domain of TRIM proteins. This would be a possibility, as coiled-coil domains are known to mediate protein-protein interactions and also to form higher order protein structures. As also NEMO possesses a coiled-coil structure, this possible interaction is important to be tested.



*: 1 = TBS; 2 = 1 % Triton X-100 + 150 mM NaCl; 3 = 1 % Triton X-100 + 200 mM NaCl

Figure 8. NEMO co-precipitates with TRIM29 in all three washing conditions. Expression plasmids for HA-TRIM29 and Myc-NEMO were transfected into HEK-293T cells. As control, expression plasmids for HA-GST, HA-p65 and Myc-IkBα were transfected. Cells were lysed in a buffer containing 1 % Triton X-100 and 250 mM NaCl. IP was performed with anti-HA magnetic beads in triplicates. The beads were then washed 6 times with three buffers of different stringencies: TBS, lysis buffer with 1% Triton X-100 and 150 mM NaCl and lysis buffer with 1% Triton X-100 and 200 mM NaCl.

NEMO non-specifically interacts with TRIM proteins

After we detected co-immunoprecipitation of NEMO with TRIM29 in this experimental setup, we wanted to test whether this is indeed a specific interaction and not just an artifact due to unspecific binding, probably also caused by the high expression levels of NEMO. To address this issue, we transfected HEK-293T cells with plasmids expressing various HA-tagged TRIM proteins which were not known to interact with NEMO (TRIM7, TRIM8, TRIM14, TRIM15 and TRIM29). These plasmids were transfected in combination with a Myc-NEMO expressing plasmid, followed by immunoprecipitations against the HA-tag (Figure 9). HA-p65 and Myc-IκBα were used as positive control.

A signal of the predicted size for NEMO could be detected with all the tested TRIM proteins and even in the empty control (Figure 9). Furthermore, the amount of co-precipitated NEMO correlated with the amount of precipitated TRIM proteins, indicating that NEMO indeed interacts with all the TRIM proteins in some way (Figure 9). In addition, as a small amount of NEMO was even detectable in our empty control, our washing steps were possibly not sufficient to get rid of all the unbound NEMO proteins. Therefore we decided to lower the amounts of plasmids in our experiments to reduce unspecific signals.

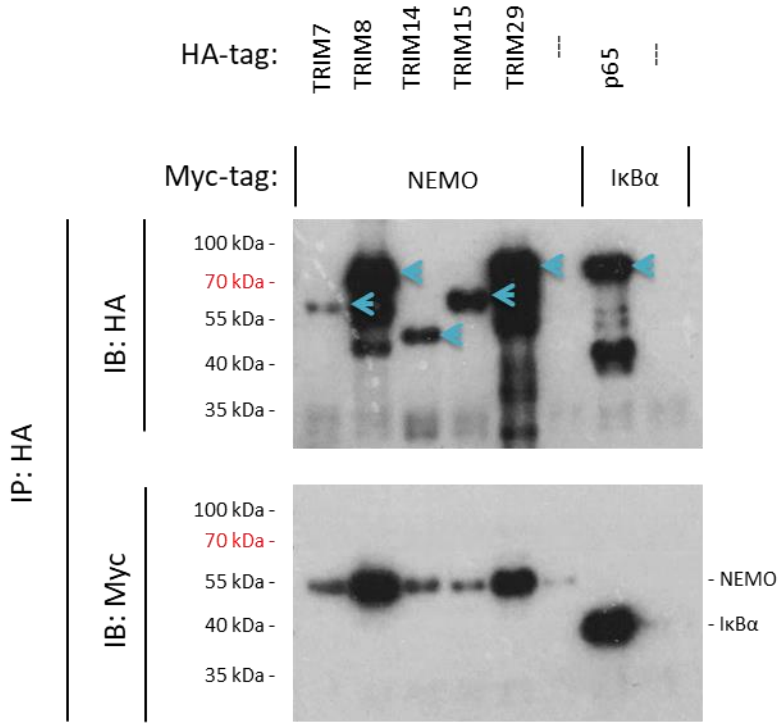


Figure 9. NEMO was co-precipitated with all transfected TRIMs, suggesting unspecific bindings. Expression plasmids for Myc-NEMO were transfected in combination with HA-tagged TRIM7, TRIM8, TRIM14, TRIM15 and TRIM29. HA-p65 and Myc-IκBα were included as positive control. Cells were lysed 48 h after transfection in a lysis buffer containing 1 % Triton X-100 and 250 mM NaCl. Pulldown was performed with anti-HA magnetic beads. Samples were run on a 10 % SDS-PAGE gel for further western blot analysis.

As a too high level of NEMO could increase the risk of unspecific interaction, we reduced the amounts of transfected NEMO encoding plasmids by a factor of ten and repeated our previous experiments. Even with this reduction, we detected a strong NEMO signal in the whole cell extract on western blot. However, we could not detect co-immunoprecipitation with TRIM29 or any of the other TRIMs anymore (data not shown). As the detected signal for NEMO in the whole cell extract was still very strong, this lower amount of plasmid was used for all further experiments.

In an attempt to find the optimal ratio between TRIM29 and NEMO we next decided to also vary the amounts of TRIM29. We speculated that a too high expression of either TRIM29 or NEMO could interfere with interaction or that only a small portion of our proteins are actually interacting, which, with a too high amount of bait protein, could result in us pulling down mainly unbound proteins. Therefore, we transfected HA-TRIM29 encoding plasmids in gradial amounts of 800 ng, 600 ng, 400 ng, 200 ng or 100 ng per 35 mm well in combination with a low amount of NEMO (35 ng) in HEK-293T cells and performed immunoprecipitations against the HA-tag.

Even though TRIM29 was expressed in a gradial manner and also NEMO was expressed at high amounts, we could not detect any co-immunoprecipitation between the two proteins (data not shown). Like in the previous and in all further experiments, HA-p65 and Myc-IκBα were used as positive controls and we detected a substantial interaction.

TRIM29 and the IKKs do not interact when overexpressed in Cos7 or HeLa cells

As overexpressed TRIM29 and the IKKs did not specifically interact in HEK-293T cells, we reasoned that other cells, especially ones with high endogenous TRIM29 expression, could express variants and amounts of co-factors more suited to mediate TRIM29-IKK interaction, therefore we decided to change our system to a different cell type. For our first set of experiments, we used HeLa and Cos7 cells for transfection. These two cell types were chosen as they are both easily transfectable and HeLa cells express high amounts of endogenous TRIM29, which could have a potential positive effect on the interaction.

We transfected both cell types with HA-TRIM29 and Myc-IKK α/β or γ encoding plasmids. All proteins were expressed in the WCE. Immunoprecipitation was performed against the HA-tag. Again, even though all HA-tagged proteins were precipitated and our positive controls did interact, we could not detect a signal for the Myc-tagged IKKs (data not shown).

As Cos7 have no substantial endogenous TRIM29 expression and HeLas are a highly mutated cancer cell type, we further reasoned that these cells may not provide a suitable environment for sufficient interaction, therefore we decided on a cell-type with high endogenous TRIM29 expression under non-cancer conditions.

Endogenous TRIM29 and NEMO do not interact in HaCaT cells

To exclude a possible interference caused by transfection and the exogenous proteins, we wanted to investigate TRIM29 – NEMO interactions with endogenous proteins in HaCaT cells and keratinocytes. We have already shown that TRIM29 is highly expressed in the keratinocytes of the skin. Therefore we concluded that this cell type would be the most relevant to study the functions of TRIM29, also in concern of its interactions with the IKKs and its potential to activate NFκB.

We chose to use the human derived keratinocyte cancer cell line HaCaT as well as primary mouse keratinocytes for those experiments. We extracted the keratinocytes from the skin of six weeks old SV/129 mice by skinning the mice and incubating the depilated skin for 45 min in a trypsin solution to part the epidermis from the dermis. Afterwards, we incubated the epidermis for 45 min in a DNAse solution to break cell-cell adhesions and get the cells in single cell suspension. These cells were filtered through a cell strainer and seeded on collagen and fibronectin coated dishes where they adhere within one hour and can be washed to remove dead cells and other remaining, non-adherent cells. Extracted keratinocytes then have a lifespan of six to seven days, before they stop proliferation and start to differentiate into corneocytes and die.

To perform these experiments we first had to test our respective anti-TRIM29 and anti-NEMO antibodies for their usability in immunoprecipitation and therefore their binding capability to the folded human and mouse proteins. At first we tested if our two anti-TRIM29 antibodies and our anti-NEMO antibody would work against both the human and the mouse protein, as they were all made by immunization with the human ortholog. For this, we made lysates of various cell lines (HEK-293T, Cos7, MEF, HaCaT, mouse keratinocytes as well as HEK-293T overexpressing HA-TRIM29 or Myc-NEMO and ran them on an SDS-PAGE gel for further western blot analysis.

NEMO was detected at the predicted size in all tested cell lines with our anti-NEMO antibody. Our polyclonal anti-TRIM29 antibody derived from rabbit detected TRIM29 in HaCaTs and HEK-293T cells overexpressing HA-TRIM29. Our anti-TRIM29 antibody derived from goat detected TRIM29 in HaCaTs, mouse keratinocytes, and HEK-293T overexpressing HA-TRIM29 at the predicted size (data not shown). From this we concluded, that our anti-TRIM29 antibody from rabbit only binds to the human protein, whereas the antibody from goat recognizes both the human and the mouse protein.

Next we tested their capability of detecting the folded proteins by immunoprecipitating overexpressed human HA-TRIM29 from HEK-293T lysates with the two anti-TRIM29 antibodies, the anti-HA antibody and, as negative control, the anti-Myc antibody (Figure 10). We detected immunoprecipitation of HA-TRIM29 with the anti-TRIM29 antibody from rabbit and the anti-HA antibody, but not with the anti-TRIM29 antibody from goat or the anti-Myc antibody (Figure 10). We are therefore only able to perform immunoprecipitations against endogenous TRIM29 in HaCaT cells, but not in primary mouse keratinocytes. Establishing immunoprecipitation of NEMO in an experiment where we overexpressed Myc-NEMO in HEK-293T cells failed due to excessive amounts of co-eluted antibodies, which masked a potential signal for NEMO on western blot (data not shown).

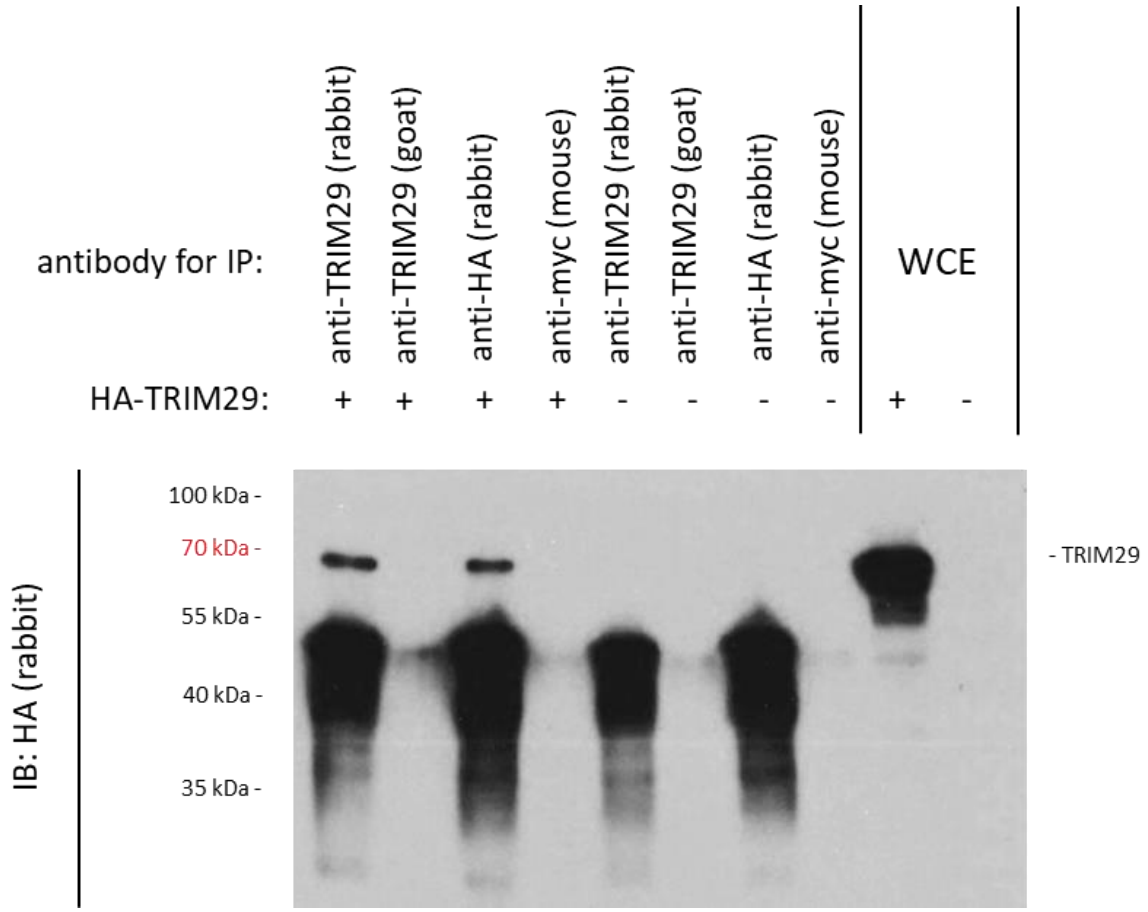


Figure 10. HA-TRIM29 can be immunoprecipitated with the anti-TRIM29 (rabbit) antibody and the anti-HA antibody, but not with the anti-TRIM29 (goat) antibody. Expression plasmids for HA-TRIM29 were transfected into HEK-293T cells. Cells were lysed 48 h after transfection with a buffer containing 1 % Tritin X-100 and 250 mM NaCl. IP was performed with an anti-TRIM29 antibody derived from rabbit, an anti-TRIM29 antibody derived from goat and an anti-HA antibody also derived from rabbit. As control, an anti-Myc antibody was included. Western blot detection was done with an anti-HA antibody derived from rabbit.

To measure the interaction between the endogenous proteins, we made cell lysates from HaCaT cells and performed an immunoprecipitation against TRIM29 with our anti-TRIM29 antibody derived from rabbit. On a whole cell lysate level, both TRIM29 and NEMO were detected at the predicted sizes. We were able to immunoprecipitate TRIM29 from these lysates, but we could not detect any co-precipitation with NEMO in this experimental setting (data not shown).

TRIM29 and IKKα/β or NEMO do not interact when overexpressed in HaCaTs or keratinocytes

While keratinocytes and HaCaTs were more promising cell types than HEK-293Ts, IP of overexpressed proteins did result in better yields than IP of endogenous proteins and enabled us to pull on the respective tags. Therefore we also wanted to immunoprecipitate overexpressed HA-TRIM29 and Myc-IKK α/β/γ in HaCaTs and mouse keratinocytes. As these cells already have high TRIM29 expression, they could potentially provide an environment more suited for this interaction, also with the exogenous proteins.

As these cells cannot be transfected, we had to switch to lentiviral transduction for delivery of our overexpression plasmids. We therefore had to produce lentivirus by transfection of high

expressing HEK-293T cells with a combination of three plasmids, containing the genes for gag-pol expression, the surface protein VSV-G and finally our packaging plasmids containing the gene for one of the tagged proteins encoded on a lentiviral pCDH-EF1 vector. As this vector contains a packaging signal, it is incorporated into the resulting virus like particles. The virus can then deliver the packed plasmid into the targeted cell. As our vectors lacked a resistance cassette, we were not able to perform selection and produce stable overexpression cell lines with this system. However, due to the fact that the transduction efficiency was overall very high (about 95% GFP positive cells in GFP expressing control), this was not problematic for short term IP experiments with these cells.

We transduced HaCaTs and keratinocytes with HA-TRIM29 expressing vectors in combination with either one of the three Myc-tagged IKKs or with all three together. HA-p65 and Myc-IκBα were used as positive controls. IP was performed against the HA-tag, the Myc-tag or TRIM29 direct in subsequent experiments. All the overexpressed proteins were detected in the whole cell extract. Pulldown of HA-p65 did result in co-precipitation of Myc-IκBα. Subsequently, pulldown of Myc-IκBα did also result in co-precipitation of HA-p65. HA-TRIM29 could be successfully precipitated with both the anti-HA and the anti-TRIM29 antibody. In addition, all three IKKs could be precipitated with the anti-Myc antibody. However, TRIM29 and the IKKs did never co-precipitate with each other in our experiments (data not shown).

TRIM29 and NEMO do not interact when stimulated with TNFα or DNA damaging reagents

We hypothesized that the TRIM29 – IKK interaction is possibly dependent on an activation stimulus and does not occur at resting conditions. We therefore also performed the experiments under stimulatory conditions. We stimulated HA-TRIM29 and Myc-NEMO expressing HaCaTs and HEK-293T cells with either TNFα or a DNA damage inducing reagent (Figure 11).

Our means of inducing DNA damage included the topoisomerase II inhibitor etoposide, the topoisomerase I inhibitor camptothecin and UV radiation. Inhibition of topoisomerase II leads to DNA double strand breaks whereas topoisomerase I inhibition is a cause for single strand breaks. To prevent a possible proteasomal degradation of one of the interaction candidates, the proteasome inhibitor MG-132 was added to some of the samples for 5 h. Treatment with TNFα was done for 15 min before lysis. Etoposide, camptothecin and UV radiation treatment were applied 1 h before lysis. Both treatments with etoposide and with camptothecin have resulted in morphological changes and also apoptosis of the cells after several hours of treatment in previous experiments. These effects did however never occur within the first hour and were therefore also not detected in this experiment.

All of the proteins were expressed at their predicted sizes and the positive controls did co-precipitate. However, none of the stimulations did result in a TRIM29 – NEMO interaction, nor did the stimulations have any effect on the protein concentrations (Figure 11). In addition, treatment with MG-132 did not show any effect on protein levels.

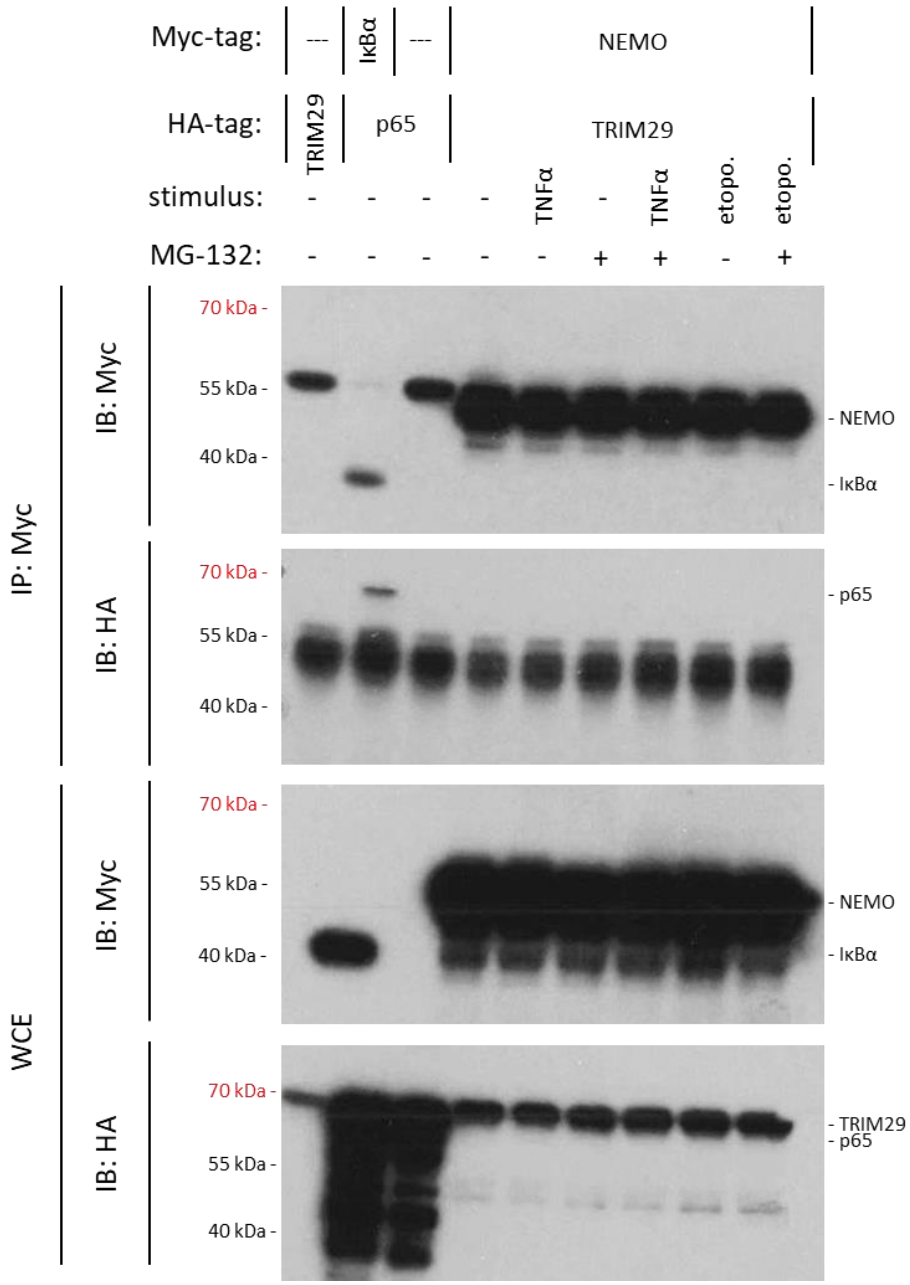


Figure 11. TRIM29 is not co-precipitated with NEMO upon stimulation with TNFα or DNA damaging reagents. Expression plasmids for HA-TRIM29 and Myc-NEMO were transfected into HEK-293T cells. As control, HA-p65 and Myc-IκBα were included. The cells were stimulated 48 h after transfection with TNFα (50 ng/ml) for 15 min or with etoposide (10 μM) for 1 h. MG-132 (1:1000) was added for 5 h to prevent proteasomal degradation. Cells were lyses after stimulation in a lysis buffer containing 1 % Triton X-100. IP was performed with anti-Myc antibodies. Proteins were eluted by boiling in disruption buffer for 10 min. Samples were run on a 10 % SDS-PAGE gel for later western blot detection. Additional bands in the IP fraction are caused by co-eluted antibodies.

TRIM29 is localized in the cytoplasm of HaCaTs, HeLas, keratinocytes and in overexpressing HEK-293T cells

In addition to identifying an interaction between TRIM29 and NEMO or the other IKKs by immunoprecipitation, we also investigated a potential interaction of these proteins through detection of co-localization by immunofluorescence. At first, we determined the localization of TRIM29 in our predominant cell-types. For these IF experiments, we used HEK-293T, HeLas, HaCaT cells and keratinocytes, either transfected or transduced with HA-TRIM29 expressing vectors (Figure 12). In addition, we also investigated localization of endogenous TRIM29 in wild-type HaCaT cells.

Identification of TRIM29 with both the anti-HA and the anti-TRIM29 antibody resulted in a strong signal located in the cytoplasm of all tested cells, both with overexpressed and endogenous proteins. This signal was absent in the secondary antibody only control and was significantly elevated from the background detection (Figure 12). Even though this was expected in most of the cells, it is

surprising for HeLas, as it has been shown in several publications that both endogenous as well as overexpressed TRIM29 has a mainly nuclear localization in these cells (Masuda et al., 2015).

However, detection of NEMO and co-localization with TRIM29 was not possible due to excessive bleed-through from the HA/TRIM29 channel into the NEMO channel, which prevented a distinction of the two signals when detecting NEMO. It would require a different set of secondary antibodies for both TRIM29 and NEMO to perform this experiment, which was not available at that time. Furthermore, even the signal detected from our NEMO only controls was barely above background level, which was probably due to low expression levels of the NEMO protein in these cells.

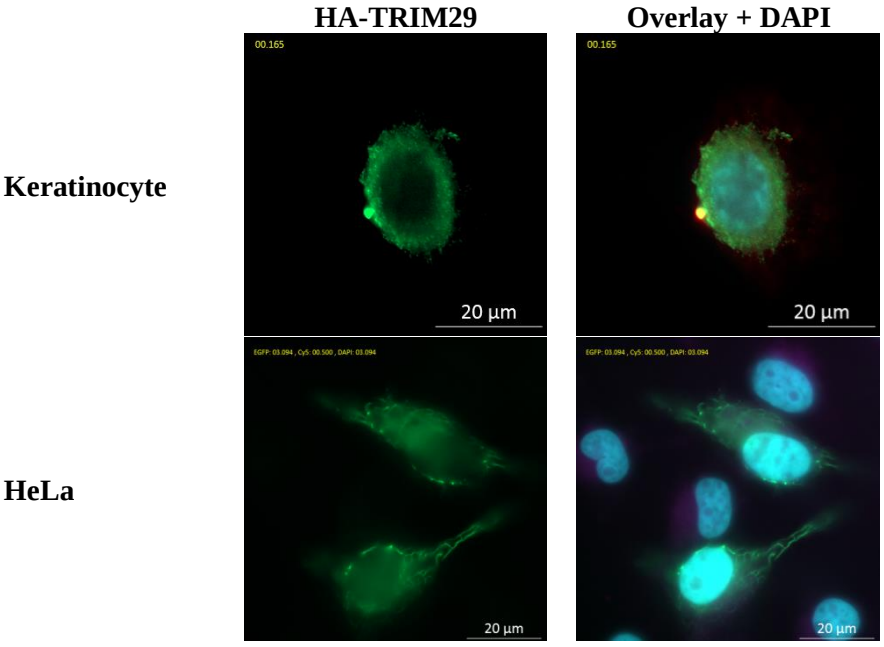


Figure 12. HA-TRIM29 is localized in the cytoplasm of mouse keratinocytes and HeLa cells. Expression plasmids for HA-TRIM29 were transduced into primary mouse keratinocytes with a lentiviral VLP. Expression plasmids for HA-TRIM29 were transfected into HeLa cells. 48 h after transfection/transduction, the cells were fixed with 3 % PFA and HA-TRIM29 was detected with anti-HA antibodies. The DNA has been visualized with DAPI.

Establishing an assay to investigate NFκB activation through TRIM29

Therefore, as all efforts aimed at investigating whether TRIM29 and the IKKs interact had proven unsuccessful, we next set out to investigate whether TRIM29 is required for NFκB activation during stimulation with pro-inflammatory cytokines or DNA damaging reagents. It has been shown before that TRIM29 promotes cell survival under DNA damaging conditions (Yang et al., 2015), as well as proliferation and NFκB activation in small lung cell cancer cells (Tang et al., 2013b) and bladder cancer cells (Tan et al., 2016). We were therefore interested if these findings hold also true for our cellular systems and if we could reproduce these findings with our methods. If we were able to reproduce these results with our methods, we would have a setup at hand to further assess this topic and search for novel functions of TRIM29 in the regulation of NFκB through different causes like bacterial or viral infections.

We chose to measure NFκB activation by making cell lysates at specific time-points after stimulation of the cells and detecting the levels of total IκBα and p-IκBα (Ser 32/36) on western blots

with specific antibodies. This approach has been used in several publications before and is an established method to assess timed NFκB activation. When NFκB is activated, p-IκBα will rapidly increase whereas total IκBα levels will decrease, which is due to the rapid phosphorylation and subsequent degradation of IκBα as an essential part of this pathway. The time point after which this activation is measureable is dependent on the activating stimulus. Whereas detectable NFκB activation after LPS or TNFα stimulation reaches its peak between 5 to 20 minutes (Lewis et al., 2006), DNA damage induced activation through etoposide reaches its maximum between 1 to 2 hours (Huang et al., 2003).

We focused our efforts to HaCaT cells and primary mouse keratinocytes as our main cellular environments. MEFs and RAW264.7 cells were used as positive controls for TNFα and LPS stimulation as we already had good knowledge and results of these cells for this kind of assay. In terms of stimulation conditions we tested LPS, TNFα and the TLR2 agonist zymosan as well as the DNA damaging reagents etoposide, camptothecin and UV radiation.

TNFα and LPS induce NFκB activation in MEFs and RAW264.7 cells

In our first experiments, we investigated NFκB activation in MEFs and RAW264.7 cells after a single stimulation to test if we can actually induce NFκB activation and measure it. Stimulation of MEFs with TNFα resulted in complete degradation of total IκBα after about 20 min, which is slowly replenished and back at wild-type levels after about 60 min (Figure 13).

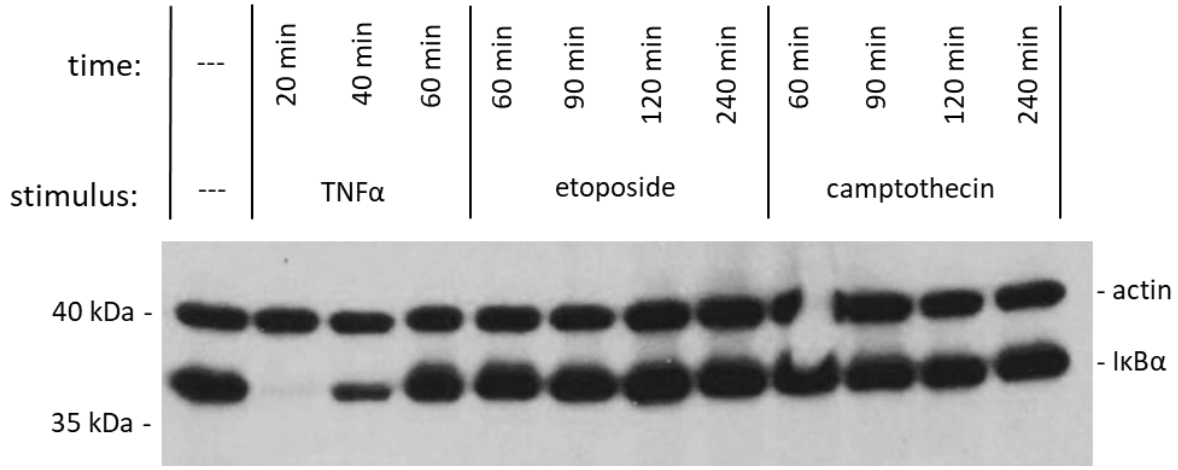


Figure 13. Treatment with TNFα causes IκBα degradation but etoposide and camptothecin have no effect. WT MEFs were stimulated with TNFα (50 ng/ml), etoposide (10 μM) or camptothecin (10 μM) for the indicated amounts of time. After the stimulation, the cells were lysed in 1.5x disruption buffer. The samples were boiled for 10 min and sheared with a 0.4 mm syringe before being run on a 10 % SDS-PAGE gel for further western blot analysis.

In a similar manner, stimulation of RAW264.7 cells with LPS resulted in complete ablation of IκBα after five minutes (Figure 14). At the same time, p-IκBα levels were significantly increased. TNF-α however did not have a major effect on NFκB activation in RAW264.7 cells, which is in contrast to the results in MEFs (Figure 14, Figure 13). Stimulations with etoposide or camptothecin did not cause NFκB activation in any of the cell lines. Stimulation of RAW264.7 cells with UV radiation resulted in significant accumulation of a 100 kDa protein which was detected with an anti-p-IκBα antibody (Figure 14).

Activation of NFκB through UV radiation leads to phosphorylation of IκBα on different residues than the canonical Ser 32/36 bases, therefore making detection with our anti-p-IκBα antibody

impossible. In addition, UV radiation is known to be able to induce NFκB activation in a NEMO independent manner, opposed to other DNA damaging factors like etoposide or camptothecin (László and Wu, 2008). This all together lets us hypothesize that UV radiation may promote phosphorylation of the p100 protein, which acts as NFκB inhibitor similar to IκBα until it is processed into the NFκB subunit p52. In our RAW264.7 cells this protein could then be detectable by our anti-p-IκBα (Ser 32/36) antibody and would therefore indicate an induction of the non-canonical NFκB pathway.

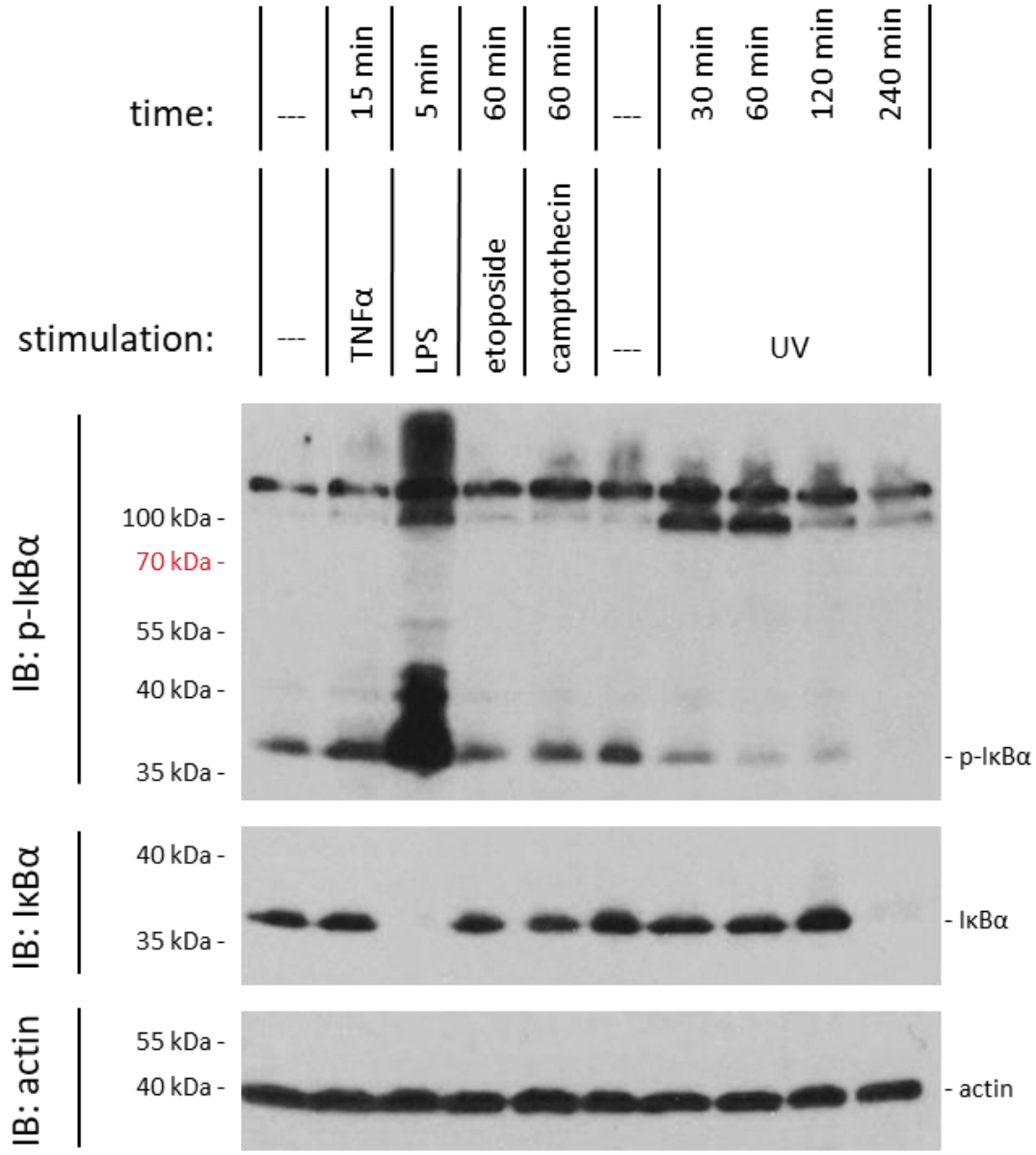


Figure 14. LPS induces NFκB activation in RAW264.7 cells. WT RAW264.7 cells were stimulated with TNFα (50 ng/ml), LPS (25μM), etoposide (10 μM), camptothecin (10 μM) or UV radiation (40 mJ/cm^2) for the indicated amounts of time. After the stimulation, the cells were lysed in 1.5x disruption buffer. The samples were boiled for 10 min and sheared with a 0.4 mm syringe before being run on a 10 % SDS-PAGE gel for further western blot analysis.

None of our tested stimuli did induce NFκB activation in HaCaTs or keratinocytes

To further investigate NFκB activation in cells naturally expressing high TRIM29 levels, we aimed to induce NFκB activation in HaCaTs and in keratinocytes. Therefore we stimulated wild-type HaCaTs and mouse keratinocytes with etoposide, camptothecin and UV radiation for certain amounts of time, from 30 minutes up to four hours, to trigger NFκB activation (data not shown). In addition, zymosan and peptidoglycan were used as positive controls to trigger NFκB activation through TLR2 activation, which has been shown before in these cell types (Mempel et al., 2003). The cells were lysed at the various time points after stimulation and the whole cell extract was run on an SDS-PAGE gel for further western blot analysis. Zymosan and peptidoglycan did not have any effect on total IκBα or p-IκBα levels.

Also up until this point, we could not detect NFκB activation through stimulation with etoposide, camptothecin or UV radiation. This was also due to fluctuations in the actin levels, which correlated with the slight differences in our total IκBα measurements and made a reliable conclusion of potential changes impossible (data not shown). All of these were preliminary experiments which aimed to establish a comparable detection of NFκB activation between wild-type HaCaTs or keratinocytes and their *Trim29* KO counterparts. The experiments for this comparison have not been done yet, as the readout was not fully established.

Trim29 is efficiently knocked-out in HaCaTs and keratinocytes using CRISPR/Cas9

In addition to the stimulation of the NFκB pathway, we used a genetic approach to investigate the functions of TRIM29. To test the influence of TRIM29 on NFκB activity, we knocked out *Trim29* in HaCaT cells and in mouse keratinocytes. The knockouts were performed with a lentiCRISPRv2 plasmid with 5 different guide RNAs (gRNAs) targeting mouse *Trim29* and 6 gRNAs targeting the human equivalent. We produced lentiviruses by transfecting high expressing HEK-293T cells with three plasmids, one containing the genes coding for gag-pol, one coding for the surface protein VSV-G and a lentiviral mini-genome which has a packaging signal and is incorporated into the resulting virus-like particles. The mini-genome contains the gene coding for the Cas9 protein as well as one of our designed gRNAs.

After transduction of the cells and expression of Cas9 and the gRNA, the gRNA is bound by Cas9 and directs the Cas9 protein to its complementary region on the hosts genomic DNA. Cas9 then cuts the DNA, which is afterwards repaired through the cellular DNA damage repair mechanism. Insertions or deletions during this error prone repair mechanism can cause frame shifts in the gene, resulting in a non-functional gene product and therefore a functional knockout.

The gRNAs were designed in a way that they target the *Trim29* gene in either exon one or two (of nine), which are both located downstream of the start-codon. Transduction of HaCaT cells followed by puromycin selection led to a nearly complete ablation of TRIM29 on western blot detection (Figure 15A). In addition, due to the high efficiency of selection, stable cell lines of *ΔTrim29* HaCaTs could be created which were still nearly completely devoid of TRIM29 after 4 weeks of culture (Figure 17).

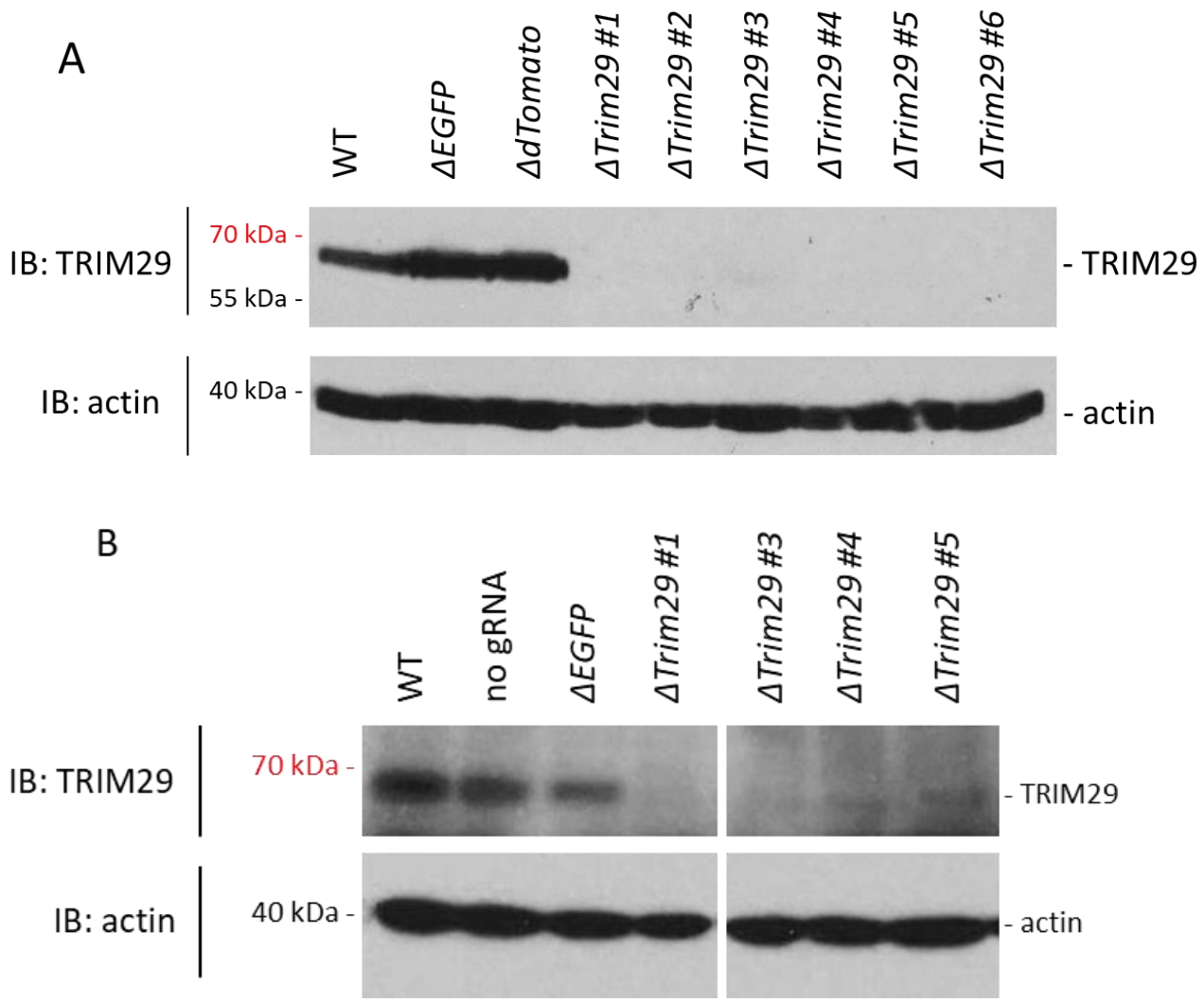


Figure 15. *Trim29* was efficiently knocked out in HaCaTs (A) and in primary mouse keratinocytes (B). HaCaTs and mouse keratinocytes were transduced with lentiviral VLPs which contain a lentiCRISPRv2 vector. The vector encodes the Cas9 protein and gRNAs against the *Trim29* gene, targeting the first two exons downstream of the start codon. 72 h after the transduction, the cells were selected with 3 µM puromycin for 72 h. After selection, the cells were lysed in 1.5x disruption buffer. The samples were boiled for 10 min and sheared with a 0.4 mm syringe before being run on a 10 % SDS-PAGE gel for further western blot analysis.

Knockout of TRIM29 in keratinocytes was more difficult due to their stop of proliferation after about 7 days in culture. Therefore, establishing a stable knockout cell line is impossible. It also provides an obstacle for selection as the whole process would greatly limit our available time for experiments. However, due to the high efficiency of the lentiviral transduction and of the CRISPR/Cas9 system itself, it was possible to efficiently target Trim29 in a timeframe which still allows for further experiments (Figure 15B). Compared to wild-type keratinocytes and control constructs, TRIM29 was reduced by over 90 % (Figure 15B, Figure 16, Figure 17).

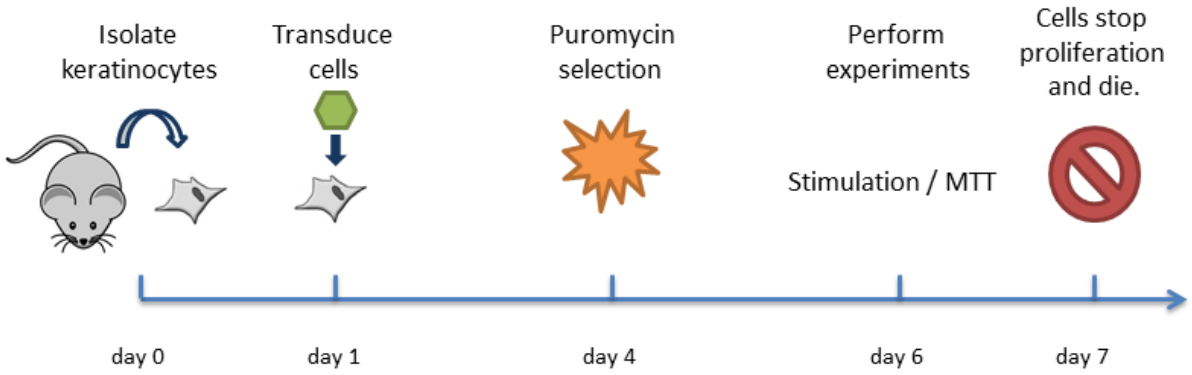


Figure 16. Timeline of the *Trim29* knockout in primary mouse kerationocytes. The kerationocytes are isolated from the skin of six weeks old SV/129 mice. 24 h after isolation, the cells are transduced with lentiviral VLPs packing a lentiCRISPRv2 vector with gRNAs targeting the *Trim29* gene. 72 h after transduction, the cells are selected by adding puromycin to the medium. 72 h after selection, experiments can be performed with the now selected *Trim29* KO cells. Keratinocytes stop proliferation and start differentiating into dead corneocytes shortly after this time-point.

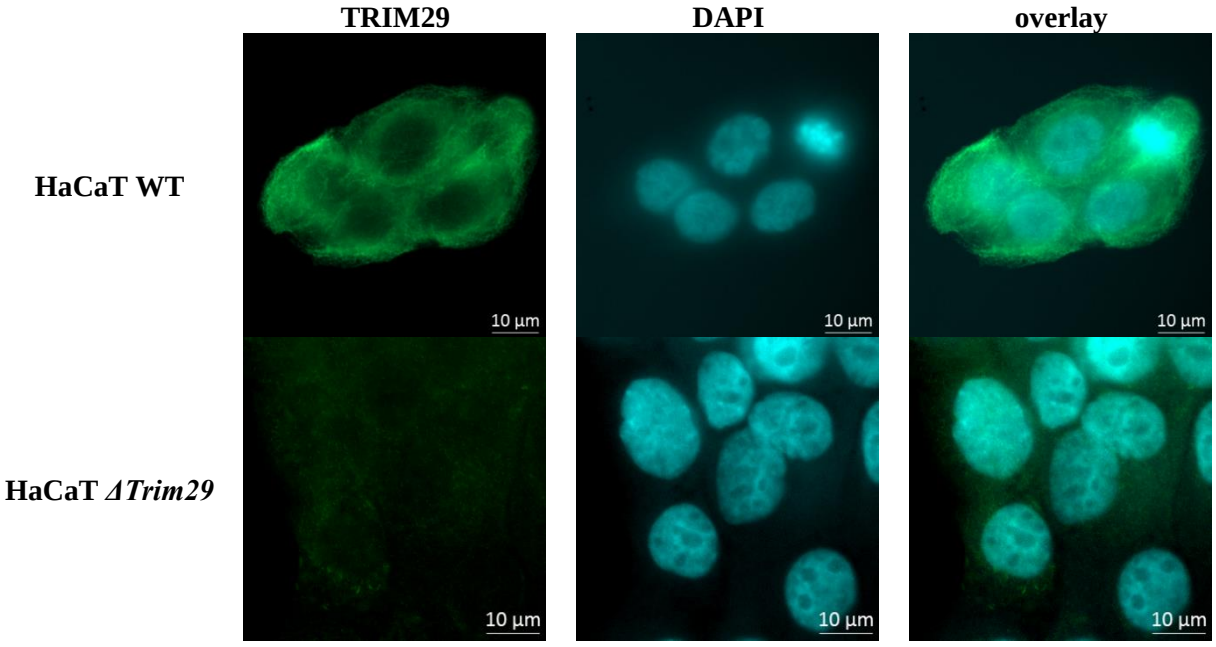


Figure 17. The TRIM29 protein is greatly reduced in *Trim29* KO HaCaTs in IF. WT HaCaTs and *ΔTrim29* HaCaTs were seeded on cover slips. The cells were fixed with 3 % PFA and TRIM29 was detected with an anti-TRIM29 antibody. The DNA has been visualized with DAPI.

TRIM29 ablation did not affect survival of HaCaT cells against UV radiation

It has been proposed that TRIM29 facilitates cell survival and proliferation, especially under DNA damaging conditions. We were interested on the one hand if we could reproduce this survival effect and on the other hand if we could identify a survival role of TRIM29 in other situations like during immunological reactions and infections.

To test this, we measured and compared the numbers of living wild-type or *ΔTrim29* cells before and after treatment with the stimuli through an MTT assay. The MTT assay is based on the

reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to unsoluble violet formazan crystals, which relies on the reduction potential of NADH and NADPH. These crystals are then brought in solution through the addition of an MTT solvent reagent. The amount of these dissolved crystals can then be measured through light absorbance at around 575 nm wavelength and as NADH and NADPH are only produced in living cells, it is an efficient measurement for the relative amount of these cells.

To test whether the MTT assay is a reliable enough tool to measure and compare cell numbers, we counted HaCaT cells and seeded them in linear decreasing amounts. In addition, we also wanted to find an optimal solvent for these experiments, as the one we used in our initial experiment did not completely dissolve the formazan crystals, which lead to inaccuracies. Therefore we tested three different solvents for each cell-number, which all differ slightly in acidity and detergents.

All of them did efficiently lyse the cells and dissolve the crystals, however they displayed differences in their color change (darker shades of violet) and also different absorbance values (Figure 18). We determined that, even though the three solvents caused different absolute absorbance values, all of them had a linear correlation of absorption with the numbers of cells (Figure 18). This result deemed the MTT assay suitable and exact enough for our experiments, and in addition, we decided to use the solvent which caused the highest absolute absorbance (10 % SDS in 0.01 M HCl) for our further experiments.

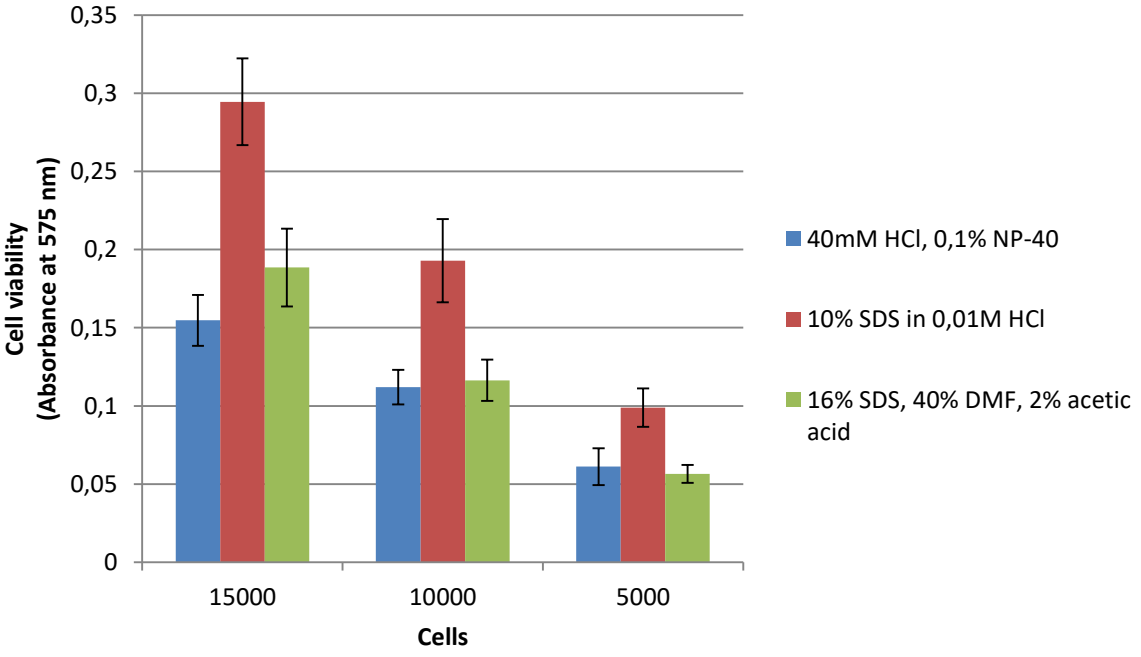


Figure 18. The MTT assay shows linear correlation with the number of living cells. HaCaT cells were counted and seeded 15000, 10000 and 5000 cells per well in octuplicates for each of the three MTT solvents. Cells were treated with the MTT reagent for two hours and then lyses with the MTT solvents for 30 min. Absorbance has been measured at a 575 nm wavelength. A 700 nm wavelength measurement was done to account for background absorbance.

Our plan for this assay was to seed wild-type and *ΔTrim29* HaCaTs in parallel and treat them with the DNA damaging stimuli. Hereby we had to treat the cells with different concentrations of etoposide, camptothecin and UV radiation. We had to find an optimal concentration of our stimuli, so that a significant number of cells are dead within 24 hours, but still enough cells are alive to measure significant differences in their amounts.

To test this, we seeded equal amounts of HaCaT cells and treated them with increasing concentrations of etoposide, camptothecin (Figure 19) or UV radiation (data not shown). After 24 hours of treatment, we performed an MTT assay with these cells. This experiment gave us an indication on the potential of our stimuli to kill the cells and let us make assumptions on the concentrations to use. In addition, it also showed camptothecin to be a much more lethal reagent for our cells (Figure 19). This is not only because of the lower absorbance values from the MTT assay (Figure 19), but also because of much faster and efficient onset of cell-death observable through the microscope.

Therefore we decided to use the lower concentrations (0.25 µM) for campthothecin, as it still caused significant cell death whereas the high concentration did result in nearly complete cell death of all the cells within our tested timeframe. Furthermore, we decided to use the high concentreations (10 µM) for etoposide in our further experiments, as it caused substantial, but not exceeding cell death.

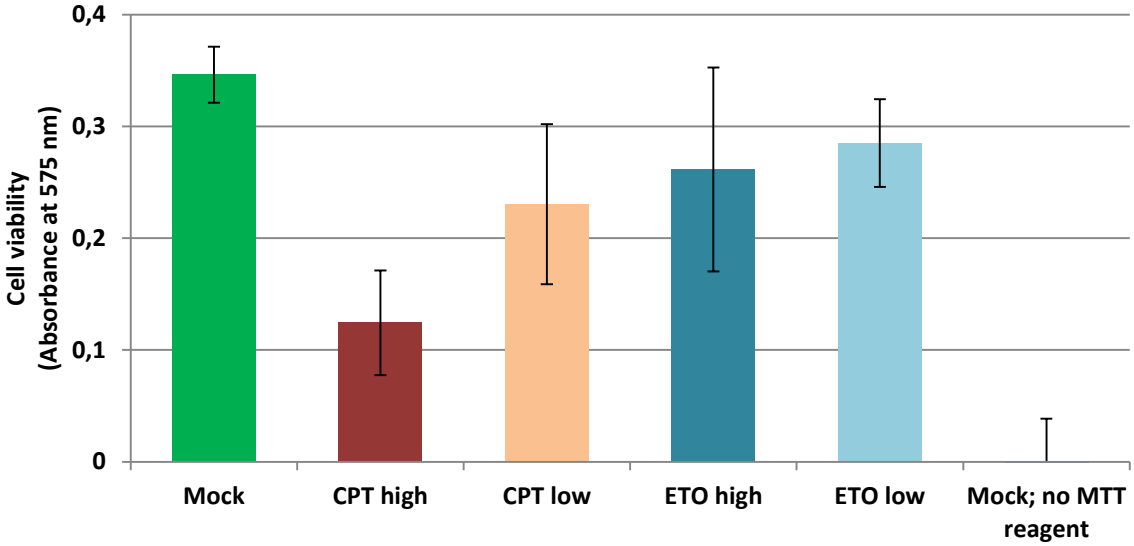


Figure 19. Camptothecin reduces cell viability to a higher degree than etoposide. HaCaT cells were seeded 5000 cells per well and treated with either 10 µM (high) or 0.25 µM (low) camptothecin (CPT), or 10 µM (high) or 1 µM (low) etoposide (ETO). Treatment was done for 24 h. MTT reagent was added for 2 h and then dissolved for 30 min in 40 mM HCl and 0.1 % NP-40. Absorption was measured at 575 nm wavelength. A 700 nm wavelength measurement was done to account for background absorbance.

To test whether TRIM29 does actually influence cell survival upon DNA damage in HaCaT cells, we counted the cells and seeded them in 5000 cells per well in a 96-well plate. The cells were then treated with various strengths of UV radiation (50 mJ/cm^2 and 200 mJ/cm^2) and then further cultured for 24 h. Afterwards, we measured the relative numbers of living cells through an MTT assay (Figure 20).

UV-radiation of both dosages lead to significant depletion of cells, but we could not conclude a significant difference between wild-type and *ΔTrim29* HaCaTs (Figure 20). This experiment was done only one time and differences in resistance against radiation were not detectable due to strong variations in the initial seeding, as seen in the untreated samples. In addition, as both treatments were too strong for an efficient readout, 30 mJ/cm^2 of UV-radiation would be a more suitable choice for further experiments. Also experiments with etoposide or camptothecin as stimulations were not yet performed and would be a topic for further experiments in this area.

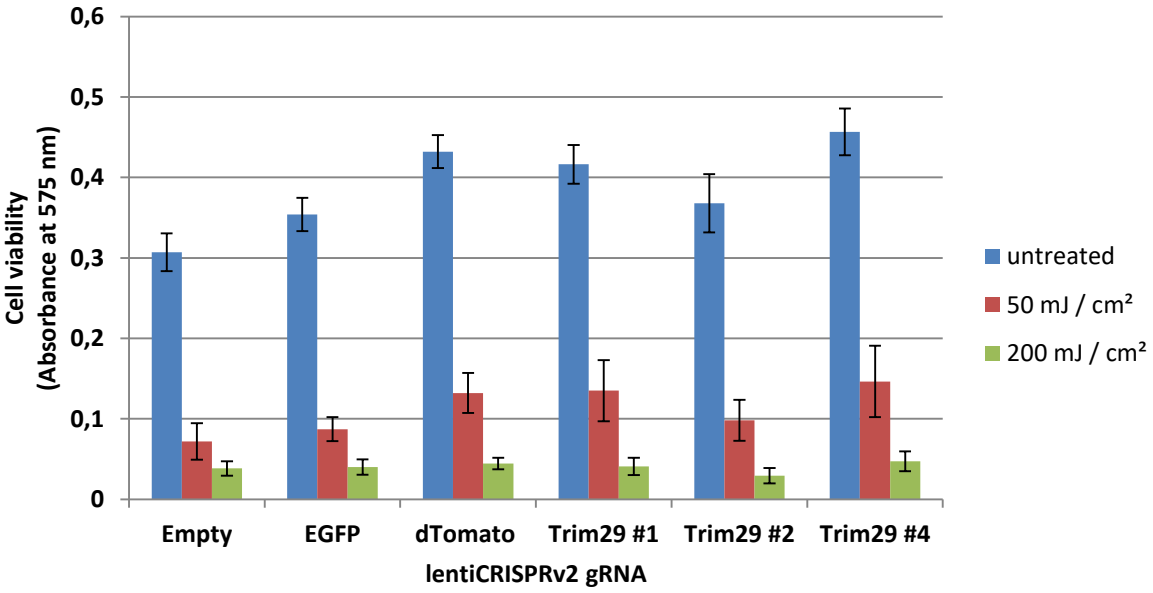


Figure 20. WT HaCaTs were not more resistant against UV radiation than *ΔTrim29* HaCaTs. HaCaT cells were seeded 5000 cells/well in heptaplicates on 96-well clusters. HaCaTs transduced with lentiCRISPRv2 vectors with either no gRNA or gRNAs targeting EGFP or dTomato were used as *Trim29*$^{+/+}$ control cells. Three different *ΔTrim29* HaCaT cell lines were used as *Trim29*$^{-/-}$ cells. The cells were either untreated or treated with 50 or 200 mJ/cm^2 UV radiation. 24 hours after treatment, the MTT reagent was added for 2 h, followed by 30 min of dissolving in 10 % SDS and 0.1 M HCl. Absorbance was measured at a 575 nm wavelength. A 700 nm wavelength measurement was used to account for background absorbance.

Discussion

In this study we investigated the tripartite-motif family protein TRIM29s function in the activation of the NFκB pathway through a possible interaction with members of the IKK complex. Our hypothesis was based on two publications which discovered an activating function of TRIM29 on NFκB in small lung cancer (Tang et al., 2013b) as well as bladder cancer cells (Tan et al., 2016). In addition, yeast-two-hybrid (Ravasi et al., 2010) and mass-spectrometry screens have detected TRIM29 to be a possible interactor with the IKK complex.

We performed a broad range of immunoprecipitation experiments to try to co-immunoprecipitate one of the IKKs together with TRIM29 to investigate this hypothesis. Furthermore, we also performed immunofluorescence experiments to determine an interaction between TRIM29 and NEMO by identifying co-localization of these two proteins. Despite our broad range of conditions, we were not able to confirm an interaction between these two proteins, which is in part the result of technical difficulties as well as of yet unknown reasons. As our approaches in finding an interaction between the proteins were unsuccessful, we further set out to investigate biological functions of TRIM29 in the regulation of NFκB under different conditions. These experiments did however not ascend above an establishing stage, as we were not able to induce NFκB activation in our investigated cells with our chosen stimuli. Therefore, even though we got several interesting insights into this topic, we were neither able to confirm, nor to dismiss our hypothesis of a TRIM29 dependent activating effect on NFκB through the IKK complex.

Our first goal was to assess the interaction between TRIM29 and one of the IKKs through immunoprecipitation experiments, but even with our multitude of experimental conditions, we were not able to reliably detect this interaction. Failing to do so can have many underlying reasons, of which we addressed some but not all during our experimental process. We were successful in our approach of performing co-immunoprecipitation experiments with interacting proteins in general, as we can conclude from our positive controls and negative controls present in our experiments, which clearly interacted and also lacked unspecific binding. The interaction between our main positive controls p65 and IκBα is however a very strong one and still stable under rather stringent buffer conditions. Assuming the interaction between TRIM29 and the IKKs is a rather weak one, the interaction could be disrupted during an experimental step while the interaction of our positive controls would stay intact.

We addressed parts of this problem by performing the experiments with a range of very mild buffers, which did however not have a major influence on the interaction. Lysis conditions were unchanged, which is due to the fact that they were already rather mild. Still, if the stringency during the lysis process was high enough to disrupt these interactions, this would be an explanation why a specific binding between our proteins was never detected.

Another possible problem concerning this matter is the intracellular localization of the interaction between TRIM29 and NEMO. As shown by us and others, TRIM29 and also NEMO is mainly localized in the cytoplasm but it has been shown for both of these proteins that they also translocate into the nucleus under certain conditions (Huang et al., 2003; Yang et al., 2015). The most notable condition under which this happens is DNA damage and especially DNA DSBs, as both of these proteins play important roles during this process.

TRIM29 and NEMO function in two separate pathways in the DNA damage repair which both are activated through ATM and even though no other role of the two proteins in the other pathway has been published, it is not unlikely that these pathways are somewhat more interconnected. In this context, it may be that the interaction between TRIM29 and NEMO happens exclusively or mainly inside the nucleus. If this would actually be the case, we couldn't detect the interaction because we never lysed the nuclei with our lysis conditions. In addition, if we really would utilize buffers which are able to lyse the nuclear membrane, the interaction between TRIM29 and NEMO could also be

disrupted in this process. If this is actually the case, we would have to utilize different methods to acquire solutions with our interacting proteins in them aside from whole cell lysates. Otherwise, it would probably be necessary to switch to other methods than immunoprecipitation to assess this interaction.

Interactions between TRIM29 and one of the IKKs could be very short lived, possibly because of a rapid modification of one of the participants, which could lead to the termination of the interaction. This could have the effect that only a small fraction of the proteins are interacting at a given time. Therefore, to detect a signal on western blot, a large amount of bait protein would probably be necessary to be pulled down to catch a small fraction of prey proteins. This would come however with its own problems, as a too high amount of the proteins can have detrimental effects on the overall processes in the cell and can potentially interfere with protein interactions. In addition, a high concentration of a protein makes the system prone to unspecific interactions or contaminations, as we have experienced in some of our experiments. We therefore decided on lower expression levels of our proteins as we wanted to exclude these situations and we deemed the residual protein amounts still high enough for conventional immunoprecipitation experiments.

Furthermore, the TRIM29 - NEMO interaction could also promote the degradation of NEMO, which would also explain a possible instability and short-liveliness of this interaction. During the writing of this manuscript, this situation has actually been described in alveolar macrophages (Xing et al., 2016), where TRIM29 has been found to K48 ubiquitinate NEMO which leads to its degradation through the proteasome. Hereby, high levels of TRIM29 expression lead to nearly complete ablation of NEMO in the tested cells. This effect could also be discovered when overexpressing both TRIM29 and NEMO in HEK-293T cells, which nearly completely lost the NEMO proteins, but only when TRIM29 was also expressed. Both of these scenarios were effectively inhibited by treatment of the cells with the proteasome inhibitor MG-132.

We ourselves have detected decreasing NEMO levels correlating with higher amounts of TRIM29 in some of our experiments (Figure 7). This was however not consistent throughout our project, but only the case in some of our experiments. Furthermore, the observed decrease in NEMO has been rather moderate and nowhere near the complete ablation that has been reported by this other group. This same group was also able to identify an interaction between TRIM29 and NEMO. However, this was only possible in an *in vitro* approach with purified proteins and never with a complete cell lysate (Xing et al., 2016).

As these data have not yet been published at the time we did our experiments and NEMO degradation did not occur persistently throughout our project, we hypothesized this effect we observed in our samples may also have been caused by decreased NEMO expression because of high TRIM29 expression. This was not unlikely as high expression of one transfected gene can decrease the expression of the other through competition for the cellular transcription and translation machinery.

We addressed the issue of a possible TRIM29 dependent NEMO degradation by treating cells with the proteasome inhibitor MG-132 (Figure 11). We could however not detect any difference in the TRIM29 or NEMO levels between the MG-132 treated and the untreated samples in these experiments. In this context it has to be noted that in the experiments where we treated samples with MG-132, we could never identify decreased NEMO levels when co-expressed with TRIM29 compared to co-expression with negative controls, even in MG-132 untreated samples. This let us speculate, that even though still a possibility in general, TRIM29 mediated NEMO degradation was not the sole cause for our lack of detectable interactions. We did not treat all of our experiments with the inhibitor, but just a few ones towards the end of the project. Therefore, proteasomal degradation could still be the underlying reason for a lack of interaction in some of the experiments where we detected decreasing NEMO levels.

In order to avoid the problems we had with co-immunoprecipitating TRIM29 and NEMO we also utilized immunofluorescence to detect co-localization of TRIM29 and NEMO. We did several

experiments in HEK-293Ts, HeLas, HaCaTs and keratinocytes, which confirmed the localization of TRIM29 in the cytoplasm of these cells, but were not successful in identifying an interaction with NEMO. The main reason why these experiments were not successful up until now was primarily a technical one. On the one hand, the signal we got from the NEMO detection was barely elevated from the background signal detected in our secondary-antibody-only controls and on the other hand, high amounts of bleed through from the other signaling channel interfered with a specific NEMO detection. This is most probably due to unspecific bindings and weak signals from our secondary antibody.

We also detected overexpressed HA-TRIM29 in combination with Myc-NEMO with our anti-HA and anti-Myc antibodies through immunofluorescence. However, the outcome of this was the same as in the endogenous detection, which is due to the fact that both our NEMO and our Myc detection had to share the same secondary antibody. To successfully perform these co-localization experiments, we would therefore have to switch to a different set of anti-NEMO and corresponding secondary antibodies.

An interesting insight we got from these experiments was, that TRIM29 is predominantly cytoplasmic in all of our tested cell lines. This was expected for HaCaTs and keratinocytes and is further also not surprising for HEK-293T cells, as it has been shown before in several publications that TRIM29 is primarily localized in the cytoplasm in most cells (Yang et al., 2015). It is however surprising for HeLa cells, as it has been published that both endogenous as well as overexpressed TRIM29 is primarily nuclear in these cells (Masuda et al., 2015). If this special localization is a feature common to certain cell-types, or if this is a mutation acquired in certain cancer cells, is not known. HeLa cells are highly mutated cancer cells which are steadily accumulating further mutations over time. Therefore, our HeLa cells could have acquired several mutations compared to the cells of other groups which could also have an impact on TRIM29s localization. Localization of proteins could also be influenced by minor environmental changes which can be differences in the growth medium as well as stimuli or stress caused somehow by different culture or treatment conditions. Whatever the reason for this effect is, it is important to keep in mind that TRIM29 might be differently regulated in our cells than in the ones from other groups.

We also aimed to reproduce some of the results which have shown TRIM29 to be an activator for NFκB and on which we based our working hypothesis. As both immunoprecipitation and immunofluorescence have not been successful in detecting an interaction between TRIM29 and the IKKs, it was important to verify if the results we built our hypothesis on hold also true in our own experimental setups. We deemed these steps important as the reproduction of these experiments would confirm that the TRIM29 dependent activation of NFκB is also a matter in our own used cells. In addition, reproducing these experiments was a useful step in establishing the necessary assays to investigate the influence of TRIM29 on NFκB in other contexts, like in immunological settings.

The assay we chose to detect NFκB activation has proven to be functional in both MEFs and RAW264.7 cells and worked, depending on the cell type, with LPS, TNF and in part even UV stimulation (Figure 13, 14). However, for us it never worked with etoposide or camptothecin as DNA damaging stimuli, even though they have been shown before by others to be a suitable stimulation for this assay (Huang et al., 2003). Furthermore, independent of the stimulus, we could never detect NFκB activation with any stimulus in our main cell types HaCaT and keratinocytes. As we were not able to detect NFκB activation in these cells even with our control-stimuli, we were not yet able to perform our planned experiments in our *Trim29* KO cells.

Concerning this, it could be that our stimulations were just insufficient in activating NFκB or that some of our cell-lines were insensitive to these stimuli. Both the etoposide and the camptothecin stimulus are known to be able to induce NFκB activation through DNA-damage, but compared to strong stimuli like LPS or TNFα, their potential in activating NFκB is rather weak. Furthermore, NFκB activation through DNA-damage is a much slower process than stimulations through the TLR or TNF receptors, taking between one to two hours to be detectable, compared to the five to twenty

minutes of a LPS stimulation. As a result of this, a less rapid and more stretched onset of the activation can result in weaker signals which may be also harder to catch time-wise. Possibly we missed the optimal time-point for this activation. We did our measurements multiple times from four hours to 30 minutes after the stimulation to hit the time-point of the activation peak, but it would still be possible that we missed it by some time. We were furthermore also not yet finished in optimizing the concentrations for our stimuli and it could be that the activation lacked sufficient strength to be detected by us at all. This could be further problematic as HaCaT cells have a constant level of constitutively active NFκB (Lewis et al., 2006). Therefore, slight additional activating imputs could probably be even harder to detect, if they do not exceed a certain threshold.

For our controls, using LPS to stimulate HaCaTs or keratinocytes is not possible as both lack a functional TLR4. We decided to use peptidoglycan or zymosan as positive controls as they have been shown to induce significant NFκB induction in these cells through induction of TLR2 (Mempel et al., 2003). We do not know why they did not suffice to induce a significant NFκB activation, but the reasons could be similar to the DNA damage stimulations. They were only used in two seperate experiments, therefore it is most likely either a problem of the strength of the activation, or again we could have been looking at the wrong time-point. TNFα was not used as a stimulus in our experiments with the HaCaTs, as we had no human TNFα available at the time we did the experiments.

Stimulations using UV radiations did only have an effect in RAW264.7 cells but not in any other of our tested cell type. This effect is however a different one than the one through the other stimuli as UV radiation does result in a phosphorylation of IκBα on different sides than the conventional Ser 32/36 residues. Furthermore, it is known that UV radiation triggers an NEMO independent path of activating NFκB. In line with this, we could not detect p-IκBα with our designated antibodies in these samples, but instead the accumulation of a not yet identified 100 kDa sized protein.

As this protein is detected with our anti-p-IκBα antibody and because of its size, it is possible our antibody bound to the phosphorylated p100 protein, even if this cross-reactivity is actually not intended. This would be an indication for the activation of the non-canonical NFκB pathway, which is also NEMO independent and would fit with what is known about UV induced NFκB activation. As this detection was not relevant for our project and also not confirmed, we resigned from using an anti-p-IκBα antibody in our other experiments with UV radiation and focused on detecting NFκB activation by detecting total IκBα. The total IκBα levels were however mainly unchanged after UV treatment, aside from a sudden decrease at the four hour detection (Figure 14). This could indicate sudden NFκB activation, even though it occurred unexpectedly late after the stimulation.

Detections in our keratinocyte samples were accompanied by strong variations in the actin levels throughout the samples, suggesting unequal cell numbers between them. These variations were primarily caused by unequal cell numbers at the beginning of the stimulations, as the cells did not adhere and proliferate equally after isolation in these few experiments. As a result of this, it was not possible to conclude an induction of NFκB from differences in the total IκBα signals as they correlated with the variations in the actin levels. As we were not jet able to optimize all these conditions, we could not yet perform our planned experiments to investigate NFκB activation in *Trim29* KO cells, which would actually give insight into an effect of TRIM29 in this pathway.

Aside from optimizing this assay, we could completely change our approach of measuring NFκB activation through western blot detections to another assay. One very promising approach which is also being used to measure DNA damage induced NFκB activation would be measuring the DNA binding activity of NFκB through an EMSA assay. Hereby NFκB activation could be directly measured by increasing DNA binding of the complex without relying on the timed measurement of IκBα levels. EMSA assays to detect NFκB activation in HaCaT cells have been done successfully by other groups before and worked even with the constant elevated NFκB activation in these cells under resting conditions (Lewis et al., 2006). Some of these experiments have also been done with UV radiation as stimulus, further underlining its potential for this project.

As another possible approach to investigate NFκB activation, a ChIP assay could be used. Also here we would stimulate wild-type or *Trim29* KO cells with various stimuli and, after mild cross-linking and sonication of the genomic DNA, we would perform immunoprecipitation on the Rel proteins. At first we would hereby focus on pulling on p65 and p50, as these are the most common NFκB subunits, but as we do not know which subunits are regulated through TRIM29, we would also have to test the other Rel proteins, foremost p52 as an indicator for the non-canonical pathway. For analysis of the co-precipitated DNA fragments, we would hereby focus on the most common NFκB target genes, but especially on genes playing roles in cell proliferation and survival. Cyclin-D1 and c-Myc have already been shown to be affected by TRIM29 dependent NFκB regulation and would therefore be prime targets for this investigation.

As an alternative to the classical ChIP approach, ChIP-seq could be used to sequence all co-precipitated DNA fragments and this way determine a broad range of target genes. This would enable us to find new TRIM29 regulated NFκB targets and possibly also whether they are dependent on a certain stimulus. Therefore, ChIP and ChIP-seq could be valuable assays to study further functions of TRIM29 in this project.

With a similar goal in mind, which is an immunological relevance of TRIM29, we also intended to test TRIM29s potential to promote cell survival during infection. Up until this point, we were still in a phase of establishing the required MTT assay for these experiments and were therefore not yet able to perform our desired key experiments. In our preliminary experiments we focused our attention on performing survival experiments with the DNA damaging reagents camptothecin and etoposide, as well as UV radiation, opposed to viral or bacterial infections which were planned for the final experiments. TRIM29 has been shown before to promote survival and cell proliferation during DNA damage conditions, which is why we focused on these during the establishing phase for these experiments. Furthermore, as similar experiments have been published before, this gave us quite good indications on some experimental conditions like concentrations.

In our first experiments, we could confirm that the MTT assay indeed seems accurate enough to work for our planned experiments, as the signal correlates well with the number of living cells. After that, we also did a first experiment to test an influence of TRIMK29 on cell survival upon UV treatment. This experiment was however accompanied by a multitude of problems which interfered with an exact readout. This assay is highly dependent on equal cell numbers between the samples at the start of the experiment to be comparable. Such a too high variation in starting cell numbers was one of the main issues in this experiment. As this was an issue in only a single experiment, we will have to take care for a more exact seeding in further ones. However, when normalizing the measured values to the initial numbers, no clear effect of TRIM29 on survival against UV radiation could be measured.

Another difficulty in this experiment concerns again the concentration of the stimuli. It is important to treat the cells with amounts that kill a significant number of cells, while at the same time enabling the survival of enough cells to measure a difference in surviving cells. Both a too low and too high concentration can impair a detection of differences between the samples, as they either kill to little cells to spot variations, or too many. In this case, the cells could be already majorly dead or the mechanism which is regulated by TRIM29 could be not strong enough to provide sufficient survival capabilities to make a difference.

We did some experiments to test various concentrations of our stimuli and their strength in killing the cells to narrow down the range of concentrations to use. For our single experiment with *Trim29* KO cells, both our 200 mJ/cm^2 and our 50 mJ/cm^2 UV treatment were chosen too high, as they killed the majority of the cells. To improve the readout, we would therefore have to lower the strength of the UV treatment in further experiments to a value of approximately 30 mJ/cm^2. As these are all technical issues, it should be relatively straight forward to fix these assays and perform the necessary control experiments before aiming at our goal of investigating survival during viral infections.

Taken together, what we know from our results, many of the experiments could not be finished or were not yet successful due to technical difficulties or because of a lack of optimization. Others, which mainly entail our interaction experiments, are far less clear and give room to a broad spectrum of speculations for underlying reasons. This is even more an issue considering some of the more recent publications on this topic. As already mentioned, an interaction between TRIM29 and NEMO which leads to the proteasomal degradation of NEMO, has recently been published (Xing et al., 2016). This same group was also able to successfully perform co-immunoprecipitation experiments with TRIM29 and NEMO. Interestingly, these immunoprecipitation experiments were only performed with concentrated and pre-purified proteins which were afterwards combined to interact *in vitro*, but never with complete cell lysates. This uncommon approach suggests that also they may have had problems performing co-immunoprecipitations in a complete cell lysate environment.

What exactly causes this potential interference with the interaction is not exactly clear. Very likely, there are some components in the lysate which interfere with prolonged TRIM29 – NEMO binding. It is also quite possible, that the K48-linked ubiquitination of NEMO through TRIM29 or another rapid modification results in the quick dissociation of the two proteins. This could lead to very short interaction times, which could be significantly prolonged with purified proteins, because of the lack of modifiers like ubiquitin and ATP as well as potential co-factors.

Interestingly, the result of this interaction between TRIM29 and NEMO was published to be the degradation of NEMO and therefore the repression of NFκB. This contradicts other publications which imply a role of TRIM29 in the activation of NFκB and is therefore also contradicting the data we based our own hypothesis on. Considering the possibility that all these publications are correct in their own sense, this leads to quite a sum of speculations concerning the role of TRIM29 in various cell types. In line with other proteins where a similar specificity has been observed, TRIM29 could perform cell-type specific functions *in vivo*. In this special case, TRIM29 could facilitate NFκB activation through a PKC dependent pathway in the investigated cancer cells and NFκB repression through K48-linked ubiquitination of NEMO in alveolar macrophages. Depending on the cell type, or on stimuli leading to the activation of certain co-factors, only one of the functions could be active at a given time.

Alternatively, it could also be that not one of the functions is only active under certain specific conditions, but rather that both functions are permanently active and one is dominant over the other. If this is the case, the weaker activating function, possibly through activation of PKC, could be permanently active, but is only really relevant after a stronger repressive function of TRIM29 has been turned off. This would also in part explain why it is so complicated to co-immunoprecipitate NEMO and TRIM29, as NEMO would be either degraded or, when inactive, the interaction would not happen at all. This would of course be different if TRIM29 would activate NFκB directly through the IKKs, but we were not yet able to find proof for such an interaction.

Strong indications that a repressive function of TRIM29 is active under resting conditions would be the facts that TRIM29 dependent NEMO depletion is also detected in transfected HEK-293T cells as well as that TRIM29 also ubiquitinates NEMO *in vitro* (Xing et al., 2016). Especially the second experiment strongly suggests that there is no activating stimulus involved and also no specific cell type required. Even though we could never co-precipitate NEMO and TRIM29, we did at times measure decreasing NEMO levels when co-expressed with TRIM29 in HEK-293Ts. As this was not a regular occurrence in our experiments, we did not consider this relevant at the time of these experiments.

Taken into account what we have seen in our experiments and what has been recently published on the topic of TRIM29, it is quite likely that even in at least some of our experiments, a TRIM29-NEMO interaction was not detectable due to rapid ubiquitination and further dissociation of NEMO. Under these circumstances, performing IPs with pre-purified proteins is an efficient solution to this issue. Certain regulatory features, also repressive in nature, still have to be necessary, as even when

occurring under resting conditions, this effect seems not to be common in all cells. Also the open question why TRIM29 is repressive in some and activating in other cell-types remains. TRIM29 may of course activate NFκB through different means than the IKK complex, like the already mentioned PKC, however, there would have to be at least another mechanism in place which turns off the repressing functions of TRIM29.

Also the fact, that TRIM29 is mainly cytoplasmic in most cell types, but nuclear in some others, like HeLa cells, could suggest a cell type or signal specific activation of TRIM29. However, a nuclear localization of NEMO has up until now only been detected in cancer cell lines, as opposed to the more common cytoplasmic localization. As TRIM29 is known to translocate into the nucleus upon certain stimuli, a mutation which causes a constant activation of TRIM29 in these cells is possible. Constantly active TRIM29 could not only permanently shuttle into the nucleus, but could also further enhance cancer progression and proliferation. We did not observe this phenotype in our HeLa cells, suggesting that our cells either lost this activating feature or that further unknown internal or external influences may be the cause.

Taken together, the available information on TRIM29 draws a very interesting picture about activating as well as repressing functions of TRIM29 in several cell types. Even though the range of function seems to be quite diverse, they all seem to center around proliferation and survival under different conditions. The most striking jet unknown open question for us concerns hereby a seemingly strong condition dependent functionality of TRIM29. Even without finding a direct activating effect of TRIM29 on the IKKs ourselves, TRIM29 seems to have both an activating as well as a repressing function on NFκB. Which one of the two has the predominant role seems hereby to depend on still unknown conditions.

Such specificity is not restricted on the NFκB pathway interacting function of TRIM29, but rather also affects other pathways. Activation of the Wnt-pathway through TRIM29 induces the transcription of similar genes as through NFκB. In addition, all publications concerning their respective pathway could show by pathway specific inhibitions, that the effect they describe is solely dependent on their stated pathway. It was hereby explicitly shown that the effect of TRIM29 dependent NFκB activation was completely independent of Wnt activation, and *vice versa*, even though they included the transcription of some of the same proteins. This means that based upon unknown conditions or situations, TRIM29 functions predominantly in either one of the two pathways. If this regulation is tied in or even identical to the switch between the activating and repressing function of TRIM29 on the NFκB pathway is also still not known.

TRIM29 functions in even more pathways, two of which are the activation of p53 and the DNA damage response. Onset of the DNA damage response function of TRIM29 is mediated through phosphorylation of TRIM29 by the ATM dependent kinase MK2. While the NEMO ubiquitinating function of TRIM29 seems to happen also under resting conditions, not much is known about the activation signals for the other pathways.

At least two papers have shown the Wnt and the NFκB activating function of TRIM29 to be mutually exclusive in their investigated cells. If this is due to a general switch between the functions of TRIM29 or separate feature due to the investigated cancer cell types, like a loss of function of an important co-factor, is yet up to speculations. The same would probably be true for the discrepancies between the activating and the repressing function of TRIM29 on NFκB. Furthermore, if the regulation of p53 and DNA damage are somehow connected to these switches, or even if the phosphorylation of TRIM29 acquired in the DNA damage response intertwines in this regulatory system, also mark an interesting topic for further investigations.

Concluding all this, there is now quite some knowledge available on the various functions of TRIM29 in several distinct biological pathways, but how these functions may interconnect and how they all are regulated is still mainly up to speculations. Determining the nature of these regulatory systems and how they affect TRIM29, through which proteins or modifications and also through

which initiating influences, would be a very interesting topic for future investigations regarding this versatile protein.

Methods

Cell culture

All used cell lines were grown in Dulbecco's modified Eagle's medium - high glucose (D5648 Sigma) supplemented with 10 % FCS (12133C Sigma) and 1 % Penicillin – Streptomycin (P4333 Sigma). Splitting of cells was performed with trypsin – EDTA (T4049 Sigma). Freezing of cells was performed with varying percentages of DMSO (D2438 Sigma): HEK-293T in 5 %, HeLa in 5 %, Cos7 in 10 %, MEF in 10 %, HaCaT in 5 %, RAW in 5 %. All cells were incubated at 37°C, 5 % CO_2.

Antibodies and reagents

Anti-HA antibody rabbit (H6908 Sigma), Anti-Flag antibody (F1804 Sigma), Anti-Myc antibody mouse (4A6 Ogris), Anti-TRIM29 antibody rabbit (ABE302 EMD Millipore), Anti-TRIM29 antibody goat (PA5-19248 Thermo Fisher Scientific), Anti-NEMO antibody (FL-419 Santa Cruz) , Anti-NEMO antibody (F-10 Santa Cruz), IκBα antibody (#9242 Cell Signaling), p-IκBα antibody (#9246 Cell Signaling), Anti-rabbit HRP-linked antibody (7074S Cell Signaling), Anti-mouse HRP-linked antibody (7076S Cell Signaling), Anti-mouse HRP-linked antibody (115-035-003 Jackson), Anti-goat HRP-linked antibody (705-035-003 Jackson), Anti-rabbit IgG – H&L (Alexa Fluor 488) (abcam ab150069), Anti-mouse – H&L (Alexa Fluor 647) (abcam ab169348), Etoposide (E1383 Sigma), Camptothecin (C9911 Sigma), h-TNF (300-01A peprotech), m-TNF (eBioscience 14-8321), LPS (Enzo Life Sciences ALX-581-008-L002), Zymosan (Z4250 Sigma), MG-132 (M7449 Sigma), Protease inhibitor cocktail (#78441 Thermo Scientific), Protein A coated beads (17-0780-01 GE Healthcare), Protein G coated beads (#10009D Thermo Scientific), Anti-HA sepharose beads (A2095 Sigma), Anti-HA magnetic beads (88836 Thermo Scientific)

Keratinocyte isolation

Primary mouse keratinocytes were obtained from 6 weeks old wild-type SV/129 mice. The mice were killed, the hair depilated and incubated in iodine surgical solution for 15 min. They were further disinfected by incubating for 2 min in 70% EtOH after 2 min washing in mQ H_2O. The mice were skinned (back skin, ears, tail) and transferred to a sterile hood where residual fat was removed. The skin was incubated for 45 min in a solution containing PBS, 0.8% trypsin (#27250-018 Invitrogen), 1x antibiotics/antimicotics (15240-062 Gibco), 1 x nystatin (N1638) and 80µg/ml gentamycin (#15750-060 Invitrogen). The dermis and epidermis were separated and the epidermis was further incubated for 45 min in a solution containing MEM (#M8167 Sigma), 8% chelated FCS, 250 µg/ml DNase (#DN-25 Sigma), 1.3mM $CaCl_2$, 1x antibiotics/antimycotics, 1 x nystatin and 80µg/ml gentamycin at 37°C (water bath). Cell suspension has been filtered through a 70µm cell strainer (BD #352350), washed once with SOS medium and further resuspended and cultured in SOS medium. SOS-medium contains MEM (#M8167 Sigma), 1µG/ml insulin (#I5500 Sigma), 2ng/ml EGF (#855731 Roche), 2µg/ml transferrin (#T8158 Sigma), 10µM phosphoethanolamine (#P0503 Sigma), 10µM Ethanolamine (#E0135 Sigma), 0.36µg/ml Hydrocortisone (#386698 Calbiochem), 1x glutamine (Invitrogen), 1x antibiotics/antimycotics, 6% chelated FCS. Seeded 1.5-2*10^6 cells/6-well coated plate. Dishes have been pre-coated with collagen (#FXP-018 Cohesion) and fibronectin (#33016-015 Invitrogen). Medium has to be changed 1h after seeding, on the next day and then every second day.

Cloning and plasmid construction

The cloned genes have been obtained either through already existing plasmids or from species appropriate cDNA. PCR reactions have been performed with appropriate primers that contain

restriction sites in their overhangs. Tags were added to the proteins by performing 2 PCR reactions, where the tag was split between the two N-terminal primers. Both the PCR amplified inserts as well as the target-vector (mainly pCDH-EF1, a lentiviral vector containing a packaging signal to enable viral transduction) has been cut with the appropriate restriction enzymes (Restriction enzymes by NEB). Purification of the desired fragments has been obtained by running the restricted samples on a 1% agarose gel, isolating the fragment of calculated size and extracting the DNA with the quiagen gel extraction kit (28704 quiagen). Ligation was performed with T4 DNA ligase (#15224-017 Thermo Scientific).

Transformation

5µl ligation has been aliquoted in eppendorf tubes on ice and mixed with 50 µl freshly thawed chemically competent E.Coli DH10b bacteria. Incubate the mixture for 20 min on ice. Heat-shock the bacteria for 45 seconds at 42°C, then cool on ice for 2 min. 500µl of LB-medium were added and the mixture further incubated at 37°C in the water bath for 60 min. The bacteria were spun down and plated on appropriate LB-agar plates containing antibiotics (ampicillin or kanamycin). Incubate at 28°C.

Transfection

For transfection of HEK-293T, HeLa and Cos7 cells, the appropriate amount of vector DNA is aliquoted in eppendorf tubes. Afterwards a mix of OptiMEM (#31985-070 Thermo Scientific) (50-100 µl per 6-well) with PEI (µg DNA : µl PEI = 1:3) is prepared. The mix is added to the DNA and incubated for 30 min. Afterwards the DNA-PEI mix is dropped onto the cells. Expression of transfected proteins should reach its peak after 48-72 h of incubation at 37°C.

Lenti-virus production and viral transduction

HEK-293T HiEx cells have to be transfected with plasmids containing Gag-Pol (coding for the structural proteins, capsid, as well as additional proteins like polymerase, integrase), VSV-G (coding for the surface glycoprotein that enables invasion in all cell types) as well as a packaging vector containing the desired packaged gene (which has to be cloned on a lentiviral vector containing a packaging signal) in a ratio of 5:1:5. The cells will produce live virus that peaks at about 48-72 hours. Virus containing supernatant is taken from the cells and either filtered or spun to avoid contamination with HEK-293T HiEx cells. The supernatant is diluted in appropriate medium and added onto the target cells. To enhance transfection efficiency polybrene (sc-134220) can be added to the mixture before addition to the cells. Infection happens rapidly, after 48h no live virus is detectable in the target cells dish. If the transduced vector contained a puromycin resistance cassette, the cells can be selected by adding puromycin to the medium.

Immunoprecipitation

Cells are harvested with trypsin, washed with PBS and pelleted inside an eppendorf tube or falcon tube. Cells have been lysed in a buffer containing 50mM Tris pH8, 150mM NaCl and 1% Triton X-100 supplemented with 1x protease inhibitor. Alternatively, a buffer containing 50mM Tris pH8, 150mM NaCl and 0.2% NP-40 (Ipegal CA-630) can be used. Let lysis proceed for 20 min, then spin down lysate to get rid of cell wall components and nuclei. Incubate the lysate with the desired antibody for 1 h on the shaker at 4°C or with pre-conjugated Antibody beads for 1-2 h at 4°C. If using pure antibodies, add protein A or protein G coated beads and incubate further 30 min. Wash the beads 4-6 times with TBS, PBS or lysis buffer. Elute precipitated proteins either by incubating for 10 min in

0.1M glycin pH 2 (gentle, does not elute all proteins, but keeps eluate antibody free) or by boiling the samples for 10 min in disruption buffer (10x contains 6.5M urea, 2M β-mercaptoethanol and 4.2% SDS, bromphenolblue; harsh, also elutes antibodies from the beads if not crosslinked). If not already boiled, boil eluates in disruption buffer for 10 min. Samples are ready to load.

SDS-Page and Western Blot

Prepared protein samples are denatured by adding disruption buffer to a 1x final concentration and boiling the samples for 10 min at 95°C. Samples are loaded onto SDS-Page gels containing 10% bisacrylamide (#3029 Roth). Samples are run in a running buffer containing 25 mM tris, 192 mM glycin and 0.1 % SDS. Western blots are performed with nitrocellulose membranes and a 3 buffer system. Anode buffer 1 (0.3 M tris, 20 % methanol; pH 10.4), anode buffer 2 (2.5 mM tris, 20 % methanol; pH 10.4), cathode buffer (0.04 M hexa-aminocaproic acid, 20 % methanol, 0.01 % SDS). The membranes were blocked by incubating for 1 h in TBS containing 5 % BSA. Incubation with the primary antibodies was performed in TBS containing 5 % BSA, 0.1% Na-azide, phenol red for 1 h. Incubation with the secondary antibody was performed in PBS containing 5 % milk. In between, blots were washed 3 times each for 5 min in TBS containing 0.01% triton X-100. Detection was performed with a western blotting luminol reagent (#sc-2048 Santa Cruz).

Immunofluorescence

Cells were seeded on cover slips, washed two times with PBS and fixed by adding 3 % PFA (Roth 30525-89-4) in PBS for 20 min. Cells were washed two times with PBS and then permeabilized with 0.1 % Triton X-100 in PBS for 15 min. After washing again with PBS, the cells were blocked with 1 % BSA in PBS for 30 min. The cells were incubated with the primary antibody for 1 h, and after washing, with the secondary antibody for 1 h. DAPI (D9542 Sigma) was used to stain the DNA. After staining, a mounting medium (Vector Laboratories, H-1000) was added onto the samples and hardened o/n at RT. The samples were analyzed with a Zeiss Axio Imager Z2 and the ZEN 2 blue software.

MTT assay

Cells are stimulated in an appropriate time frame prior to MTT assay. 5 mg/ml MTT solution (Thiazolyl Blue Tetrazolium Bromide; #M2128 Sigma) is added to the cells and incubated for 3 h. After the incubation time, the MTT solvent is added (4 mM HCl, 0.1 % NP40, in isopropanol). Incubating in the solvent for cell lysis and dissolving the crystals is performed for 1 h at 37°C. Readout for absorption by the crystals is performed at 570 nm wavelength. Readout for background absorbance and normalization is performed at 650 nm.

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