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The Role of Rip2 Protein in the Nod Mediated Innate Immune Response

A Dissertation Presented

By

Yibin Yang

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April 16, 2010

Cancer Biology

Role of Rip2 Protein in the Nod Mediated Innate Immune Response

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Abstract

The Rip2 kinase contains a caspase recruitment domain (CARD) and has been implicated in the activation of the transcriptional factor NF- κ B downstream of Nod-like receptors. However, how Rip2 mediates innate immune responses is still largely unclear. We show that Rip2 and IKK- γ become stably polyubiquitinated upon treatment of cells with the Nod2 ligand, muramyl dipeptide. We demonstrate a requirement for the E2 conjugating enzyme Ubc13, the E3 ubiquitin ligase Traf6 and the ubiquitin activated kinase Tak1 in Nod2-mediated NF- κ B activation. We also show that *M. tuberculosis* infection stimulates Rip2 polyubiquitination. Collectively, this study revealed that the Nod2 pathway is ubiquitin regulated and that Rip2 employs a ubiquitin-dependent mechanism to achieve NF- κ B activation.

We also demonstrate that intraphagosomal *M. tuberculosis* stimulates the cytosolic Nod2 pathway. We show that upon Mtb infection, Nod2 recognition triggers the expression of type I interferons in a Tbk1 - and Irf5-dependent manner. This response is only partially impaired by the loss of Irf3 and therefore, differs fundamentally from those stimulated by bacterial DNA, which depends entirely on this transcription factor. This difference appears to result from the unusual peptidoglycan produced by mycobacteria, which we show is a uniquely potent agonist of the Nod2/Rip2/Irf5 pathway. Thus, the Nod2 system is specialized to recognize bacteria that actively perturb host membranes and is remarkably sensitive to Mycobacteria, perhaps reflecting the strong evolutionary pressure exerted by these pathogens on the mammalian immune system.

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Chapter I

Introduction

Cells within an organism face constant stresses from both outside and inside the cell. These stresses allow communications between cells and signal changes in the environment to which the cells must adapt. To respond to these stresses, cells activate a series of pathways which ultimately lead to changes in gene expression, enzyme activity, or the localization of cellular components. For example, binding of pro-inflammatory cytokines to their receptors, stimulation of pathogen recognition receptors by pathogen-associated molecular patterns, or events that induce DNA damage activate the transcription factors NF- κ B and AP-1, as well as mitogen-activated protein kinases and interferon regulatory factors, resulting in specific cellular responses [1,2].

However, these cellular stress pathways must be precisely regulated, since they regulate cell proliferation, motility, communication and survival. Deregulation of these pathways can result in chronic inflammatory diseases such as inflammatory bowel disease (IBD), Crohn's disease (CD), autoimmune disease, etc [3,4]. This dissertation focuses on the receptor-interacting protein (RIP) family, which are critical regulators/mediators of several cellular stress pathways.

I. Overview of the Rip protein family

The human genome contains about 500 protein kinase genes, which transmit signals and control complex processes in cells [5,6]. One branch of the serine/threonine kinase family includes the receptor-interacting proteins (RIP). Rip kinases are closely

related to members of the interleukin-1-receptor-associated kinase (IRAK) family. IRAK family members mediate interleukin-1 β (IL-1 β) and Toll-like receptor (TLR) signaling pathways [7], suggesting that the Rip family proteins may also be involved in the response to cell stress induced signaling pathways.

Receptor interacting protein (RIP) kinases constitute a family of seven members (Figure 1), all of which contain a serine/threonine kinase domain (KD) but diverge at their C-termini. Rip kinases mediate cellular responses to infection, inflammation, differentiation and DNA damage. Although these stimuli are diverse, they activate similar pathways including activation of the transcription factors NF- κ B or AP-1 [8].

Rip1 and Rip2 are the Rip family members which have been most extensively studied. Besides the serine/threonine kinase domain, Rip1 and Rip2 (CARDIAK/RICK) contain a C-terminal domain belonging to the death domain (DD) superfamily, namely, a DD and a caspase recruitment domain (CARD), respectively. Both the DD domain and CARD domains recruit large protein complexes through domain oligomerization, initiating different signaling pathways. Rip1 and Rip3 also contain a RIP homotypic interaction motif (RHIM). This RHIM domain is sufficient for the interaction of Rip1 with the TLR3/4 adapter protein Trif and Rip3 [9], which suggests that Rip3 and Rip1 may regulate Trif dependent anti-viral responses. Rip4 (DIK/PKK) and Rip5 (SgK288) are characterized by the presence of ankyrin repeats in their C-terminal domains, which are required for keratinocyte differentiation[10,11]. Rip6 (LRRK1) and Rip7 (LRRK2)

contain a leucine-rich repeat (LRR) motif in the N-terminal domain, which may be involved in recognition of pathogen-, damage- or stress-associated molecular patterns (PAMP, DAMP, SAMP). In addition, they contain Roc/COR domains (Ras of complex proteins/C-terminal of Roc) adjacent to the LRR motif. Binding of GTP to the GTPase-like Roc domain leads to the stimulation of Rip6 kinase activity. Mutational analysis indicated that the COR domain might be important for transmitting an stimulating signal to the kinase domain. The function of Rip6 and Rip7 remains unclear, though, mutations in the human Rip7 gene are associated with both familial and sporadic Parkinson's disease[12,13].

II. Protein Ubiquitination and Signal Transduction

Protein ubiquitination is an essential and ubiquitous mechanism used by eukaryotic cells to regulate responses to cellular stress [14]. This process involves a covalent modification of cellular proteins by ubiquitin, a highly conserved, 76-amino-acid protein [15]. Ubiquitin is linked to specific lysine residues of the substrate, and the long polyubiquitin chains are formed as additional ubiquitins are attached to a lysine residue in ubiquitin itself. Originally, ubiquitin modification was primarily considered a mechanism of targeting proteins for proteasomal degradation. In the past several years, a number of new cellular functions have been associated with stable protein ubiquitination.

The protein ubiquitination process initiates with ubiquitin activation by an E1

(ubiquitin-activating enzyme), followed by the transfer of an activated ubiquitin to an E2 (ubiquitin-conjugating enzyme, also known as Ubc), and ends with the conjugation of ubiquitin to a target protein through the activity of a ubiquitin-protein ligase (E3) to form an isopeptide bond between the carboxyl terminus of ubiquitin and an epsilon amino group of a lysine residue on the protein substrate. The E3 ubiquitin ligase, sometimes together with E2s, usually determines substrate specificity. E3 ubiquitin ligases are a large protein family that can be divided into three categories, depending on the nature of the E3 ligase domain: HECT (homology to E6AP C-terminus), RING (really interesting new gene) domain and U Box domain. The HECT domain E3s contain an active-site cysteine, which can accept ubiquitin from an E2 and transfer the ubiquitin to a target protein. In contrast, the RING domain E3s do not contain a conventional enzyme active site, but they promote ubiquitination by binding to both protein substrates and E2s, facilitating the conjugation of ubiquitin to specific protein targets. Also, similar to phosphorylation, the ubiquitination reaction is a reversible covalent modification, regulated by a large family of deubiquitination enzymes (DUBs, also known as isopeptidases) [16].

There are seven lysines in ubiquitin, each of which can be conjugated by another ubiquitin to form a polyubiquitin chain [17]. Recent evidence suggests that the topology of polyubiquitin chains may have specific functions. Generally speaking, polyubiquitin chains linked through lysine 48 (K48) of ubiquitin normally target a protein for proteasomal degradation, whereas K63-linked polyubiquitin chains have functions

independent of proteolysis, and are usually involved in responses to cellular stress [18]. Monoubiquitination, where a protein substrate is modified with a single ubiquitin, usually does not lead to proteasomal degradation, but appears to regulate chromatin remodeling, vesicle trafficking and cellular localization.

A recent study showed that a ubiquitin ligase complex composed of two RING domain proteins, RNF31 (also known as HOIP) and RBCK1 (also known as HOIL-1L), and UbcH5C catalyze the synthesis of linear ubiquitin chains. These linear ubiquitin chains can bind NEMO and are believed to be important for IKK activation [19,20]. This study suggests that in addition to K63-linked ubiquitin chains, linear ubiquitin chains may also have the specific function of mediating signaling pathways. However, another report indicated that the RNF31-RBCK1 synthesized linear polyubiquitin chains did not activate IKK or Tak1 and only K63-linked ubiquitin chain can activate IKK and Tak1. Furthermore, the results from this report indicate that unanchored polyubiquitin chains directly activate IKK and Tak1 *in vitro*, suggesting a potentially new mechanism of kinase activation [21].

III. The NF- κ B pathway

Both proteolytic and nonproteolytic protein ubiquitination reactions contribute to the regulation of nuclear factor kappa B (NF- κ B). There are five different Rel family members that have been identified in mammals: RelA (p65), RelB, c-Rel, NF- κ B1 (p50 and its precursor p105) and NF- κ B2 (p52 and its precursor p100), which can form either

hetero- or homodimers. Active NF- κ B transcription factors are composed of dimeric combinations of members of the Rel transcription factor family [22,23]. Rel family members have a 300-amino-acid long N-terminal domain, called the Rel homology domain (RHD), which is responsible for DNA binding, dimerization and interaction with inhibitor of κ B (I κ B) proteins. Interaction with I κ B blocks the nuclear localization signal (NLS) present in the RHD and sequesters NF- κ B in an inactive form in the cytoplasm. In addition, RelA/p65, RelB and c-Rel also have transcriptional activation domains (TAD) which are required for their transcriptional activity. In most cells, the p50/p65 (p50/RelA) heterodimer is the major transcriptional activator which regulates diverse gene expression. p50 and p52 lack a TAD domain and p50 or p52 homodimers are thought to repress transcription of NF- κ B-dependent target genes. The NF- κ B transcription factor family is involved in the host response to diverse cellular stresses, including cytokines, bacteria, viruses, oxidative, genotoxic, physiological or chemical stress factors [24]. Three major NF- κ B activating pathways have been identified [23,25,26] (Figure 2).

The canonical or classic NF- κ B pathway, is typically activated in response to pathogen-associated molecular patterns (PAMPs) or pro-inflammatory cytokines. During infection or an inflammatory response, the IKK signalosome complex, consisting of two kinases (IKK α and IKK β) and a regulatory scaffolding subunit IKK γ /NEMO (NF- κ B essential modifier), is activated by an upstream kinase. The activation of the IKK complex results in phosphorylation, ubiquitination, and degradation of NF- κ B inhibitor I κ B α , which releases the prototypical NF- κ B dimer RelA/p50 from its inhibitor I κ B α .

I κ B α degradation exposes the NLS of NF- κ B, allowing the nuclear translocation of the RelA/p50 heterodimer and subsequent induction of specific gene transcription. The canonical NF- κ B pathway can be activated rapidly in response to acute stress situations.

The noncanonical or alternative pathway is predominantly active in B cells and is induced by a particular subset of TNFR family ligands, such as B-cell-activating factor of the tumour necrosis factor (TNF) family (BAFF), CD40 or lymphotoxin (LT) β . In this pathway, the IKK signalosome complex consists exclusively of IKK α homodimers, without IKK β and IKK γ /NEMO. Upon stimulation, NF- κ B-inducing kinase (NIK), a kinase implicated in the phosphorylation of IKK α , is recruited to the receptor and activates IKK α . IKK α then targets p100 for phosphorylation and K48 linked ubiquitination, resulting in the limited proteolysis of its ankyrin-like C-terminus, generating the mature p52 subunit. p52 dimerizes with RelB, and the dimeric complex enters the nucleus to activate the expression of genes important for B-cell maturation and activation.

The third NF- κ B activation pathway, also known as the atypical pathway is induced by stimuli such as DNA damage or oxidative stress. Compared to the canonical pathway, which usually induces a rapid and strong NF- κ B-activating signal, the atypical NF- κ B activators induce a slower and weaker NF- κ B signal (with peak activities reached after 2–4 hours). The detailed mechanism of how genotoxic stress activates NF- κ B remains unclear, and each different stimulus may use a completely unrelated mechanism. For example, in the case of DNA damage induced NF- κ B activation, ultraviolet

(UV)-induced NF- κ B signaling appears to be IKK-independent [27], while most other genotoxic stress agents activate a pathway that require classic IKK activation and I κ B α degradation. No consensus has been reached as to how oxidative stress induces NF- κ B activation, but most likely this involves phosphorylation of I κ B α [28].

IV. Rip1 and TNF-induced NF- κ B Activation

Rip1, the first member of the Rip family, was identified in a yeast two-hybrid screen in 1995 as an interaction partner of the Fas death receptor through a homotypic DD–DD interaction [29]. Subsequent work revealed that Rip1 interacted with other death receptors, such as tumor necrosis factor (TNF)-R1, TRAIL-R1 and TRAIL-R2, and other DD-containing adaptor proteins such as TNF-receptor-associated death domain (TRADD) and FADD. The recruitment of Rip1 to the TNFR1 complex upon TNF stimulation indicated that Rip1 plays an important role in TNF-induced signaling [29,30].

Rip1 is constitutively expressed in many tissues, and also shows inducible expression upon T-cell activation or after stimulation with TNF [29]. Rip1 expression in HEK293 cells activates an NF- κ B–luciferase reporter plasmid and induce apoptosis, suggesting that Rip1 may regulate both survival and death via the TNF pathway[30]. These dual functions of Rip1 can be explained by the observation of a caspase-8-dependent cleavage of Rip1 in treated cells, which generates a C-terminal cleavage product during stimulation by TNF, Fas or TNF-related apoptosis-inducing ligand (TRAIL) [31]. The C-terminal cleavage product of Rip1 blocks NF- κ B activation

and promotes cell death, whereas a Rip1 non-cleavable mutant activates NF- κ B and protects the cells against TNF-induced apoptosis [31]. Thus, the cleavage of Rip1 controls the survival/death decision.

The physiological role of Rip1 in the TNF pathway was addressed with the generation of Rip1-deficient cells. A Rip1 deficient Jurkat leukemic T cell line was selected for its inability to activate NF- κ B. When these cells were stimulated with TNF α , they failed to activate NF- κ B [32]. Reconstitution of the Rip1-deficient Jurkat T cell line with a Rip1 expression plasmid restored TNF signaling. Similarly, studies of Rip1-deficient MEFs revealed that Rip1 mediates the TNF α induced NF- κ B pathway [33]. Rip1 deficient mice die during the postnatal period and display massive cell death of lymphoid and adipose tissues [33]. Although the NF- κ B response to TNF α stimulation is impaired in the absence of Rip1, TNF induced c-Jun N-terminal kinase (JNK) activation appears less affected [33]. Rip1 deficiency also sensitizes to TNF-induced cell death [33]. The kinase activity of Rip1 is not required for the activation of NF- κ B because reintroduction of a kinase-dead form of Rip1 into the Rip1-deficient Jurkat cells or MEFs restored full NF- κ B activation [32,34].

More recent studies have demonstrated that Rip1 is ubiquitin modified in TNF stimulated cells and its polyubiquitination appears essential for TNF induced NF- κ B activation. Upon TNF stimulation, Rip1 is polyubiquitinated in lipid rafts [35]. Rip1 can be modified by both K48 and K63 linked polyubiquitin chains [34,36]. K63-linked polyubiquitination of Rip1 is required for TNF α -induced activation of NF- κ B. Although

K63-linked ubiquitination can occur on several lysine residues in the intermediate region of Rip1, a critical lysine residue in the intermediate region of Rip1 (Lys377 in hRip1 and Lys376 in mRi1) was shown to be critical for the function of Rip1 in TNF pathway [37]. Expression of a Rip1 K377R mutant in Rip1-deficient cells failed to activate NF- κ B upon TNF treatment. Rip1 ubiquitination may stimulate recruitment of the Tak1-binding protein 2 (Tab2) and thereby recruit Tak1-Tab2 complex to TNFR1 associated polyubiquitinated Rip1. K63-linked polyubiquitinated Rip1 also binds polyubiquitinated NEMO and thereby brings the IKK complex and activated Tak1 into close proximity with IKK β [37]. Some studies suggest that K63 polyubiquitin chains are able to directly activate Tak1 by binding to the ubiquitin receptor Tab2. This binding leads to autophosphorylation and activation of Tak1 [21]. Thus, binding to K63 polyubiquitinated Rip1 stimulates formation of NEMO/Tak1/IKK β complex and ensures Tak1 and IKK activation [37] (Fig. 3).

Multiple E3 ubiquitin ligases have been implicated in the ubiquitin modification of Rip1. The Traf2 and Traf5 Ring domain-containing E3 ligases have been suggested to be required for Rip1 K63-linked ubiquitination following TNF α stimulation [34,36]. In the absence of Traf2 and Traf5, Rip1 polyubiquitination and NF- κ B activation is impaired. cIAP1 and cIAP2, another group of Ring domain containing E3 ligases, have also been shown to be required for Rip1 polyubiquitination in the TNF pathway [38]. Whether these E3 ligases cooperate to regulate Rip1 polyubiquitination remains unclear and requires further investigation.

The deubiquitinating enzyme (DUB) A20 terminates the TNF-induced NF- κ B response by regulating Rip1 polyubiquitination [36]. The amino-terminal domain of A20, which is a de-ubiquitinating (DUB) enzyme of the OTU (ovarian tumour) family, removes K63-linked ubiquitin chains from Rip1. The carboxy-terminal domain of A20, composed of seven C2/C2 zinc fingers, then functions as a ubiquitin ligase to polyubiquitinate Rip1 with K48-linked ubiquitin chains, thereby targeting Rip1 for proteasomal degradation, terminating signaling to NF- κ B. The polyubiquitin chain editing on Rip1 in the TNF pathway has been confirmed by using the linkage-specific polyubiquitin antibodies [39]. Consistently, binding of NEMO to polyubiquitinated Rip1 stabilizes Rip1 by inhibiting its degradation, possibly by interrupting the interaction of Rip1 with A20.

V. Rip1 and necroptosis

Patterns of cell death have been divided into apoptosis, which is actively executed by caspases, and programmed necrosis. Although death receptor ligands predominantly induce apoptosis, a form of programmed necrosis called necroptosis occurs in the absence of caspase activation [40]. Necroptotic cells lack chromatin condensation and form large lysosome-derived cytosolic vacuoles which lead to cellular disintegration and release of cytoplasmic materials. Caspase 8-deficient Jurkat leukemic cells undergo cell death upon TNF α treatment, suggesting that TNFR1 activation stimulates an alternative form of cell death [41]. Furthermore, treatment with TNF α or

FasL induced cell death in Jurkat T cells and L929 murine fibroblasts pretreated with the caspase inhibitor zVAD-fmk [42]. Although the mechanism for this necroptotic pathway is unknown, Rip1-deficient Jurkat cells are resistant to TNF α - and FasL- induced cell death in the presence of the caspase inhibitors, suggesting that Rip1 may regulate necroptosis [43].

Recent studies demonstrate that Rip1, as well as another Rip family member Rip3, play essential roles in the decision between tumor necrosis factor (TNF)-induced necroptosis and survival when apoptotic signaling is blocked [44,45,46]. Indeed, both Rip1 and Rip3 are activated catalytically by phosphorylation, and interact with each other via the RIP homotypic interaction motif, RHIM. This phosphorylation and interaction is essential for the initiation of programmed necrosis *in vitro* and *in vivo*. In cells that express Rip3 and cannot properly activate caspases due to the presence of the pancaspase inhibitor zVAD-fmk, TNF stimulation leads to the assembly of a multiprotein complex that contains caspase-8, FADD, Rip1 and Rip3. In this complex, Rip1 and Rip3 are phosphorylated in an interdependent manner. Phosphorylated Rip3 then interacts with several bioenergetic enzymes including glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL) and glutamate dehydrogenase 1 (GLUD1), thereby enhancing their catalytic activity. Enhanced glycogenolysis and glutaminolysis provide additional respiratory substrates and ultimately result in an increase of reactive oxygen species (ROS). Excess ROS, in turn, can trigger mitochondrial membrane permeabilization (MMP), thereby mediating TNF-induced programmed necrosis.

VI. Rip1 and the anti-viral innate immune response

In addition to a kinase and death domain, a short region of 35 amino acids located in the intermediate region of Rip1 was also identified in the TIR-domain-containing adaptor protein Trif (also called TICAM-1), known to mediate TLR3 and TLR4 signaling [47]. This region is called the RIP homotypic interaction motif (RHIM) and also present in the intermediate region of Rip3 and DAI (DNA-Dependent Activator of IFN Regulatory Factors) [47]. Trif binds to TLR3 and TLR4, two TIR-domain-containing receptors that recognize double stranded RNA (dsRNA) from viruses and lipopolysaccharide (LPS) from Gram-negative bacteria, respectively. Rip1 has been shown to interact with Trif through the RHIM domain, implicating Rip1 in TLR3 and TLR4 signaling [9,48]. Studies in Rip1 deficient cells demonstrated that the activation of NF- κ B and MAPKs, induced by TLR3 and TLR4 ligands, depends on Rip1 [9,48]. TRIF-induced activation of NF- κ B can also proceed independently of the RHIM domain, through N-terminal binding of Traf6. Upon TLR3 activation, Rip1 is also modified by polyubiquitin chains and is recruited to TLR3 along with Traf6 and the ubiquitin-activated kinase Tak1 [49]. Rip1 polyubiquitination is also been observed in TLR3 stimulated cells, and the E3 ubiquitin ligase Peli1 has been reported to mediate ubiquitin modification of Rip1 in the TLR3 pathway [50]. These studies suggest that Rip1 uses a similar, ubiquitin-dependent mechanism to activate IKK β in response to TNF- α and TLR3 ligands. Rip1 is dispensable for TRIF-dependent activation of IRF3, which occurs in a TRIF/TRAF3/TBK1/IKK ϵ -dependent manner.

Viral replication intermediates, including dsRNA, are potent stimuli that trigger host responses when they are recognized and engaged by specific pathogen recognition receptors (PRRs). TLR3, TLR7 and TLR9 participate in anti-viral innate immunity and recognize dsRNA, ssRNA and CpG DNA, respectively. However, dsRNA is also recognized by the cytoplasmic RNA helicases retinoic acid-inducible gene I (Rig-I) [51] and melanoma differentiation-associated gene (Mda) 5 [52,53]. These helicases mediate the activation of NF- κ B, ATF2-c-Jun, IRF-3 and IRF-7 via recruitment of a CARD-like-domain-containing adapter, IFN promoter stimulator (IPS-1) [54] (also called MAVS, CARDIF, VISA). The IPS-1 adapter interacts with Fadd and Rip1 to activate NF- κ B and type I IFN promoters. Cells deficient for FADD or Rip1 are impaired in their type I IFN response to infection with VSV (vesicular stomatitis virus) [55]. A Fadd-Rip1 pathway has also been described in *Drosophila* where the Rip1 homolog Imd binds dFadd to activate an NF- κ B-like pathway and induce expression of anti-microbial genes [56].

How do Rip1 and FADD the mediate anti-viral innate immune response? It has been implied that Rip1 mediates Rig-I anti-viral signaling by facilitating IRF-7 activation. Rip1 interacts with IRF7 and LMP1 (latent membrane protein 1) in EBV-positive Burkitt's lymphoma cells [57]. LMP1 stimulates the K63-linked ubiquitin modification of Rip1 and IRF7. Although the IPS-1 adapter interacts with Rip1, it does not associate with TBK-1 or IKK- ϵ , suggesting that Rip1 may link TBK-1/IKK- ϵ kinase to IRF-7. Thus, Rip1 may contribute to Rig-I anti-viral signaling by mediating both NF- κ B and IRF-7

activation.

Most recently, Rip1 has been implicated in DAI (DNA-dependent activator of IFN regulatory factors) mediated NF- κ B activation [58]. DAI is a cytosolic DNA sensor that stimulates the IRF3 and NF- κ B pathways leading to type I IFN and cytokine production. DAI, also contains a RIP homotypic interaction motif (RHIM), and binds to and colocalizes with endogenous Rip1 in characteristic cytoplasmic granules. Rip1 deficient cells are impaired in their NF- κ B activation mediated through DAI. The role of Rip1 in DAI-mediated NF- κ B activation in response to immunostimulatory DNA appears analogous to the sensing of dsRNA by TLR3. Both pathways involve RHIM-dependent signaling, reinforcing a central role for Rip1 in anti-viral innate immune responses.

VII Overview of Rip2 and the NLR pathway

The Rip1-related Rip2 protein also responds to cellular stress brought on by bacterial infection. Rip2, also called RIP-like-interacting CLARP kinase (RICK) and caspase-recruitment domain (CARD)-containing IL-1 β converting enzyme (ICE)-associated kinase (CARDIAK), is a novel Rip1-like kinase also capable of inducing NF- κ B activation and cell death [59,60,61]. Similar to Rip1, Rip2 is expressed in many tissues and the kinase activity of Rip2 appears dispensable for the NF- κ B response [61]. Rip2 also contains an N-terminal kinase domain followed by an intermediate region. Instead of a DD domain, Rip2 contains a C-terminal CARD domain in the C-terminal. Rip2 interacts with many CARD-domain containing proteins.

In transfected cells, Rip2 also interacts with several TRAF family members, including TRAF1, TRAF2, TRAF5, TRAF6 and the anti-apoptotic proteins cFLIP and cIAP1 (cellular inhibitor of apoptosis 1) [59,60,61].

Overexpression of Rip2 stimulates the NF- κ B, p38 MAP kinase, JNK and ERK2 pathways [61,62]. Upon overexpression, the CARD domain of Rip2 interacts with the CARD domain of caspase-1, a pro-inflammatory caspase implicated in IL-1 β maturation [63]. Rip2 was therefore proposed to play a role in the generation of active IL-1 β , but this could not be confirmed in a mouse model, as caspase-1 activation occurs normally in Rip2 deficient macrophages [64]. It remains to be seen whether Rip2 itself might be regulated by the activity of caspase-1, since Rip1 itself might be a caspase target.

An important hint regarding the physiological function of Rip2 came from the observation that the CARD of Rip2 interacts with CARDS of Nod1 and Nod2 (also called CARD-4 and CARD-15, respectively), two cytoplasmic pathogen-recognition receptors that sense intracellular peptidoglycans [65]. In a model of invasive *Shigella* infection, Nod1 was shown to sense the presence of intracellular bacteria and to subsequently activate JNK and NF- κ B. *Shigella* infection or self-association of Nod1 induces the transient formation of a complex containing Nod1, Rip2, and the IKKs [66]. Rip2 also interacts with the second Nod family member, Nod2 [67]. Mutations in NOD2 or CARD15 are associated with a subset of Crohn's disease patients [68,69]. Hence, Rip2 is likely to participate in the elaboration of the innate immune response to pathogens,

downstream of the intracellular Nod receptors.

VIII. NLR and innate immunity

As the first line of defense, the innate immune system is involved in the initial detection and removal of harmful microbes. While immune reactions induced through the adaptive immune system may take days, innate immune responses are activated immediately or within several hours following an encounter with an invading organism. Thus the signaling pathways triggered by innate immune system must be regulated with exquisite precision [70].

Unlike adaptive immunity, the innate immune system does not have the capability to recognize specific antigens. Rather, it is designed to recognize molecules shared by groups of related microbes that are essential for the survival of those organisms and are not found associated with mammalian cells. These unique microbial molecules are called pathogen-associated molecular patterns or PAMPS [71]. These PAMPS include lipopolysaccharide (LPS), a major component of the outer layer of Gram-negative bacteria, peptidoglycan (PGN), the main component of the cell wall of Gram-positive bacteria, flagellin, and microbial nucleic acids. In order to recognize PAMPS, cells express a variety of pattern-recognition receptors or PRRs capable of binding specifically to conserved portions of these molecules. PRRs comprise an array of proteins present at the plasma membranes, endosomes, and cell cytosol. A single microorganism can be detected by multiple sensors. The existence of multiple PRRs

capable of recognizing a single microorganism ensures the induction of immune responses when one signaling pathway is blocked by the pathogen. In addition, the activation of multiple PRRs in response to a pathogen often results in a combinatorial activation that specifically stimulates the host response to a particular class of microbes [72,73]. There is evidence that certain PRRs are involved not only in recognizing microorganisms but also in recognizing endogenous non-microbial 'danger' signals such as uric acid, ATP and HSP70 [74]. The activation of PRRs by microbial or endogenous danger stimuli results in the activation of multiple signaling pathways including nuclear factor- κ B (NF- κ B), mitogen-activated protein kinases (MAPKs), and IRF family proteins to induce production of cytokines and type I interferon (IFN) [75].

There are two major groups of PRRs in the cells: membrane-bound PRRs and cytoplasmic PRRs. The first group usually refers to Toll-like receptors (TLRs), the first class of cellular PRRs identified. TLRs monitor the extracellular environment and endosomal compartments, and recognize a variety of microbial components including bacterial lipoprotein, peptidoglycan, CpG DNA, and double- and single-stranded RNA [75,76]. In contrast, the nucleotide oligomerization domain (Nod)-like receptors (NLRs) and the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are intracellular cytosolic sensors. NLRs were first identified in plants where they play a critical function in disease resistance (R genes) against microbial and parasitic pathogens [77,78]. RLRs are helicases that sense viruses [79]. Homologues of the NLRs are present in vertebrates (at least 22 was been found) as well as in primitive organisms, such as the sea urchin,

indicating that NLR responses are conserved through evolution [80].

NLRs are multi-domain proteins and all have a centrally located NOD domain that is critical for activation. The NOD domain (also referred as the NACHT cassette) is closely related to the oligomerization module found in the AAA+ family of adenosine triphosphatases (ATPases). The NOD domain mediates NLR protein oligomerization and may also contain ATPase activity [81]. The NLR N-terminal domain is composed of a variable effector region consisting of caspase recruitment domain (CARD), pyrin domain (PYD), acidic domain, or baculovirus inhibitor repeats (BIRs). Based on the class of N-terminal effector domain, the NLR family has been divided into five subfamilies including the caspase-recruiting domain (CARD)-containing NODs, the pyrin (PYR) domain-containing NALPs and the baculovirus-inhibitor-of-apoptosis-repeats (BIRs)-containing NAIPs.

VIII. Rip2 and Nod signaling

First discovered as mammalian members of the Ced4/Apaf-1 family of apoptosis regulators, Nod1 and Nod2 are critical pathogen-recognition receptors that sense intracellular peptidoglycans [67,82]. Nod1 and Nod2 are multi-domain proteins consisting of one or two CARD domains in the N-terminal region, a centrally located NOD domain followed by a number of C-terminal LRRs. Nod1 and Nod2 are found expressed in almost all higher vertebrates but not in insects or worms. Nod2 expression is restricted to macrophages, dendritic cells, Paneth cells, keratinocytes, and epithelial cells

of the intestine, lung, and oral cavity [67,83,84,85]. Nod1 is more widely expressed in many cell types and organs. Both Nod1 and Nod2 are expressed in the cytosol, but more recently Nod1 and Nod2 proteins have been found associated with the plasma membrane [86,87]. In the case of Nod2, point mutants that prevent membrane association are impaired in their ability to activate downstream signaling [87]. However, the same mutations are also known to affect microbial recognition and secondarily Nod2 activation; therefore, the role of membrane localization in Nod2 signaling remains unclear.

In cells, Nod1 and Nod2 are thought to be kept in an inactive state by intra-molecular interactions. Truncation or mutation of the C-terminal LRR region of a variety of NLRs results in a constitutively active form of the proteins [88]. When co-expressed in cells, the Nod2 C-terminal fragment consisting of only the LRRs interacts with an N-terminal fragment consisting of the CARD and NOD domains. The LRR/CARD interaction can be disrupted by the presence of the Nod2 ligand MDP [89]. Collectively, these results suggest a model whereby ligand recognition results in a conformational change in the Nod1/2 protein, relieving the autoinhibitory intramolecular interactions and allowing NOD domain-dependent nucleotide binding and oligomerization. However, evidence for a direct interaction between NLRs including Nod1 and Nod2 and their putative ligands is still lacking; so, it is possible that the activation of Nod1 and Nod2 by microbial stimulation is indirect. Clearly, more structural, biochemical, and functional studies are needed to test these models.

While the functions of most NLR's remain undefined, the Nod1 and Nod2

proteins have been shown to respond to bacterial cell wall fragments. The Nod1 protein recognizes a fragment of peptidoglycan (PGN) containing the dipeptide γ -d-glutamyl-*meso*-diaminopimelic acid (iE-DAP) produced by Gram-negative and some Gram-positive bacteria [90,91]. Nod2 recognizes muramyl dipeptide (MDP) present on most types of PGN [90,91,92,93]. While the recognition of these common forms of peptidoglycan has been extensively studied, bacteria modify their cell walls in a myriad of ways and the effects of these modifications on Nod1/2 recognition are only beginning to be appreciated [94,95,96]. For example, *Listeria monocytogenes* removes a common *N*-acetyl moiety from the glucosamine of its peptidoglycan, which renders the cell wall resistant to host lysozyme and thereby inhibits bacterial recognition by Nod1 [97]. In contrast, mycobacteria, replace the *N*-acetyl group of the muramic acid of MDP with an *N*-glycolyl moiety [98,99], and this modification significantly increases the potency of this compound as a Nod2 agonist.

Nod1- and Nod2- deficient cells are unable to activate NF- κ B and produce cytokines and chemokines in response to ligand stimulation [100,101]. Rip2, known to interact with Nod1 and Nod2, has been implicated in the pathway. The original work on Rip2 deficient mice suggests that Rip2 may mediate multiple adaptive and innate immune pathways. Rip2-deficient cells displayed an impaired cytokine response to the TLR ligands LPS, lipoteichoic acid and poly(I:C), but not bacterial DNA, suggesting a role for Rip2 downstream of TLR-4, -2, and -3, respectively [100,101]. Rip2-deficient cells also appeared hyporesponsive to signaling through interleukin (IL)-1, IL-18 and Nod1/2

ligands [64,102]. While studies implicating Rip2 in multiple TLR pathways appear to reflect the presence of contaminating peptidoglycan [103], the role of Rip2 in Nod signaling was the subject of this thesis research.

Following microbial detection, Rip2 is directly recruited to Nod1 or Nod2 proteins through CARD–CARD interactions [67,82]. Our work presented in chapter II demonstrated that Rip2 is modified with K63-linked ubiquitin chains in MDP treated cells [104]. Although the E3 of Rip2 is still unclear, it is clear that the polyubiquitination of Rip2 is critical for Nod1/2 response. A Rip2 lysine mutant (K209R) can not restore NF- κ B activation in Nod stimulated cells [104]. Our work demonstrated that K63-linked ubiquitin modification of Rip2 recruits the ubiquitin activated transforming growth factor β -activated kinase (Tak1), which is essential for IKK activation [105,106]. Tak1, a serine–threonine kinase that forms a complex with the ubiquitin-binding proteins Tak1-binding protein 1 (Tab1), Tab2, and/or Tab3, activates IKK by phosphorylating IKK β . Tak1 binds to polyubiquitinated Rip2 via its N-terminal ubiquitin-binding CUE domain [107]. Rip2 also binds NEMO and promotes its ubiquitination, and thereby brings the IKK complex and Tak1 into close proximity [108]. IKK activation results in phosphorylation, ubiquitination and degradation of NF- κ B inhibitor I κ B α , exposes the NLS of NF- κ B, and allows its nuclear translocation (Fig. 4).

Both Rip2 and Tak1 also contribute to Nod1 - and Nod2-mediated p38 MAPK and JNK1/2 activation, although the intermediate steps in this pathway are less well characterized [105,106]. Stimulation of Nod1 or Nod2 results in the secretion of

proinflammatory cytokines and chemokines including IL-6, IL-8, KC, macrophage inflammatory protein-2 (MIP-2), CCL2, CCL5, and presumably secreted (RANTES) [109,110]. Nod1 activation induces neutrophil recruitment *in vivo* [110], and both Nod1 and Nod2 signaling lead to the production of anti-microbial peptides [111].

The deubiquitinase A20, known to remove K63-ubiquitin chains from Rip1 protein, also regulates Rip2 polyubiquitination and downregulates Nod- induced NF- κ B signaling [112]. A20 has a conserved role in the regulation of Rip proteins in the TNF and NLR pathways [36], indicating that A20 acts broadly to suppress proinflammatory pathways. Also, caspase-12 and MEKK4 have been shown to downregulate Nod2 signaling by disrupting the Nod2/Rip2 CARD–CARD interaction [113,114]. Additionally, an alternatively transcribed CARD domain-only isoform of Nod2 appears to function as a dominant negative regulator of Nod2 signaling and works by similar mechanisms to inhibit the Nod2/Rip2 interaction [115]. Recently, a negative regulatory role for the cell polarity protein, Erbin, has been proposed based on its ability to associate with Nod2 and inhibit NF- κ B activation and subsequent inflammatory cytokine secretion [116]. Whether Rip2 and Erbin associate is unclear.

X. Nod mediated pathogen recognition

The Rip2 and Nod proteins are involved in the sensing of numerous pathogenic bacteria. Although TLRs might be the primary sensor of a bacterial infection, several studies suggest that Nod1 and Nod2 play non-redundant roles with TLRs in pathogen

recognition and production of proinflammatory and anti-microbial molecules [70]. Consistently, the functions of Nod1 and Nod2 appear to be important when TLR signaling is absent or reduced.

Nod1 detects a variety of Gram-negative pathogens including *Shigella flexneri*, *Chlamydia*, *Pseudomonas aeruginosa*, enteroinvasive *Escherichia coli*, *Campylobacter jejuni*, and *Helicobacter pylori* [66,109,111,117,118,119]. However, most of the evidence that Nod1 mediates pathogen sensing comes from *in vitro* experiments. For example, while Nod1 senses *Chlamydia in vitro*, studies using vaginally infected mice fail to reveal a requirement for Nod1 *in vivo* [109]. Nod1 has been implicated in the host defense against a human enteric pathogen, *H. pylori*, which is involved in the development of gastritis, duodenal ulcers, and gastric cancer. Following intragastric infection with *H. pylori*, Nod1 deficient mice exhibit decreased expression of the anti-microbial peptide β -defensin 4 and an increased bacterial load. Although *H. pylori* is a non-invasive bacterium, it activates the Nod1 pathway by injecting PGN fragments into epithelial cells via a bacterial type IV secretion system. Consistently, mutant *H. pylori* lacking the *cag* pathogenicity island that encodes the secretion apparatus are not sensed by Nod1 [119]. The requirement of the bacterial secretion system for cytosolic Nod activation revealed a possible mechanism whereby PGN fragments enter the host cells cytosol to induce Nod1 and Nod2 activation. This feature will be discussed in greater detail in Chapter III.

Similar to Nod1, *in vitro* studies have identified a variety of pathogens that activate Nod2, including *S. pneumoniae*, *S. aureus*, *Salmonella typhimurium*, and *S.*

flexneri and *Mycobacterium tuberculosis* [120,121,122,123]. Oral infection of Nod2-deficient mice with *Yersinia pseudotuberculosis*, revealed in a slight increase in susceptibility [124]. Differences were observed in the cytokine responses of infected wildtype and Nod2-deficient macrophages with *Yersinia pseudotuberculosis* *in vitro* [125]. Meanwhile, when Nod2-deficient mice were orally infected with Gram-positive *L. monocytogenes*, the infected mice had increased bacterial loads, and exhibited diminished expression of certain anti-microbial α -defensin peptides relative to wildtype controls [100].

Nod1 and Nod2 are primarily located in the cytosol. How do the PGN fragments gain access to the cytosol to induce Nod1 and Nod2 activation, especially when the bacterium is located in membrane-bound vesicles such as a phagosome? One way is through the bacterial secretion system, discussed in the case of *H. pylori* infection [119]. Another potential mechanism could involve the pore-forming toxins produced by bacteria. For example, *Staphylococcus aureus*, *Bacillus anthracis*, and *Streptococcus* all produce toxins that mediate cytosolic sensing of Nod1 agonists [126]. Nod1 or Nod2 ligands produced by bacteria may also enter the cell via endocytosis or via epithelial transporters. For example, the Nod2 ligand MDP is internalized by macrophages and localized to acidified vesicles, suggesting that MDP uptake can occur via endocytosis [127], although the precise mechanism involved remains unclear. The epithelial transporters PepT1 and PepT2 have been shown to selectively deliver Nod1/2 ligands iE-DAP and MDP to the cytosol [128]. However, the expression of PepT1 is largely

confined to intestinal epithelial cells, while Nod2 is also expressed in monocytes and macrophages [128]. So, it is unclear whether this transporter regulates MDP uptake in cells of the innate immune system.

XI. The Nod pathway and *Mycobacterium tuberculosis*

Mycobacterium tuberculosis (Mtb), the causative agent of human tuberculosis, is thought to persist within as many as one billion individuals worldwide [129]. Mtb is an extremely successful intracellular pathogen that infects and causes disease by manipulating the host immune response [130,131]. Following uptake by phagocytic cells such as macrophages and dendritic cells (DC), Mtb resides in a modified phagosomal compartment with the characteristics of an early endosome. Mtb inhibits phagosomal maturation and lysosomal fusion, and survives within modified phagosomal compartments. The ability of Mtb to survive and replicate inside a modified phagosomal compartment of host macrophages is central to the pathogenesis of this disease [132]. Thus, like many other pathogens that cause chronic infections, the long-term survival of Mtb depends on a delicate balance between bacterial virulence and host immunity.

The immunity to Mtb depends on both the host innate and adaptive response. During infection, Mtb triggers an innate immune response mediated by pattern recognition molecules (PRMs) [133]. Genetic studies in mice have shown that multiple TLRs have important roles in protective immunity to mycobacterial infection, including TLR2, TLR4, TLR6 and TLR9 [133,134,135,136,137]. Moreover, in the absence of

MyD88, the common adaptor for two IL-1R and most TLRs pathways, mice were profoundly susceptible to Mtb infection, indicating that instead of one TLR, multiple TLR pathways are required for the innate immune response to Mtb [138]. However, a large fraction of the transcriptional response to Mtb, including many immunologically important proteins, such as the chemokines RANTES and IP-10, and the inducible nitric oxide synthase enzyme NOS2 that are critical for mycobacterial immunity, are induced independently of TLR2/4 and the adapter proteins MyD88, MAL and TRIF. Instead, these responses rely on autocrine or paracrine signaling via type I interferons (IFN α/β), which are induced largely through undefined pathways [139]. Also, the adaptive immune response is not significantly impaired in Mtb infected MyD88 deficient mice [140,141]. These findings suggest that other PRMs, signaling through a MyD88-independent pathway, are involved in shaping the adaptive immune response to mycobacterial infection.

The cytosolic Nod pathway has been implicated in the MyD88-independent Mtb response. Several studies have shown that Nod2 pathway activation results in an optimal Mtb response in both human and murine derived macrophages *in vitro* [121,142,143]. Also, human *NOD2* variants were found to be associated with susceptibility to tuberculosis in an African American population [144]. Moreover, the Nod2 ligand MDP has been shown to synergize with cytosolic DNA to enhance type I IFN production during both *L. monocytogenes* and Mtb infection [143]. Collectively, these studies indicate that the Nod pathway may mediate immune responses upon Mtb

infection. Also, in light of these findings, the importance of Nod-mediated immunity in Mtb pathogenesis requires further study and is a focus of the work described in chapter III.

XII. Type I Interferon and Interferon regulatory factors

Interferons, so called because of their activity to interfere with (inhibit) virus replication in a cell [145,146], are divided into at least three distinct groups (types): types I, II, and III [147]. Type I IFNs include IFN- α , - β [148], and others, such as IFN- ω , - ϵ , and - κ [147]. In human and mouse cells, the IFN- α genes are composed of multiple subfamily genes (13 in humans and 14 in mice), whereas only a single IFN- β gene has been found [149].

Type I IFN production is primarily controlled at the gene transcription level. The promoter region of the IFN- β gene contains at least four regulatory cis elements, called the positive regulatory domains (PRDs) I, II, III, and IV [150], whereas the promoter regions of IFN- α genes contain PRD I- and PRD III-like elements (PRD-LEs) [151]. PRD I and PRD III contain the binding sites for IRF family members, whereas PRD II and PRD IV elements are the sites for NF- κ B and the activating transcription factor 2 (ATF2) / c-JUN heterodimer (AP-1) binding, respectively. Upon viral infection, PRDs direct the assembly of IRFs, NF- κ B, AP-1, and the high-mobility group protein HMG-I (Y) to form a complex known as the “enhanceosome” [150] to stimulate IFN- β

gene expression. However, the IFN- β promoter is activated only weakly, if not at all, by tumor necrosis factor- α (TNF- α), which strongly activates NF- κ B and AP-1, but not the IRFs [152], suggesting that activation of IRFs is the major mediator of robust type I interferon induction.

The IRF family of transcription factors is comprised of nine members: IRF1, IRF2, IRF3, IRF4 (also known as PIP or ICSAT), IRF5, IRF6, IRF7, IRF8 (also known as ICSBP), and IRF9 (also known as ISGF3 γ) [153,154] (Figure 5). These family members are characterized by a well-conserved amino (N)-terminal DNA binding domain (DBD). The DBD is a helix-turn-helix DNA binding domain. The DBDs of all IRFs recognize very similar DNA sequences. Except of IRF1 and IRF2, the carboxy (C)-terminal regions of all the IRFs have an IRF association domain (IAD) that is responsible for homo- and heteromeric dimer formation/interaction with other family members or transcription factors such as PU.1 and signal transducer and activator of transcription (STAT) [153,154]. Mouse genetic studies of most of the IRF genes have revealed that IRFs have distinct roles in development and immune responses [154,155,156].

Among the IRFs, four IRFs—IRF1, IRF3, IRF5, and IRF7—have been implicated as positive regulators of type I IFN gene transcription. IRF3 and IRF7, which are highly homologous, have been well established as the key regulators of type I IFN gene expression induced upon viral infection. IRF3 is constitutively expressed and resides in the cytosol in the inactive form. Upon viral infection, IRF3 is phosphorylated

in the C-terminal region by TBK1/IKK ϵ . Phosphorylated IRF3 interacts with the hydrophobic pocket within the IAD of another IRF3 or potentially IRF7, resulting in the formation of either an IRF3 homodimer or an IRF3/IRF7 heterodimer [157]. The IRF3 homodimer or an IRF3/IRF7 heterodimer then translocates to the nucleus, forms a complex with the coactivators CBP and/or p300, and binds to the target DNA promoter sequence in type I IFN genes as well as certain cytokine and chemokine genes [158,159]. Unlike IRF3, constitutive expression of IRF7 is limited to peripheral blood lymphocytes and dendritic cells and is strongly induced upon type I IFN mediated signaling [160,161]. Similar to IRF3, IRF7 resides in the cytosol and, on viral infection, undergoes serine phosphorylation in its C-terminal region, allowing its dimerization and nuclear translocation. IRF7 forms a homodimer or a heterodimer with IRF3, and each of these dimers differentially acts on the type I IFN gene family members. IRF3 is more potent in activating the IFN- β gene than the IFN- α genes, whereas IRF7 efficiently activates both IFN- α and IFN- β genes [160,161]. The cellular events controlling the activity of IRF3 and IRF7 have been studied in recent years. They mediate essential antiviral pathways that are triggered by the detection by at least two PRRS derived from viruses: transmembrane TLRs and cytosolic retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5).

IRF1 is the first family member discovered to activate type I IFN gene promoters [162]. The overexpression of IRF1 results in the induction of endogenous type

I IFN genes. However, the induction of type I IFN was unaffected in viral-infected *irf1*^{-/-} fibroblasts, although IRF1 participates in type I IFN gene induction in some facets of TLR signaling [163]. Mouse genetic studies also revealed that IRF5 is dispensable for type I IFN gene induction by viruses or TLR agonists and that IRF5 regulates the expression of inflammatory cytokine genes, such as interleukin (IL)-12 and TNF- α [164]. Hence, the precise role of IRF1 and IRF5 in type I IFN induction still remains unclear. In this thesis work, we reveal a specific role for IRF5 in the Nod2 mediated anti-bacterial type I IFN response.

XIII. Overview of work to be presented in this thesis

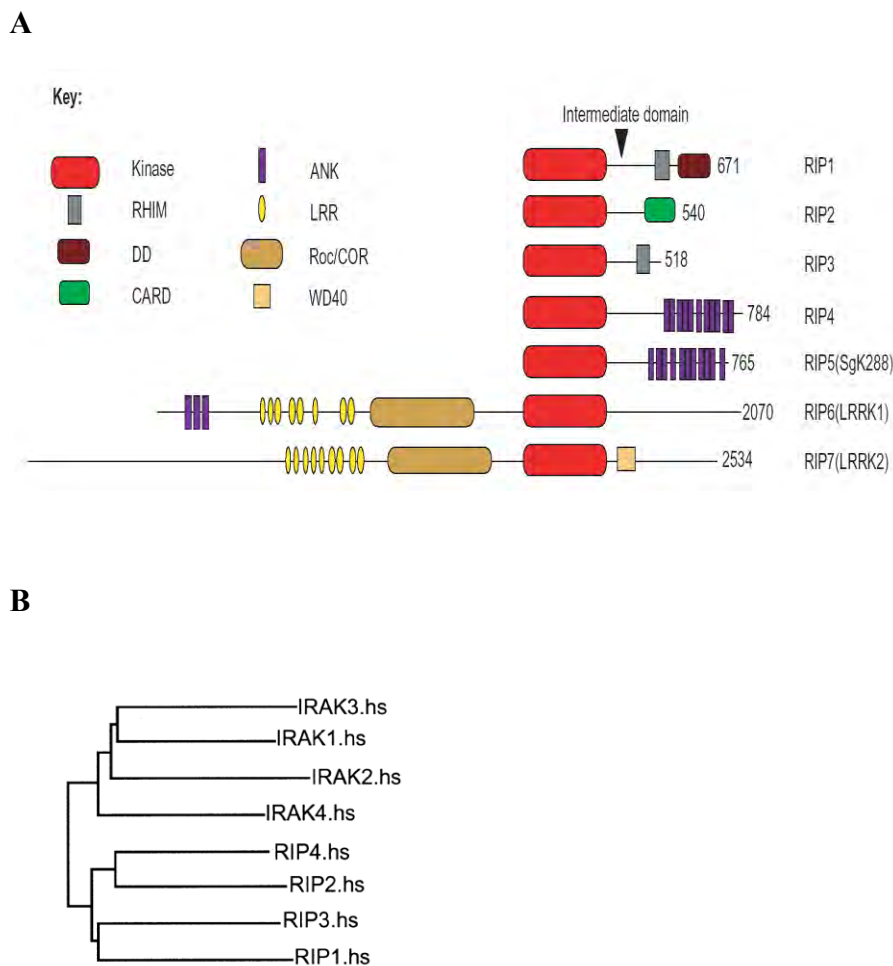
Despite recent advances in our understanding of cellular stress responses, a number of questions remain. This thesis research reveals detailed mechanisms whereby Rip2 mediates Nod pathway activation. Furthermore, the role of the Nod-Rip2 pathway in the innate immune response to *Mycobacterium tuberculosis* infection will be extensively examined.

In chapter 2, we demonstrate that Rip2 and the IKK regulatory subunits NEMO/IKK- γ become stably polyubiquitinated upon treatment of cells with the NOD2 ligand, muramyl dipeptide. We demonstrate a requirement for the E2 conjugating enzyme Ubc13, the E3 ubiquitin ligase Traf6 and the ubiquitin-activated kinase Tak1 in Nod2-mediated NF- κ B activation. We also show that bacterial infection stimulates Rip2 polyubiquitination. Collectively, this study revealed that the NOD2 pathway is

ubiquitin regulated and that Rip2 employs a ubiquitin-dependent mechanism to achieve NF- κ B activation.

In chapter 3, we demonstrate that infection with the bacterial pathogen *M. tuberculosis* stimulates the cytosolic Nod2 pathway. We show that upon Mtb infection, Nod2 recognition triggers the expression of type I interferons in a Tbk1- and Irf5-dependent manner. This response is only partially impaired by the loss of Irf3 and therefore, differs fundamentally from those stimulated by bacterial DNA, which depends entirely on this transcription factor. This difference appears to result from the unusual peptidoglycan produced by mycobacteria, which we show is a uniquely potent agonist of the Nod2/Rip2/Irf5 pathway. Thus, the Nod2 system is specialized to recognize bacteria that actively perturb host membranes and is remarkably sensitive to Mycobacteria, perhaps reflecting the strong evolutionary pressure exerted by these pathogens on the mammalian immune system.

Figure 1. RIP family of proteins. A. Domain organization of RIP family members. All members share a homologous N-terminal kinase domain. RHIM: RIP homotypic interaction motif; DD: death domain; CARD: caspase recruitment domain; ANK: ankyrin repeats; LRR: leucine-rich repeat; Roc/COR: Ras of complex proteins/C-terminal of Roc domain; WD40: WD40 repeats. **B. Phylogenetic tree of the kinase domain of human RIPs and IRAKs.** RIP5-7 are not represented here.



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Trends Biochem Sci. 30, 3, 151-159 (2005)

Figure 2. Schematic representation of three NF- κ B signaling pathways. The canonical or classic NF- κ B pathway (depicted in the middle) is typically activated in response to pathogen-associated molecular patterns (PAMPs) or proinflammatory cytokines. The noncanonical or alternative pathway (depicted on the right) is predominantly active in B cells and is induced by a particular subset of TNFR family ligands, such as B-cell-activating factor of the tumour necrosis factor (TNF) family (BAFF), CD40 or lymphotoxin (LT) β . The third NF- κ B activation pathway, also known as the atypical pathway (depicted on the left) is induced by stimuli such as DNA damage or oxidative stress.

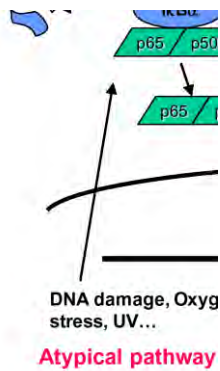


Figure 3. Model of IKK activation by TNF α . Trimeric TNF α stimulation leads to the trimerization of TNF-R1 (TNF receptor). Multiple signaling proteins, including TRADD, TRAF2, TRAF5, and RIP1 recruit to the TNF-R1 after stimulation. The TRAF proteins TRAF2/TRAF5, together with Ubc13/Uev1A polyubiquitinate RIP1 at K377. Polyubiquitinated RIP1 then recruits the TAK1 kinase complex through the interaction between the polyubiquitin chains and the NZF domain of TAB2. The polyubiquitin chains on RIP1 can also bind to NEMO, resulting in recruitment of the IKK complex. IKK β in the complex is then phosphorylated and activated by TAK1, leading to the phosphorylation, ubiquitination, and degradation of I κ B. NF- κ B subsequently translocates into the nucleus to activate gene expression.

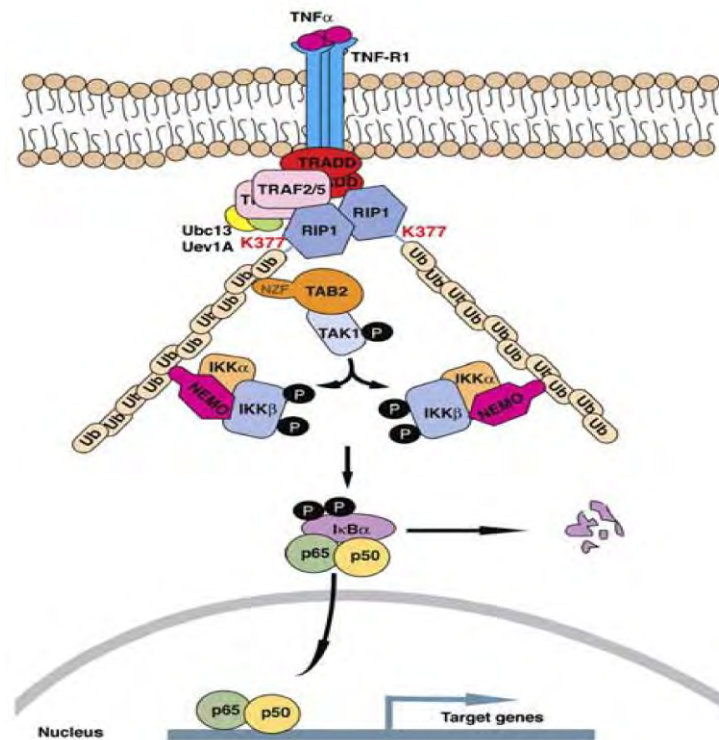


Figure 4. Model of IKK activation by MDP. Stimulation of cells with MDP leads to the conformational change of NOD2 and subsequent recruitment of signaling protein RIP2 through its CARD domain. RIP2 is polyubiquitin modified by cIAP1/2 in the complex. Polyubiquitinated RIP2 then recruits the TAK1 kinase complex. RIP2 can also bind to NEMO, resulting in recruitment of the IKK complex. IKK β in the complex is then phosphorylated and activated by TAK1, leading to the phosphorylation, ubiquitination, and degradation of I κ B. NF- κ B subsequently translocates into the nucleus to activate gene expression.

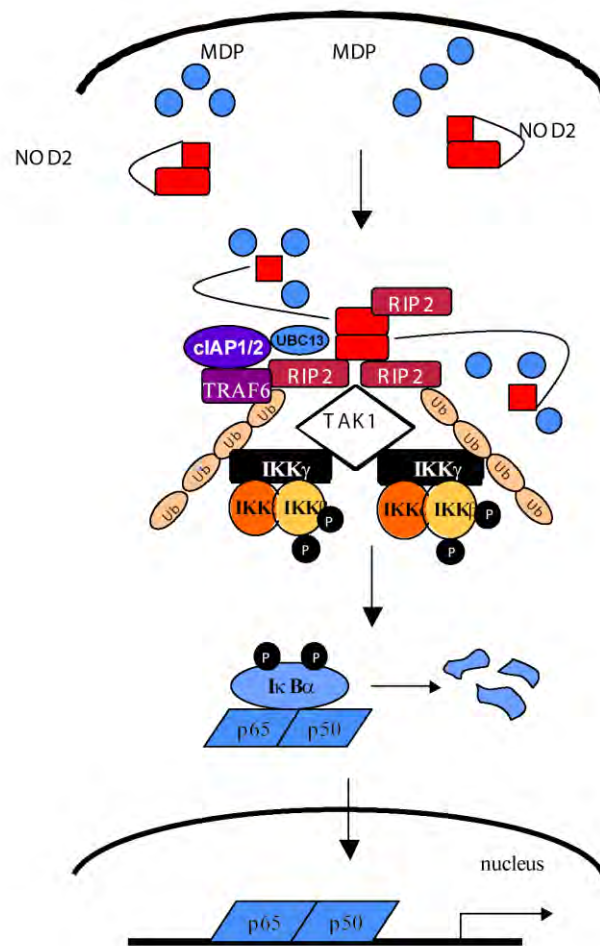
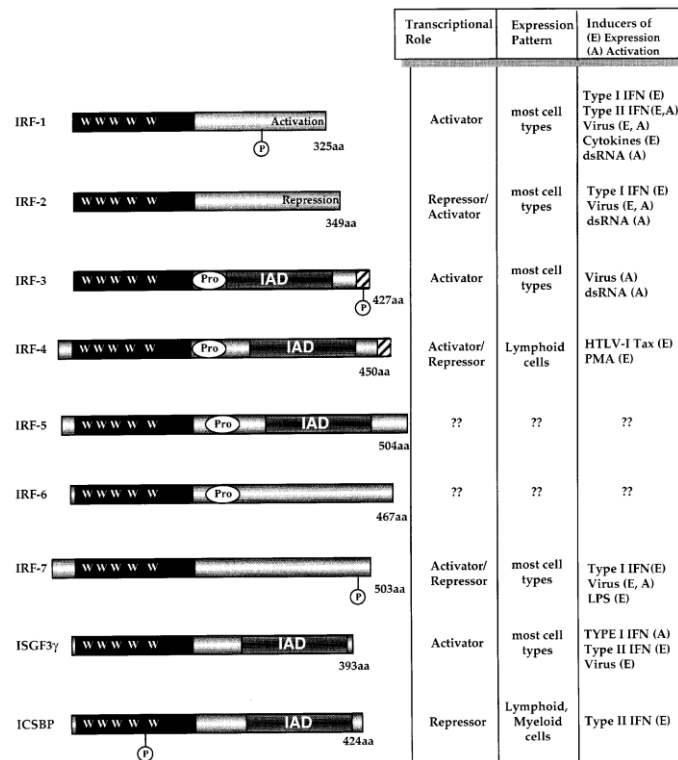


Figure 5. Overview of the IRF family members and their expression patterns and transcriptional roles. The conserved tryptophan repeats in the DNA binding domain (DBD) (black bar) are represented by W. Certain IRF family members possess a proline-rich domain shown by Pro. IAD presents an IRF association domain, hatched bars present a C-terminal autoinhibition domain, and phosphorylation sites designated by P.



Chapter II

NOD2 pathway activation by MDP or *Mycobacterium tuberculosis* infection involves the stable polyubiquitination of Rip2

This chapter is based on work from the following publications:

Yang Y, Yin C, Pandey A, Abbott D, Sasseti C, Kelliher MA. NOD2 pathway activation by MDP or *Mycobacterium tuberculosis* infection involves the stable polyubiquitination of Rip2. *J Biol Chem.* 2007 Oct 18; PMID: 17947236

Abbott DW, **Yang Y**, Hutti JE, Madhavarapu S, Kelliher MA, Cantley LC. Coordinated regulation of Toll-like receptor and NOD2 signaling by K63-linked polyubiquitin chains. *Mol Cell Biol.* 2007 Sep;27(17):6012-25. PMID: 17562858

Yang Y contributed the work of Fig. 7, 8, 9, 10B, 10C, 11, 12, 13; Yin C contributed the work of Fig. 6; Abbott DW contributed the work of Fig. 10A; Pandey A contributed the work of Fig. 11.

Introduction

Conserved structures on pathogens are recognized by specific host receptors such as Toll-like receptors (TLRs) or nucleotide binding oligomerization domain (NOD)-like receptors (NLRs). In contrast to TLRs which recognize pathogens at the cell surface or within the endosome, NLRs induce innate immune responses by recognizing bacterial products released into the cytosol. Several NLRs, including Nod1 and Nod2 proteins, induce cytokine production by activating the transcription factor NF- κ B and by stimulating MAP kinase activation [165]. The Nod1 protein recognizes a form of peptidoglycan (PGN) containing the amino acid meso-diaminopimelic acid (IE-DAP) produced by Gram-negative and some Gram-positive bacteria [91]. The Nod2 pathway recognizes muramyl dipeptide (MDP) present on most types of PGN [93]. Transfection studies have shown that expression of Nod proteins stimulates NF- κ B activity and the ability to stimulate NF- κ B reporter activity is dependent on the co-expression of the adapter protein Rip2 [67]. Yet how Rip2 proteins achieve Nod-mediated NF- κ B or MAP kinase activation remains undefined.

We found Rip2 required for Nod2 pathway activation in macrophages, however we did not find Rip2 required for TLR 2, 3 or 4-mediated IKK activation. Similar data have recently been published by Gabriel Nunez and colleagues who find Rip2 required for cytokine production induced through NLRs, but not TLRs [103]. We show that treatment of macrophages with the Nod2 ligand MDP stimulates the ubiquitin modification of endogenous Rip2 and IKK- γ . The ubiquitin modification of Rip2 is

stable and involves the conjugation of K63-linked polyubiquitin chains. MDP-induced Rip2 polyubiquitination and NF- κ B activation requires the E2 conjugating enzyme Ubc13, the E3 ubiquitin ligase Traf6 and the ubiquitin activated kinase Tak1.

The Nod2 pathway has been implicated in the innate immune response to *Mycobacterium tuberculosis* (*Mtb*) infection, as Nod2-deficient macrophages are impaired in their cytokine response to infection with *Mtb* [121]. Moreover, a quantitatively small fraction of the transcriptional changes induced by *Mtb* infection of macrophages can be attributed to TLR2/4- or MyD88-dependent pathways [139]. Collectively, these studies suggest nonredundant roles for NOD2 and TLR in the innate immune recognition of *Mtb*. We find that infection with live, but not heat killed, Wt strains of *Mtb* stimulates Rip2 polyubiquitination. Therefore, we hypothesize that Nod proteins recognize *Mtb* components that are translocated into the cytosol and consistent with this model, Rip2 polyubiquitination induced by live *Mtb* infection appears MyD88 independent, implicating NLRs in the cytosolic recognition of *Mtb*.

Results

Rip2-deficient macrophages are impaired in their response to the NOD2 ligand MDP, but respond normally to the TLR4 ligand, LPS.

The CARD domain kinase Rip2 has been implicated in signaling from multiple TLRs including TLR 2, 3 and 4, the T cell receptor and the cytosolic NOD1/2 pathways. To understand how Rip2 mediates such diverse signals, we isolated macrophages from Rip2-deficient mice and stimulated the cells with the NOD2 ligand muramyl dipeptide (MDP) or with purified LPS to stimulate the TLR4 pathway. We found that MDP and LPS induced NF- κ B, p38 MAP kinase and JNK1/2 activation when wild type macrophages were stimulated (Figure 6A and 6B). Rip2-deficient macrophages also respond upon LPS stimulation, but appear impaired in their ability to mediate NF- κ B activation when treated with MDP (Figure 6A). Similarly, the MDP-induced phospho-p38 and phospho-JNK1/2 activity was consistently impaired but not abrogated, suggesting that Rip2 contributes to MAP kinase activation but is not absolutely required (Figure 6A and 6B). This data suggests that Nod-mediated MAP kinase activation may involve other CARD domain containing adapter proteins. One possibility may be CARD9, as Card9-deficient mice exhibit defects in p38 MAP kinase and JNK activation in Nod stimulated cells.

NOD2 pathway activation stimulates the stable K63-linked polyubiquitination of Rip2.

We hypothesized that Nod mediated IKK activation may involve the ubiquitin modification of Rip2 or IKK- γ /NEMO and tested this possibility by stimulating the mouse macrophage cell line RAW 264.7 with the Nod2 ligand MDP and assayed for the presence of polyubiquitinated Rip2 or NEMO/IKK- γ . We observed a ligand-dependent induction of Rip2 and NEMO/IKK- γ polyubiquitination and interestingly, Rip2 polyubiquitination appears to precede the ubiquitin modification of NEMO/IKK- γ . Nod2 pathway activation by MDP appears to induce the stable modification of Rip2 and IKK- γ , suggesting that Rip2 and IKK- γ may be conjugated by K63-linked polyubiquitin chains (Figure 7A). To test this possibility, HEK293 cells were transfected with epitope-tagged versions of Rip2 and NOD2 in the presence of wild type or mutant forms of ubiquitin where only the lysine(s) at positions 63 or 48 are available for conjugation. In this setting, Rip2 appeared preferentially modified by K63-linked polyubiquitin chains and this activity appeared dependent on the presence of NOD2 (Figure 7B). The K63-specific polyubiquitin modification of Rip2 in MDP-NOD2 pathway was confirmed by using the linkage-specific polyubiquitin antibodies [39] (Figure 8). These data suggest that physiologic activation of the NOD2 pathway might stimulate the stable K63-linked polyubiquitination of Rip2.

MDP-induced Rip2 polyubiquitination is dependent on the expression of the E2 conjugating enzyme Ubc13 and the E3 ubiquitin ligase Traf6.

Our studies suggest that NOD2-induced NF- κ B activation may be mediated by

the ubiquitin conjugation of Rip2 and IKK- γ and the transfection studies suggest that K63-linked polyubiquitin chains may stably modify both proteins. Therefore, we were interested in testing whether MDP-induced NF- κ B activation and Rip2/IKK- γ polyubiquitination require expression of the E2 conjugating enzyme Ubc13, known to conjugate K63-linked polyubiquitin chains. To address a requirement for Ubc13, we infected the MDP responsive mouse macrophage cell line RAW 264.7 with lentiviruses expressing shRNAs specific for the Ubc13 enzyme. These stable pools of RAW 264.7 macrophages were impaired in their ability to phosphorylate I κ B α in response to MDP (data not shown). Rip2 polyubiquitination was detected in RAW 264.7 macrophages expressing a control shRNA, however, treatment with the Nod2 ligand MDP failed to stimulate Rip2 polyubiquitination when the Ubc13-deficient cells were stimulated (Figure 9A). The level of Rip2 polyubiquitination and MDP-induced NF- κ B activation correlated with the reduction of Ubc13 expression achieved in the RAW 264.7 pools.

In transfected cells, Rip2 has been shown to interact with Traf 2, 5 and 6 proteins [60], suggesting that one of these Traf proteins may modify Rip2 upon Nod2 pathway stimulation. To identify the E3 ubiquitin ligase responsible for the modification of Rip2, we transfected HEK293 cells with epitope-tagged versions of NOD2 and Rip2 and tested whether MDP- or NOD2-dependent Rip2 polyubiquitination is observed in cells where Traf2 or Traf6 expression was reduced. In HEK293 cells transfected with NOD2 and stimulated with MDP, Rip2 polyubiquitination was detected, as expected (Figure 9B). Similarly, in cells where Traf2 expression is reduced via transfection of siRNAs specific

for Traf2, MDP stimulated Rip2 polyubiquitination and this modification appeared NOD2-dependent. In contrast, Rip2 polyubiquitination was reduced in MDP-stimulated HEK293 cells in which Traf6 expression was reduced (Figure 9B), thereby implicating Traf6 as a potential Rip2 E3 ubiquitin ligase in the Nod2 pathway. We also examined whether MDP or NOD2 pathway activation stimulates an association between Rip2 and Traf6. Using co-immunoprecipitation, MDP treatment stimulated interaction between endogenous Rip2 and Traf6 between 30 and 60 min following MDP treatment. Consistently, polyubiquitinated Rip2 proteins are detected at similar time periods following MDP treatment (Figure 7A).

To further test a requirement for Traf6, we attempted to knock down the expression of Traf6 in the RAW 264.7 mouse macrophage cell line and then stimulated the cells with IL-1 or MDP. As expected [166], no IL-1-induced NF- κ B activation is detected in the Traf6 knocked down cells, however, the control shRNA treated cells activate NF- κ B upon IL-1 treatment. MDP-induced NF- κ B activation is clearly impaired in cells where Traf6 expression is reduced, suggesting that Traf6 is required for optimal NF- κ B responses to MDP (Figure 9D). These data are consistent with a model whereby Traf6 modifies Rip2 upon activation of the Nod2 pathway, raising the possibility that this enzyme may be targeted in human inflammatory disease.

Rip2 and the ubiquitin activated kinase Tak1 interact upon MDP stimulation and Tak1 expression is required for MDP-induced IKK activation.

Our data suggest that Rip2 polyubiquitination may contribute to NOD2-mediated NF- κ B activation by recruiting the ubiquitin activated kinase Tak1. Therefore, we tested whether Rip2 interacts with Tak1 and whether MDP stimulates an endogenous Rip2/Tak1 interaction. A Rip2/Tak1 interaction could be readily demonstrated in transfected cells (Figure 10A) and an endogenous Rip2/Tak1 interaction was detected following MDP treatment (Figure 10B), during a time period when a Rip2/Traf6 interaction was also observed (Figure 9C). Collectively, these data suggest that MDP stimulation or NOD2 pathway activation stimulates the formation of a Rip2/Ubc13/Traf6/Tak1 complex that results in the stable ubiquitin modification of Rip2.

These data predict that MDP-induced NF- κ B activation will be dependent on the expression of the ubiquitin-dependent kinase Tak1. We reduced Tak1 expression level in the RAW 264.7 macrophage cell line and tested its effects on MDP- and TNF-induced NF- κ B activation. Consistent with the transfection studies, NF- κ B activation was reduced in both the MDP- and TNF-stimulated Tak1 silenced cells, whereas it remained unaffected when the control cells were stimulated with either MDP or TNF- α (Figure 10C). These data led us to propose that Tak1 is critical for NOD2 signaling to NF- κ B. Thus, stimulation of the NOD2 pathway results in the recruitment and subsequent ubiquitin modification of Rip2 by an Ubc13/Uev1a and Traf6 complex. Polyubiquitinated Rip2 then signals the recruitment and activation of the Tak1 kinase.

Live *Mycobacterium tuberculosis* infection stimulates Rip2 polyubiquitination.

Although MDP stimulates NOD2-mediated NF- κ B activation and mutations in the *CARD15* gene are associated with human inflammatory disease, MDP has not, as yet, been shown to bind to the NOD2 protein [122]. Hence, we were interested in testing whether infection with live bacteria stimulates the ubiquitin modification of Rip2. The cytosolic NOD2 pathway has been implicated in the cytokine response to *Mycobacterium tuberculosis* (*Mtb*) infection *in vitro* and recent studies reveal important, nonredundant roles for NOD2 and TLRs in the *in vivo* recognition of *Mtb* [121,140]. To test whether *Mtb* infection stimulates Rip2 polyubiquitination, we infected bone marrow derived macrophages with a wt strain of *Mtb* (H37Rv) or with heat-killed *Mtb* and assayed for polyubiquitinated Rip2 proteins at 1 and 4 hours post infection. Infection with live *Mtb* stimulated Rip2 polyubiquitination, whereas heat killing destroyed the ability of *Mtb* to induce the modification of Rip2 (Figure 11A).

TLR2/4 have been implicated in the innate immune recognition of *Mtb* and published studies place Rip2 downstream of TLR2/4 [64,102,167]. Thus, it was important to test whether *Mtb*-induced Rip2 polyubiquitination was TLR-dependent. To test this idea, we isolated wild type and MyD88-deficient macrophages and left the cells uninfected or infected them with *Mtb*. Rip2 polyubiquitination was observed in *Mtb*-infected wild type and MyD88-deficient macrophages, suggesting that *Mtb* recognition is TLR independent (Figure 11B). We also examined Rip2 polyubiquitination in macrophages lacking both TLR2 and TLR4 to directly test the contribution of the TLR4, MyD88 independent pathway, but observed no differences in

the ability of *Mtb* infection to stimulate Rip2 polyubiquitination (Figure 11B). Although it remains unclear precisely how the cytosolic NOD2 pathway is activated by *Mtb* infection, several observations suggest that the Mycobacterial phagosome may be permeable to some compounds [168,169,170], suggesting a mechanism whereby *Mtb* products are released into the cytosol where they stimulate Rip2 recruitment and polyubiquitination.

Identification of critical lysine residues critical for Rip2 polyubiquitination.

It remains unclear from our studies, however, whether polyubiquitinated Rip2 is required for Nod2-mediated IKK activation. To map the ubiquitination site(s) on Rip2, we focused on the intermediate domain of Rip2, since like Rip1, expression of the intermediate domain of Rip2 activates an NF- κ B reporter construct, whereas expression of the kinase domain does not [171]. To identify the activated lysine residue(s) within the intermediate domain, we aligned the sequence of the intermediate domains of human, rat and mouse Rip2 and found that lysines 310, 311, 313, 326, 327 and 383 are conserved among the different species (Fig. 12A). We hypothesized that the critical ubiquitin site is likely to be conserved among species and therefore constructed 2 mutant forms of Rip2. One in which the lysine residues 310/311/313 (Rip2 3K) were mutated to arginines, designated Rip2 3K. The other mutant was designated Rip2 2K since lysine residues 326/327 were mutated to arginines.

To test if these lysine residues are critical for Rip2 polyubiquitination in the Nod2

pathway, wild type Rip2 and the Rip2 lysine mutants were overexpressed in HEK293 cells. Wild type and mutant Rip2 protein were immunoprecipitated and Rip2 polyubiquitination measured by blotting with ubiquitin antibody. Compared to wild type Rip2 protein, the Rip2 K326/327R form failed to be ubiquitin modified in this assay (Figure 12B). Polyubiquitination of Rip2 3k protein was detected, suggesting that K310/311 and K313 may not be essential. Co-expression of Rip2 wild type and Rip2 2K with Nod2 followed by MDP stimulation revealed that the Rip2 2k mutant unable to undergo stable polyubiquitination compare to wild type Rip2 (Figure 12C). This experiment suggested that Rip2 lysine 326/327 might be critical for Rip2 polyubiquitination in MDP pathway.

Rip2 polyubiquitination is required for Nod2-mediated IKK activation.

Recently, Inohara and colleagues identified lysine 209 in Rip2 kinase domain required for Nod1-mediated NF- κ B activation in MEFs [104], suggested that lysine at position 209 may contribute to Rip2 polyubiquitination. To determine whether polyubiquitinated Rip2 is required for Nod2-mediated IKK activation, we generated *rip2*^{-/-} macrophage cell lines stably express wild type Rip2, Rip2 K209R, Rip2 K326/327R (Rip2 2K) and Rip2 K310/311/313 (Rip2 3K). We cloned wild type Rip2 and the lysine mutants into the MSCV2.2.IRES-GFP retroviral vector and prepared high titer, replication incompetent retroviral stocks. The *rip2*^{-/-} transformed macrophage cell line was infected with the retroviral vector alone or with retroviruses expressing wild

type or mutant forms of Rip2. Transduction efficiency was reported as percentage of GFP positive cells for each reconstituted line (Figure 13A). Rip2 proteins in reconstituted macrophage cell line were measured by immuno blotting of Rip2 antibody to ensure that comparable levels of Rip2 expression were achieved (Figure 13A).

Using this approach, we found conserved lysines 326 and 327 (Rip2 2K) required for MDP-induced NF- κ B activation and cytokine production (Fig 13B and 13C). Expression of this Rip2 2K lysine mutant in our *rip2*^{-/-} macrophage cell line failed to restore responsiveness to MDP. In contrast, expression of wild type Rip2 or Rip2 3K restores Nod2-mediated NF- κ B activity and cytokine production. The Rip2 K209R mutant also failed to restore IKK activation upon MDP treatment (Figure 13D), suggested that Rip2 K209 and K326/327 might all contribute to Rip2 function in Nod pathway. Together with our previous results that Rip2 2K protein cannot be ubiquitin modified upon MDP stimulation (Figure 12C), these results indicate that Rip2 polyubiquitination is essential for MDP or Nod2 mediated IKK activation.

Discussion

Our work demonstrates that Rip2 primarily mediates NF- κ B activation but is not required for MAP kinase activation in MDP-stimulated peritoneal macrophages. MDP treatment of macrophages or infection with the human pathogen *Mycobacterium tuberculosis* stimulates Rip2 polyubiquitination, suggesting that Rip2 polyubiquitination may be required for NF- κ B activation by NLRs. Our work also implicates the E2 conjugating enzyme Ubc13, the E3 ubiquitin ligase Traf6 and the ubiquitin-activated kinase Tak1 in Nod2-mediated NF- κ B activation. In collaboration with Abbott and Cantley, we also recently showed that IKK- γ is ubiquitin modified in NOD2 transfected cells [172]. These studies are limited however, as they involve transfected proteins and the NOD2 pathway is activated not by ligand, but rather by NOD2 or Rip2 overexpression. In the work herein, macrophages are stimulated with ligand (MDP) or infected with the intracellular pathogen *Mtb*, and the endogenous Rip2 and IKK- γ proteins were examined for evidence of polyubiquitination. Collectively, both studies provide strong evidence that the NOD2 pathway is ubiquitin regulated and that IKK- γ and Rip2 polyubiquitination is mediated by an Ubc13/Traf6/Tak1 complex. Abbott et al also suggest that in contrast to wild type NOD2, expression of the Crohn's disease (CD) associated *NOD2* allele, L2007insC fails to stimulate IKK- γ polyubiquitination [172]. Yet NOD2 L2007insC is impaired in its ability to stably interact with Rip2 ([108] and Yang and Kelliher, unpublished data), suggesting that Rip2 and potentially polyubiquitinated Rip2 stimulates IKK- γ recruitment and its subsequent ubiquitination.

These findings also raise the intriguing possibility that human inflammatory diseases like Crohn's Disease or Blau syndrome may reflect deregulated ubiquitin mediated signaling, a hypothesis that remains to be tested by asking whether polyubiquitinated IKK- γ or Rip2 proteins are detected in the relevant cell types isolated from patients.

Although we observed a defect in MDP-induced NF- κ B activation in Rip2-deficient macrophages, MDP-induced p38 MAP kinase or JNK1/2 activation appears only modestly affected. These data suggest that other CARD containing adapter proteins may contribute to NOD2-mediated MAP kinase activation. One possibility may be CARD9, that is expressed in RAW 264.7 macrophages and whose expression has been shown to preferentially activate the p38 MAP kinase and JNK1/2 pathways. Consistent with this model, MDP-induced MAP kinase responses and cytokine production are impaired in CARD9-deficient cells, whereas MDP-induced NF- κ B activation appears unaffected [173].

The cytosolic Nod2 pathway has been implicated in innate immune responses to *Streptococcus pneumoniae*, *Listeria monocytogenes* and *Mycobacterium tuberculosis* (*Mtb*) [100,120,121]. Rip2-deficient mice have been shown to be susceptible to *Listeria* infection [102], however the contribution of Rip2 to the innate immune response to *Mtb* has not yet been examined. We demonstrate that *Mtb* infection results in the ubiquitin modification of Rip2. Rip2 polyubiquitination is observed in macrophages infected with wt strains of *Mtb*, but is not observed if the bacilli are killed prior to infection (with heat,) (Figure 11A). These data indicate that Rip2 polyubiquitination and Nod2

pathway –activation” requires bacterial viability and suggests that *Mtb* components may gain entry into the host cell cytosol to stimulate the Nod2/Rip2 pathway.

Although Rip2 and NLRs have been implicated in the innate immune recognition of *Listeria monocytogenes*, we did not observe Rip2 polyubiquitination following *Listeria* infection. It remains unclear why *Listeria* infection did not stimulate Rip2 polyubiquitination, but recent work by Girardin and colleagues suggests that the *Listeria monocytogenes* genome encodes a peptidoglycan N-deacetylase (*pgdA*) gene and inactivation of the bacterial *pgdA* gene results in reduced bacterial growth and a massive host IFN- β response [97]. Thus, our inability to detect Rip2 polyubiquitination in *Listeria*-infected macrophages may reflect the ability of the bacteria to deacetylate peptidoglycan and thereby avoid NLR recognition.

In summary, this study provides genetic and biochemical evidence that Nod2-mediated bacterial recognition is mediated by the stable ubiquitin modification of Rip2, raising the possibility that the enzymes responsible for the ubiquitin modification of Rip2 may serve as potential therapeutic targets in certain infectious diseases and potentially in chronic inflammatory disease.

Materials and Methods

Isolation of Macrophages and Mycobacterium tuberculosis infection

To isolate peritoneal activated macrophages, wild type and *rip2*^{-/-} mice (gift of Dr. Vishva M. Dixit, Genentech, Inc. South San Francisco, CA) were injected with 3ml of thioglycolate by intraperitoneal injection. Three days later, macrophages were isolated by gentle flushing of the peritoneal cavity. Peritoneal macrophages were left unstimulated or treated with MDP or purified LPS (Sigma). NF- κ B activation was measured using phospho-I κ B α antibodies (Cell Signaling Technology), p38 MAP kinase and JNK1/2 activity was measured using anti-phospho-p38 and phospho-JNK1/2 antibodies (Cell Signaling Technology), respectively. Bone marrow derived macrophages for *Mycobacterium tuberculosis* (*Mtb*) infection were harvested from wild type or MyD88-deficient mice as described in [174]. *Mtb* (strain H37Rv) was cultivated in 7H9 broth to exponential phase and washed thoroughly in phosphate buffered saline prior to infection. Bacterial clumps were removed by passing the washed suspension through a 5 μ M syringe filter. “Heat-killed” bacteria were inactivated by heating to 80°C for 30 minutes. Macrophages were infected for 1 or 2 hours after which filtered cell lysates were immunoprecipitated with anti-Rip2 antibody (Santa Cruz) or immunoblotted with anti-phospho-I κ B α antibody (Cell Signaling Technology).

Plasmids and Constructs

The full-length Rip2 constructs was provided by Dr. Vishva M. Dixit (Genentech,

South San Francisco, California). The wild type NOD2 construct has been described previously [108]. The HA-ubiquitin, HA-K48-only ubiquitin and HA-K63-only ubiquitin constructs were generously provided by Dr. Zhijian James Chen (University of Texas Southwestern, Dallas, TX).

Antibodies, siRNA and Reagents

Anti-Rip2 (Rabbit), anti-Nemo (Rabbit), anti-Traf2 (Rabbit), anti-Traf6(Mouse), anti-Ubiquitin (Mouse), anti-Omni (Rabbit), anti-Tak1 (Rabbit), anti-p38 (Goat) and anti-JNK (rabbit) antibody were obtained from Santa Cruz Biotechnology. Anti-phospho-I κ B- α , anti-phospho-p38 and anti-phospho-JNK antibodies were obtained from Cell Signaling Technology. Anti-UBC13 antibody was obtained from Zymed, anti-FLAG, and anti- β -actin antibody were obtained from Sigma and anti-HA antibody was obtained from Boehringer Mannheim. Human Traf2 and Traf6 siRNA and mouse Tak1 siRNA were obtained from Santa Cruz Biotechnology. MDP was obtained from InvivoGen. Mouse TNF- α were obtained from Sigma. LPS derived from *Escherichia coli* strain 0111.B4 was purchased from Sigma, dissolved in deoxycholate, and re-extracted with phenol/chloroform as described in [175].

Cell Culture, DNA transfection and SiRNA transfection

HEK293T cells and the mouse RAW 264.7 macrophage cell lines were obtained from ATCC and were grown in DMEM containing 10% FBS. For all

transfection studies, HEK293T cells were plated at 5×10^5 cells/plate on 60mm plates. Constructs were transfected to HEK293T cells using FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer's instructions. SiRNAs were transfected to HEK293T cells using Lipofectamine 2000 (Invitrogen). Mouse control and Tak1 specific siRNAs were transiently transfected into the mouse RAW 264.7 macrophage cell line using a two step transfection procedure and Lipofectamine 2000 (Invitrogen).

Generation of Ubc13 stably knock down RAW246.7 cell lines

The mouse macrophage cell line RAW264.7 was infected with lentiviruses containing shRNA sequences specific for Ubc13 (Open Biosystems) and selected with 2.5 μ g/ml puromycin. Selected puromycin resistant pools of cells were left unstimulated or treated with MDP and Rip2 polyubiquitination assayed by immunoprecipitation with anti-Rip2 antibody (Santa Cruz) followed by immunoblotting with anti-ubiquitin antibodies. The expression of the E2 conjugating enzyme Ubc13 in the lentiviral infected pools was determined by immunoblotting with an anti-Ubc13 antibody (Zymed).

Immunoprecipitation and Western Blot Analysis

For all immunoprecipitation and ubiquitination assays, cell lysates were prepared in radioimmune precipitation assay (RIPA) buffer (150mM NaCl, 50mM Tris-HCl (pH 7.5), 1% NP40, 0.25% deoxycholate, 0.1% SDS, 1mM EDTA),

supplemented with a protease mixture inhibitor (Roche Applied Science) and 5mM N-ethylmaleimide (Sigma), immunoprecipitated with anti-Rip2 or anti-NEMO antibody (Santa Cruz), Polyubiquitinated Rip2 or NEMO/IKK- γ proteins were detected by immunoblotting with an anti-ubiquitin antibody (Santa Cruz). To examine endogenous protein interactions, cells were lysed in an endogenous lysis buffer (0.5 M Tris (pH 7.6), 0.5 M NaCl, 0.1 M EDTA, 1% Triton X-100, 0.5 M NaF, and 0.5 M sodium pyrophosphate) supplemented with a protease mixture inhibitor (Roche Applied Science), immunoprecipitated with anti-Rip2 antibody, and then immunoblotted with either anti-Tak1 or anti-Traf6 antibody (Santa Cruz Biotechnology). Expression of epitope-tagged, transfected proteins or endogenous proteins was detected by immunoblotting with corresponding antibodies.

Figures and Legends

Figure 6. Rip2-deficient macrophages are impaired in their response to NOD2 ligand MDP, but respond normally to the TLR4 ligand, LPS. A. Rip2 is required for NF- κ B and p38 MAP kinase activation in response to MDP or NOD2 pathway stimulation. Macrophages from wild type and *rip2*^{-/-} mice were grown to confluence on 6 well plates and treated with 10 μ g/ml MDP or 10ng/ml LPS for the indicated times. The IKK and p38 MAPK activity were measured by immunoblotting with anti-phospho-I κ B α and anti-phospho-p38 MAP kinase antibody (Cell signaling). Total p38 protein level was measured by immunoblotting with anti-p38 antibody (Santa Cruz). **B. MDP-induced JNK1/2 activation is modestly impaired in the absence of Rip2.** Macrophages from wild type and *rip2*^{-/-} mice were grown to confluence on 6 well plates and treated with 10 μ g/ml MDP or 10ng/ml LPS for the indicated periods of time. The JNK MAPK activity was measured by immunoblotting with anti-phospho-JNK antibody (Cell signaling). Total JNK protein level was measured by immunoblotting with anti-JNK antibody (Santa Cruz).

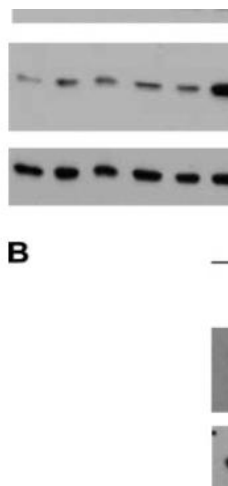


Figure 7. NOD2 pathway activation stimulates the stable K63-linked polyubiquitination of Rip2. **A. MDP treatment stimulates the ubiquitin modification of endogenous Rip2 and NEMO/IKK- γ .** The RAW 264.7 macrophage cell line was left untreated or treated with the NOD2 ligand, MDP (10 μ g/ml) for the indicated times. Cells were lysed, and an equal amount of total protein was immunoprecipitated with anti-Rip2 or anti-NEMO antibody (Santa Cruz). To detect ubiquitinated proteins, immunoprecipitated proteins were immunoblotted with an anti-ubiquitin antibody (Santa Cruz). Total immunoprecipitated Rip2 or NEMO/IKK- γ protein was measured by immunoblotting with anti-Rip2 or anti-NEMO antibodies (Santa Cruz). **B. NOD2 expression stimulates the stable, K63-linked polyubiquitination of Rip2.** Omni-tagged NOD2 and FLAG-tagged Rip2 constructs were expressed in HEK293T cells in the presence of HA-tagged K63-only or HA-tagged K48-only forms of ubiquitin. Rip2 was immunoprecipitated with anti-Rip2 antibody (Santa Cruz), and western blots were performed with the indicated antibodies. Expression levels of both Flag-Rip2 and Omni-NOD2 was measured by immunoblotting total cell lysates with anti-Flag or anti-Omni antibodies.

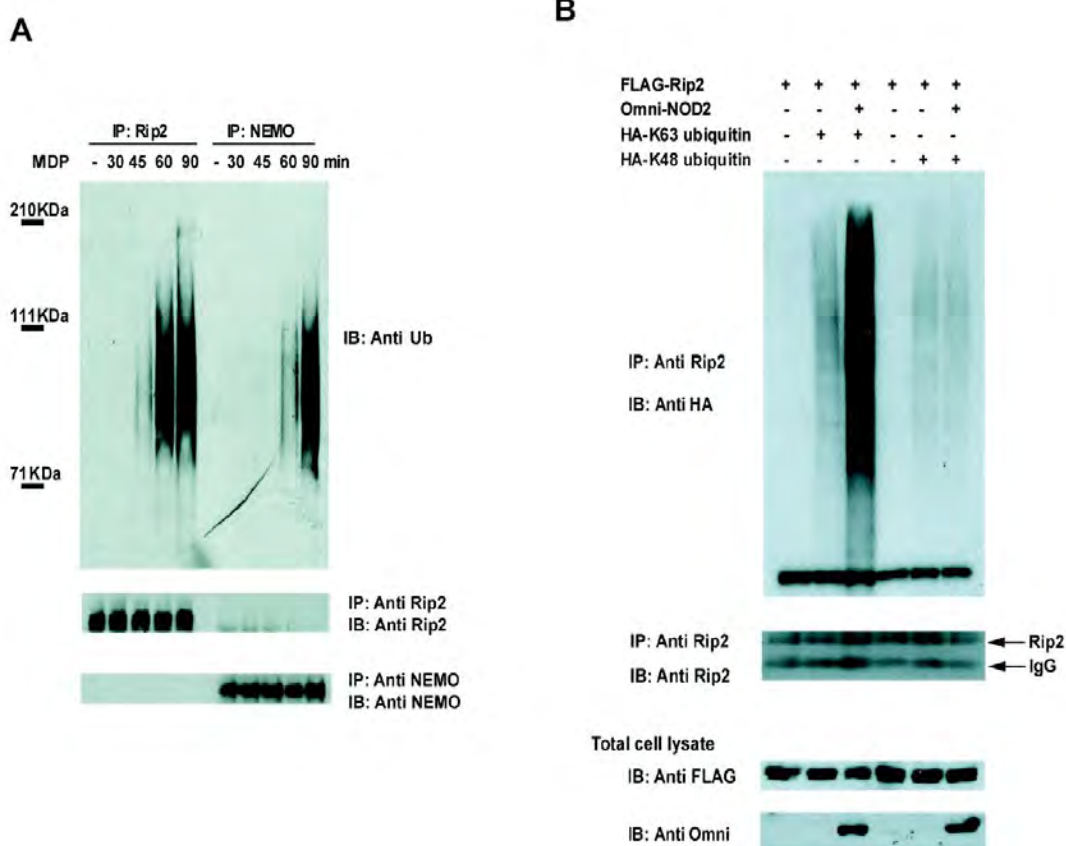


Figure 8. MDP treatment stimulates the K63-specific ubiquitin modification of endogenous Rip2. The RAW 264.7 macrophage cell line was left untreated or treated with the NOD2 ligand, MDP (10 μ g/ml) for the indicated times. Cells were lysed, and an equal amount of total protein was immunoprecipitated with anti-Rip2 antibody (Santa Cruz). To detect K63-specific ubiquitinated proteins, immunoprecipitated proteins were immunoblotted with an anti-K63-ubiquitin antibody (Millipore and original from Genentech). Total immunoprecipitated Rip2 protein was measured by immunoblotting with anti-Rip2 antibody (Santa Cruz).

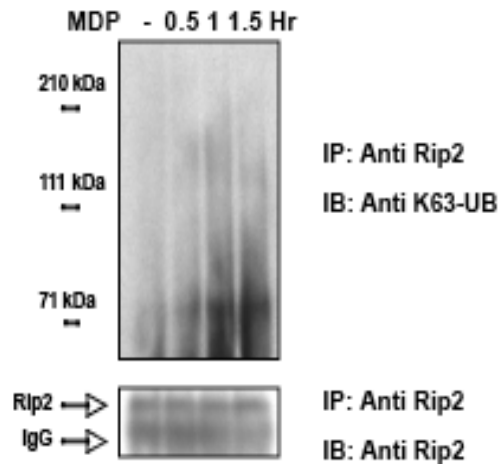


Figure 9. MDP-induced Rip2 polyubiquitination is dependent on the expression of the E2 conjugating enzyme Ubc13 and the E3 ubiquitin ligase Traf6. **A. MDP fails to stimulate Rip2 polyubiquitination in Ubc13-deficient RAW 264.7 macrophages.** The mouse macrophage cell line RAW 264.7 was infected with lentiviruses containing shRNA sequences specific for Ubc13 or GFP as a control and stable puromycin resistant pools selected. These lines were left untreated or stimulated with MDP (10 μ g/ml) for the time periods indicated. Cells were lysed, and an equal amount of total protein was immunoprecipitated with anti-Rip2 antibody (Santa Cruz) followed by immunoblotting with an anti-ubiquitin antibody (Santa Cruz). Total immunoprecipitated Rip2 protein was measured by immunoblotting with anti-Rip2 antibody (Santa Cruz). Expression of Ubc13 level was determined by immunoblotting with an anti-Ubc13 antibody (Zymed). **B. Rip2 polyubiquitination requires the expression of the E3 ubiquitin ligase Traf6, but not Traf2.** Omni-tagged NOD2 was expressed in HEK293T cells in the presence of either a control siRNA or siRNA specific for Traf2 or Traf6. Rip2 was immunoprecipitated with anti-Rip2 antibody (Santa Cruz) followed by immunoblotting with anti-ubiquitin to detect polyubiquitinated Rip2 proteins. Expression levels of Omni-NOD2 and Traf2 or Traf6 protein levels was measured by immunoblotting the total cell lysates with anti-Omni, anti-Traf6 and anti-Traf2 antibodies (Santa Cruz). **C. MDP stimulates interaction between endogenous Rip2 and Traf6.** RAW 264.7 macrophages were left untreated or treated with 10 μ g/ml MDP for the indicated periods of time. Cells were lysed, and an equal amount of total protein was immunoprecipitated with anti-Rip2 antibody (Santa Cruz) followed by immunoblotting with an anti-Traf6 antibody (Santa Cruz). The total immunoprecipitated Rip2 protein was measured by immunoblotting with anti-Rip2 antibody (Santa Cruz). **D. Traf6 expression is required for MDP- and IL-1 β induced NF- κ B activation.** The RAW 264.7 macrophage cell line was transfected with control siRNA or a pool of Traf6 siRNAs. Forty-eight hours later, the cells were left untreated or stimulated with MDP (10 μ g/ml) for the time periods indicated or with IL- β (5ng/ml) for 10 minutes. NF- κ B activation was measured by immunoblotting with a phospho-I κ B α antibody (Cell Signaling Technology). Traf6 and β -actin levels were determined by immunoblotting with the indicated antibodies.



Figure 10. Rip2 and the ubiquitin activated kinase Tak1 interact upon MDP stimulation and Tak1 expression is required for MDP-induced IKK activation.

A. Rip2 and Tak1 interact in transfected cells. Flag-tagged Tak1 and HA-tagged Rip2 were either co-expressed or expressed alone in HEK293T cells. Tak1 was immunoprecipitated with an anti-Flag antibody and Rip2 detected by immunoblotting with anti-HA antibody or Rip2 was immunoprecipitated with an anti-HA antibody followed by immunoblotting with anti-Flag antibody to detect associated Tak1 proteins. Relative expression of each construct was measured by immunoblotting the total cell lysate with anti-HA or -Flag antibodies.

B. MDP stimulates interaction between Rip2 and Tak1. The RAW 264.7 macrophage cell line was left untreated or treated with 10 μ g/ml MDP for the indicated periods of time. Cells were lysed, and an equal amount of total protein was immunoprecipitated with anti-Rip2 antibody (Santa Cruz) followed by immunoblotting with an anti-Tak1 antibody (Santa Cruz). Total immunoprecipitated Rip2 protein was measured by immunoblotting with anti-Rip2 antibody (Santa Cruz).

C. Tak1 expression is required for TNF- α and MDP-induced NF- κ B activation. Mouse RAW 264.7 macrophages cell line was transfected with control siRNA or a pool of Tak1 siRNAs. Forty-eight hours later, the cells were left untreated or stimulated with MDP (10 μ g/ml) for the time periods indicated or with TNF- α (10ng/ml) for 10 minutes. NF- κ B activation was measured by immunoblotting with a phospho-I κ B α antibody (Cell Signaling). Tak1 and β -actin levels were determined by immunoblotting with the indicated antibodies.

Figure 11. Live *Mycobacterium tuberculosis* infection stimulates Rip2 polyubiquitination. **A. Live, wt strains of *Mtb* (Rv) stimulate Rip2 polyubiquitination but heat killed strains fail to stimulate Rip2 polyubiquitination.** Wildtype bone marrow-derived macrophages were infected with live virulent (Rv) or heat killed (HK-Rv) strains of *M. tuberculosis* (*Mtb* H37Rv). Polyubiquitinated Rip2 proteins were detected by immunoprecipitating the cell lysates with an anti-Rip2 antibody followed by immunoblotting with an anti-ubiquitin antibody. Immunoprecipitates were immunoblotted with a Rip2 antibody to insure that equal amounts of Rip2 protein were immunoprecipitated. **B. *Mtb*-induced Rip2 polyubiquitination is MyD88 and TLR2/4 independent.** Wildtype, *MyD88*^{-/-} and *TLR2/4*^{-/-} bone marrow-derived macrophages were left uninfected (-) or infected with live wt *M. tuberculosis* (H37Rv) for 1 hour. Polyubiquitinated Rip2 proteins were detected by immunoprecipitating the cell lysates with an anti-Rip2 antibody followed by immunoblotting with an anti-ubiquitin antibody.

IP: Anti-Rip2

IB: Anti Ub

Figure 12. Identification of critical lysine residues critical for Rip2 polyubiquitination. A. Sequence alignment of the intermediate domains of human, rat and mouse Rip2. Lysines 310, 311, 313, 326, 327 (in Red Box) are conserved among the different species. **B. Rip2 2K mutant is unable to undergo polyubiquitination when overexpressed in HEK293 cells.** FLAG-tagged Rip2 wt, 3K or 2K constructs were expressed in HEK293T cells. Rip2 was immunoprecipitated with anti-FLAG antibody and western blots were performed with the indicated antibodies. Expression levels of Flag-Rip2 was measured by immunoblotting total cell lysates with anti-Flag antibodies. **C. Rip2 2k polyubiquitination is impaired in MDP treated cells.** FLAG-tagged Rip2 wt or 2K constructs and Omni-tagged NOD2 were expressed in HEK293T cells. Cells were treated with MDP (10 μ g/ml) for the indicated time. Rip2 was immunoprecipitated with anti-FLAG antibody (Santa Cruz) followed by immunoblotting with anti-ubiquitin to detect polyubiquitinated Rip2 proteins.

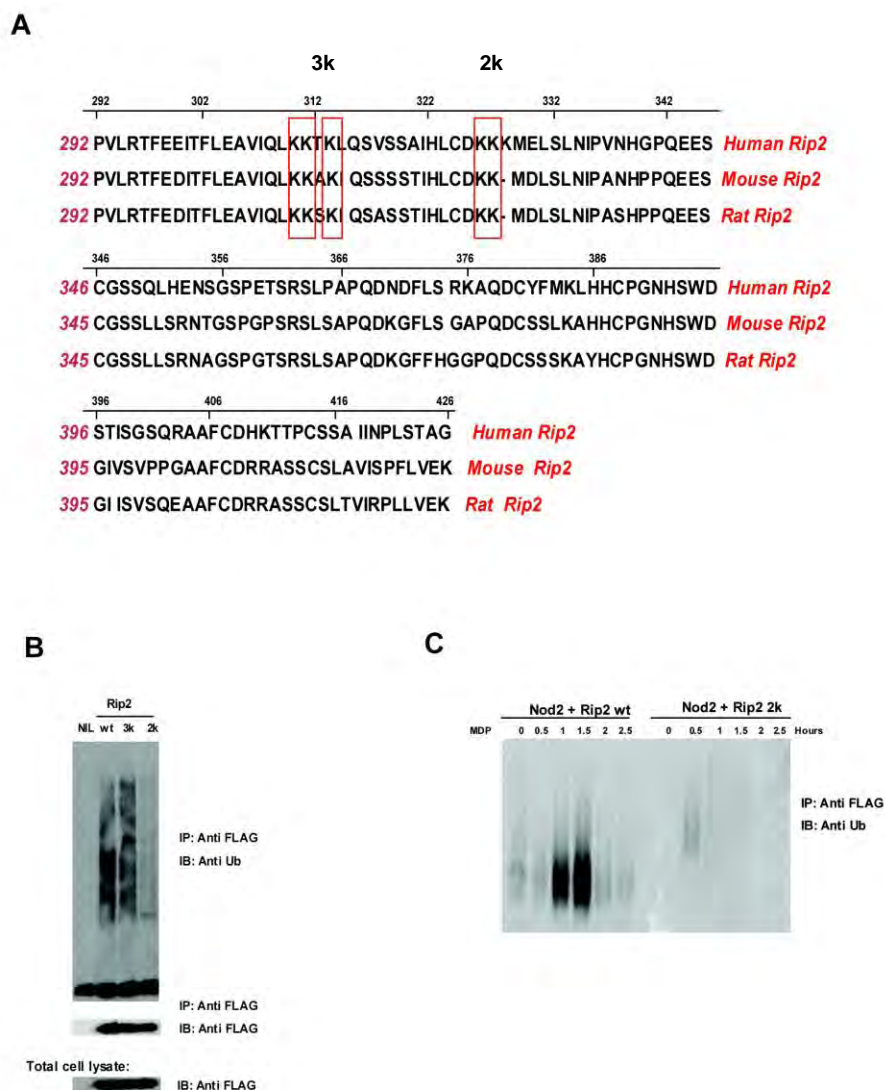
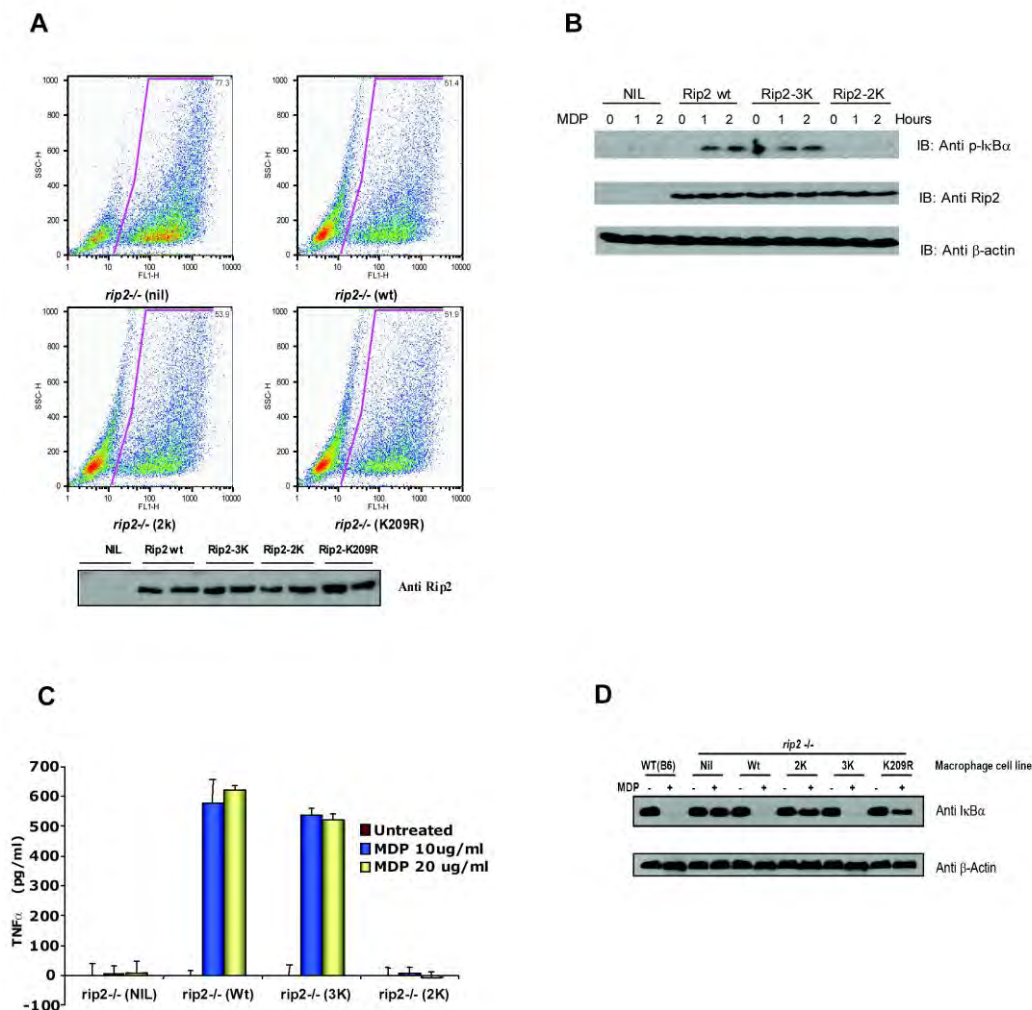


Figure 13. Rip2 polyubiquitin modification is required for Nod2-mediated IKK activation. **A. Generation of *rip2*^{-/-} reconstituted macrophage cell lines stably expressing wild type Rip2, Rip2 2K, Rip2 3K and Rip2K209R.** Rip2 wild type and lysine mutants was cloned into an MSCV2.2.IRES-GFP retroviral vector. The *rip2*^{-/-} transformed macrophage cell line was infected with the retroviral vector alone or with retroviruses expressing Rip2. Rip2 protein expression level was measured by GFP positive cells and Rip2 immuno blot to ensure comparable levels of Rip2 expression were achieved. **B and C. Conserved lysines 326 and 327 (Rip2 2K) are required for MDP-induced NF- κ B activation and cytokine production.** *rip2*^{-/-} reconstituted macrophage cell lines were left untreated or stimulated with MDP (10 μ g/ml) for the time periods indicated. NF- κ B activation was measured by immunoblotting with a phospho-I κ B α antibody (B). TNF α expression was measured by TNF α ELISA (C). **D. Rip2 K209R mutant reconstituted cell line also failed to restore IKK activation upon MDP treatment.** *rip2*^{-/-} reconstituted macrophage cell lines were left untreated or stimulated with MDP (10 μ g/ml) for the time periods indicated. NF- κ B activation was measured by immunoblotting with a phospho-I κ B α antibody.



Chapter III

NOD2, RIP2 and IRF5 Play a Critical Role in the Type I Interferon Response to *Mycobacterium Tuberculosis*

This chapter is based on work from the following publications:

Pandey AK*, **Yang Y***, Jiang Z, Fortune SM, Coulombe F, Behr MA, Fitzgerald KA, Sasseti CM, Kelliher MA. NOD2, RIP2 and IRF5 play a critical role in the type I interferon response to *Mycobacterium tuberculosis*. *PLoS Pathog.* 2009 Jul;5(7):e1000500.

*indicates both authors contributed equally to this work

Coulombe F, Divangahi M, Veyrier F, de Léséleuc L, Gleason JL, **Yang Y**, Kelliher MA, Pandey AK, Sasseti CM, Reed MB, Behr MA.J. Increased NOD2-mediated recognition of N-glycolyl muramyl dipeptide. *J Exp Med.* 2009 Aug 3;206(8):1709-16.

Yang Y and Pandey A contributed the work of Fig. 14, 15, 16, 17, 18, 19, 21A, 21D, 22, 23, 24, 26, 27; Yang Y and Coulombe F contributed the work of Fig. 20, 25; Jiang Z contributed the work of Fig. 21B, 21C.

Introduction

Mycobacterium tuberculosis (Mtb), the causative agent of human tuberculosis, is an exquisitely adapted obligate human pathogen that is thought to persist within as many as one billion individuals worldwide [129]. While residing inside a modified phagosomal compartment of host macrophages, Mtb is able to persist for decades. However, a robust cell-mediated immune response effectively inhibits bacterial replication in approximately 90% of otherwise healthy individuals, and the infection can be controlled indefinitely. Deficits in this immune response result in progressive bacterial replication, necrosis of infected lung tissue, and spread to other individuals. Thus, like many other pathogens that cause chronic infections, the long-term survival of Mtb, depends on a delicate balance between bacterial virulence and host immunity.

Immunity to tuberculosis depends on both the innate and adaptive responses of the host. Initial recognition of the bacterium is mediated by pattern recognition receptors (PRR) such as Toll-like receptors (TLRs) [74,165] or nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) [176,177], both of which recognize conserved microbial structures known as pathogen associated molecular patterns (PAMPs). Although the functions of most NLR's remain undefined, the Nod1 and Nod2 proteins have been shown to respond to bacterial cell wall fragments. While the recognition of these common forms of peptidoglycan have been extensively studied, bacteria modify their cell walls in a myriad of ways and the effects of these modifications on Nod1/2 recognition are only beginning to be appreciated (reviewed in [94,95,96]). For example,

Listeria monocytogenes removes a common *N*-acetyl moiety from the glucosamine of its peptidoglycan, which renders the cell wall resistant to host lysozyme and thereby inhibits bacterial recognition by Nod1 [97]. In contrast, mycobacteria, replace the *N*-acetyl group of the muramic acid of MDP with an *N*-glycolyl moiety[98,99], and this modification significantly increases the potency of this compound as a Nod2 agonist (Coulombe et al, unpublished data).

Nod1 and Nod2 functions depend on the serine threonine kinase Rip2. Rip2 has been shown to be essential for cytosolic Nod1/2 signaling, and its overexpression stimulates NF- κ B activity and induces apoptosis [60,67]. We have shown that Rip2 is stably modified with ubiquitin in cells treated with the Nod2 agonist MDP (Figure 7). This modification is required for Nod1-mediated NF- κ B activation [104], indicating that stable polyubiquitination is a critical component of this signaling cascade.

Intact *Mtb* bacilli are recognized by both TLRs and NLRs, which cooperatively respond to infection and synergistically induce NF- κ B activation [121]. However, a large fraction of the transcriptional response to *Mtb*, including many immunologically important proteins are induced independently of TLR2/4 and the adapter proteins MyD88, MAL and TRIF. Instead these responses rely on autocrine or paracrine signaling via type I interferons (IFN α/β), which are induced through largely undefined pathways [139].

Despite the ability of cell surface localized TLR4 to trigger IFN α and IFN β transcription, existing evidence indicates that during genuine bacterial infections, this response instead requires the recognition of bacterial products in the cytosol. This has

been most clearly demonstrated for *Listeria monocytogenes*, which must disrupt the phagosomal membrane and escape into the cytosol in order to trigger the type I IFN response in resting macrophages [178,179]. Despite its residence in the phagosome, Mtb still induces rapid and robust IFN α/β transcription, and this response depends on a specialized secretion system of the bacterium, ESX1 [169]. This system has been suggested to contribute to the perturbation of the phagosomal membrane [180,181,182], indicating that cytosolic recognition might be critical for IFN α/β responses to diverse bacterial pathogens including Mtb.

The primary pathways leading to IFN α/β induction upon bacterial infection remain obscure. Since transfection of DNA into the cytosol of macrophages can induce a Tbk-1 and Irf3-dependent IFN α/β response similar to that seen upon *L. monocytogenes* infection, bacterial DNA has been implicated as the eliciting stimulus [183]. Two different cytosolic DNA sensors have been identified, DAI [184] and AIM2 [185], but their importance during bacterial infections remains to be demonstrated. While Nod2 recognition of MDP is not absolutely required for IFN α/β production [186], it has been shown to synergize with the cytosolic DNA response and enhance IFN production during both *L. monocytogenes* and Mtb infection [143]. However, Nod2 stimulation alone is thought to be insufficient to induce type I IFN production [143].

In sum, while a large fraction of the macrophage response to Mtb infection depends on type I IFN [139], and therefore is likely to rely on a cytosolic signaling pathway, the bacterial products recognized and the pathways involved remain unknown.

We previously found that *Mtb* infection of macrophages triggers Rip2 polyubiquitination in a TLR and MyD88 independent manner [187]. We now show that this stimulation is due to the ESX1-dependent entry of bacterial products into the cytosol where they are recognized by Nod2, implicating MDP as the relevant PAMP. Unexpectedly, this results in IFN α/β production that is dependent on a novel pathway consisting of Nod2, Rip2, Tbk1, and Irf5. This work is the first to implicate NLRs in IRF activation and to suggest a role for Irf5 in anti-bacterial innate immune responses. Furthermore, we found that the unusual *N*-glycolyl MDP produced by *Mtb* was 10-100 fold more potent than the commonly studied *N*-acetylated MDP produced by most bacteria, and that only *N*-glycolyl MDP could stimulate Rip2-dependent IFN α/β transcription in the absence of other stimulants. Thus, the mammalian Nod2 pathway appears to be remarkably sensitive to mycobacterial MDP and responds to infection by triggering the production of type I interferon, which is responsible for a significant component of the transcriptional response to *Mtb* infection.

Results

***Mycobacterium tuberculosis* infection stimulates the ubiquitin modification of Rip2 via the Nod2 protein.**

The ability of Mtb to rapidly modify macrophage signaling and vesicular sorting pathways [132] suggests that bacterial products gain access to the cytosol soon after phagocytosis. These products are, in turn, likely to be sensed by the host and trigger the innate immune response. Previously, we demonstrated that Mtb rapidly induces the TLR2/4 independent polyubiquitination of the Rip2 protein [187], an event that could represent the initiation of cytosolic recognition. To characterize these events in more detail, we infected the mouse macrophage cell line RAW 264.7 or primary bone marrow derived macrophages (BMDM) with live or heat killed Mtb. In both cell types, we observed that infection with live, but not heat-killed, Mtb stimulated the rapid polyubiquitination of Rip2. The Mtb-induced ubiquitin modification reached maximal levels within 1 hour post-infection and declined by 4 hours (Figure 14A). Furthermore, pretreatment of cells with cytochalasin D to inhibit phagocytosis reduced Rip2 polyubiquitination in a dose-dependent manner (Figure 14B), indicating that the bacteria must be both live and intracellular to initiate this response.

Since Nod1 and Nod2 have been implicated in the cytosolic recognition of mycobacterial components [121], we sought to determine if Rip2 polyubiquitination depended on these proteins. In contrast to cells from wild type mice, inducible Rip2 polyubiquitination was not observed in macrophages derived from mice lacking Nod1

and Nod2, and was greatly reduced in cells lacking only Nod2 (Figure 14C). These data confirmed that intracellular Mtb is recognized by a Nod2-dependent pathway and that this protein is required for the stable ubiquitination of Rip2.

The mycobacterial ESX1 system is required for Nod recognition.

Live intracellular Mycobacteria were required to stimulate the Nod-Rip2 pathway, indicating that the bacterium actively participated in this process, likely via the translocation of bacterial products into the cytosol. A specialized protein secretion system, encoded by the ESX1 locus, has been implicated in the perturbation of the host membranes [180,181,188] and for stimulation of the type I IFN response [169] and inflammasome activation [189], suggesting that this system might contribute to cytosolic recognition via Nod proteins. In order to test this hypothesis, we infected the mouse macrophage cell line RAW 264.7 with wild type Mtb or mutants lacking ESX1 function. No induction in Rip2 polyubiquitination was observed upon infection with a strain of Mtb harboring the $\Delta RD1$ mutation, which deletes a portion of the ESX1 locus [190]. Similarly, a mutant lacking *espA*, a distally-encoded gene that is required for ESX1-mediated secretion [191], also failed to elicit this response (Figure 15). The phenotype of the latter mutant could be complemented by the expression of *espA* from a plasmid vector, demonstrating that the inability to stimulate Rip2 polyubiquitination was linked to the *espA* mutation. Furthermore, *M. bovis* BCG, an attenuated vaccine strain carrying the RD1 deletion and therefore lacking ESX1 function [190], was unable to

stimulate Rip2 polyubiquitination. While all of these ESX1 mutants are less virulent than wild type bacteria, the lack of Nod2-Rip2 stimulation did not appear to be a nonspecific effect of attenuation. Two unrelated bacterial mutants that are unable to grow intracellularly, a biotin auxotroph ($\Delta bioF$ [192]) and a small molecule efflux mutant (TN::*rv1410c* [193]), robustly stimulated this response (Figure 15). Taken together, these observations indicate that a functional ESX1 secretion system is specifically required for Nod2 stimulation.

Membrane damage allows Nod2-mediated recognition of ESX1 mutants.

Since the Mtb-induced Rip2 polyubiquitination required ESX1, we hypothesized that this system might be responsible for the release of Nod2 ligands into the cytosol, perhaps via the disruption of vacuolar membrane integrity. However, it also remained possible that ESX1-deficient strains simply lacked a critical PAMP or other Nod2 stimulating activity. To distinguish between these possibilities, we investigated whether ESX1 function could be complemented by two exogenous membrane-disruptive activities. Streptolysin O (SLO) is a cholesterol-dependent toxin that introduces pores directly into mammalian membranes. Pores can also be introduced by adding ATP to macrophages, resulting in stimulation of the P2X7 receptor and the subsequent opening of the hemichannel, pannexin-1 (PANX1) [194]. We observed that membrane perturbation by either of these two methods resulted in robust Rip2 polyubiquitination upon infection with *espA*-deficient bacteria, which were otherwise unable to induce this response (Figure

16A). The involvement of PANX1 in the ATP-facilitated Rip2 ubiquitination was verified by the addition of a competitive inhibitory peptide of the PANX1 pore. This peptide, but not a scrambled control peptide, inhibited Rip2 polyubiquitination to levels observed in cells infected with the $\Delta espA$ mutant (Figure 16). While the K^+ flux subsequent to membrane damage has been found to stimulate NLRs in some circumstances [176], we found that the addition of ATP or SLO alone resulted in a minimal response. These data indicate that SLO, PANX1 and ESX1 are all likely to promote Nod2 pathway activation via a similar mechanism, by facilitating the release of a stimulatory mycobacterial component into the cytosol. Since this pathway appears to be specific for peptidoglycan fragments, mycobacterial MDP-containing fragments were the most likely candidates.

The cytosolic Nod2-Rip2 pathway contributes to type I IFN production upon Mtb infection.

The inability of ESX1 mutants to stimulate either the Nod2-Rip2 pathway or the type I IFN response [169] led us to hypothesize that the Nod2 pathway may mediate type I IFN expression in this system. To investigate a potential link between Nod2 and IFN α/β , we infected Nod2- or Rip2-deficient macrophages with Mtb, and measured the induction of IFN α and IFN β mRNAs using real time PCR (qRT-PCR). In the absence of Rip2, IFN β induction was reproducibly reduced approximately 3-fold, whereas IFN α induction was almost completely abrogated (Figure 17A and B). Nod2 deficiency

had a similar effect on both IFN α and IFN β transcription, consistent with its requirement for Rip2 polyubiquitination. Nod1 appears to play no role in this pathway, as *nod1*^{-/-} macrophages produced wild type levels of IFN β (Figure 18). The decreases in mRNA abundance observed in *rip2*^{-/-} and *nod2*^{-/-} cells were reflected in a similar decrease in protein production, as measured by ELISA (Figure 17C and D).

In order to assess the importance of Nod2 and Rip2 to the downstream IFN α/β -dependent macrophage response, we quantified the induction of RANTES mRNA, which depends on type I IFN secretion and signaling via the IFN α receptor (IFNAR1) in this infection model [139]. We found that in the absence of Rip2 or Nod2, Mtb infection failed to induce RANTES expression (Figure 17E). These data suggest that the effect of a Rip2 deficiency on downstream type I IFN responses may be even more pronounced than the IFN β mRNA levels indicate. In contrast, TNF α mRNA levels were unaffected by Nod2- or Rip2-deficiency (Figure 17F) indicating that other pattern recognition pathways remained responsive to Mtb in these cells.

Consistent with previous work [169], we found that infection with ESX1 mutant bacteria induced significantly less IFN β and RANTES expression than wild type bacteria (Figure 19). To test whether ESX1-mediated type I IFN expression was mediated solely via Rip2, we infected Rip2-deficient macrophages with ESX1 mutants and quantified IFN β and RANTES mRNA levels. We found that in the absence of Rip2, the loss of ESX1 function resulted in a further decrease in IFN β mRNA levels (Figure 19A), suggesting the presence of an additional host pathway(s) that contribute to IFN β

induction. However, Rip2 deletion had no effect in the absence of ESX1 (Figure 19B), supporting our biochemical evidence that NOD2 stimulation depends entirely on the ESX1-dependent delivery of stimulants into the cytosol.

The *N*-glycolylated MDP produced by *Mtb* is a potent stimulator of the Nod2-mediated type I IFN response.

While our data indicated that a significant fraction of the IFN α/β response could be attributed to the Nod2-Rip2 pathway, it has been suggested that MDP stimulation alone is unable to induce type I IFNs and can only augment responses triggered by other pathways [143]. Indeed, we also found that the *N*-acetylated MDP that is commonly used to stimulate Nod2 was a very poor inducer of IFN β and RANTES expression (Figure 20A and B). However, our preliminary studies investigating Rip2 polyubiquitination indicated that *Mtb* was a particularly potent stimulator of this response [187], and therefore we reasoned that this could be due to the *N*-glycolylated form of MDP produced by *Mtb*. To determine if this form of MDP was sufficient to induce type I IFN responses, we compared the ability of *N*-acetyl- and *N*-glycolyl-MDP to stimulate IFN β expression. In contrast to *N*-acetyl MDP, treatment with the *N*-glycolylated form stimulated a robust IFN β response, which was entirely dependent on Rip2 and Nod2 (Figure 20). In addition, at least 30-fold less *N*-glycolyl-MDP was necessary to stimulate the IFN β transcription. Thus, the Nod2/Rip2 pathway alone is sufficient to induce the production of the IFN response when stimulated with this potent form of

MDP.

Induction of the host type I IFN response upon Mtb infection requires the Tbk1 kinase and Irf5.

Listeria monocytogenes infection induces a potent host type I IFN response mediated by the Tbk1 kinase and Irf3 [179,183,186,195]. To test whether Mtb infection triggered similar pathways, we infected Irf3-deficient and Tbk1/Tnfr1-deficient macrophages with Mtb and measured IFN induction. The Tnfr1 deficiency was necessary to suppress the embryonic lethality of Tbk1 deletion [196]. Similar to the *L. monocytogenes* model, we found that IFN β induction by Mtb infection was completely dependent upon Tbk1, and the loss of Tnfr1 had little effect (Figure 21A). However, in contrast to the complete dependence on Irf3 observed for *L. monocytogenes* [179,183,186], we found IFN β expression was reduced, but not ablated when Irf3-deficient macrophages were infected with *M. tuberculosis* (Figure 21A). This partial dependence on Irf3 was not changed by varying the multiplicity of infection (Figure 22). These data prompted us to test whether other IRFs mediate Nod2-dependent type I IFN responses.

Induction of IFN β expression is dependent on the formation of the enhancosome which includes the NF- κ B, ATF-2, c-jun, Irf3 and Irf7 transcription factors [197]. Irf5 is a related family member that has also been shown to contribute to induction of type I IFN responses triggered by TLRs, and overexpression of MyD88 has been shown to synergize

with Irf5 to induce IFN β expression [198]. Based on these studies, we tested whether RIP2 collaborates with IRF5 or IRF3 to stimulate IFN β luciferase reporter activity. HEK293 cells were transfected with an IFN β -luciferase reporter construct, along with increasing amounts of expression plasmids encoding RIP2, MyD88, IRF3 or IRF5. RIP2 and IRF5 coexpression stimulated IFN β promoter activity in a dose dependent manner and to a similar extent as MyD88 and IRF5 (Figure 21B). In contrast, RIP2 and IRF3 expression failed to induce this robust response (Figure 21C). RIP2 and IRF5 expression also stimulated IFN α 4 promoter activity as well as a reporter construct containing multimerized ISRE elements (data not shown).

To further investigate the contribution of Irf5 to the anti-bacterial type I IFN response, we infected macrophages from Irf5-deficient mice and control littermates with either Mtb or *L. monocytogenes*, and measured IFN β expression. Consistent with the luciferase reporter studies, we found that Mtb-induced IFN β (Figure 21D) and IFN α (Figure 23) expression was impaired in the absence of Irf5. In contrast, the response to *Listeria* was unaffected by the loss of Irf5 (Figure 21D and Figure 23). While the related Rip1 adaptor protein regulates Irf7 activity in innate anti-viral signaling [57], we found that IFN β induction after Mtb infection was unaffected by Irf7 deficiency (data not shown). To rule out the possibility that Irf3 expression levels may also be affected in *irf5*^{-/-} macrophages, we verified that the Irf3 protein level was unchanged in Irf5-deficient cells (Figure 24). These results indicated that Mtb infection stimulates type I IFN expression via a pathway that depends on Nod2, Rip2, Tbk1, and Irf5. This

contrasts with the pathway triggered by *L. monocytogenes*, which depends entirely on Irf3 and not Irf5. We reasoned that this dependence on different Irf proteins might be explained by the preferential stimulation of a Nod2-Rip2-Irf5 pathway by mycobacterial peptidoglycan. Consistent with this model, we found that the IFN β induction triggered by *N*-glycolyl MDP was entirely dependent on Irf5 and independent of Irf3 (Figure 25), functionally linking Irf5 with the Nod2 pathway.

Discussion

Mammals first detect microbial infections via an array of PRRs that include both cell surface TLRs and cytosolic NLRs. However, not all microbial interactions represent a pathological state, and the immune system must be able to discriminate to some degree between colonization by commensal organisms and dangerous infection. One level of discrimination is provided by the desensitization or anatomical sequestration of TLRs at sites of chronic stimulation, such as the gut, which presumably allows for tolerance to normal flora [199,200]. Bacterial pathogens can still be recognized at these sites via NLRs, since these systems rely on the specific ability of pathogens to translocate PAMPs into the host cytosol.

The concept that NLRs are specific for pathogenic organisms that disrupt host membranes is supported in a number of bacterial systems in which the loss of specific virulence functions abrogates NLR signaling. For example, in resting macrophages, cytosolic recognition of *L. monocytogenes* requires the pore-forming toxin, listeriolysin O [178,179]. Similarly, *Helicobacter pylori* [119] and *Legionella pneumophila* [183] mutants lacking a functional type IV secretion system (T4SS), and *Shigella flexneri* [201] or *Salmonella enterica serovar typhimurium* [201] mutants lacking a functional type III secretion system (T3SS) fail to stimulate NLR pathways. In each case, the virulence system in question is responsible for host membrane damage and the likely translocation of bacterial products into the cytosol where they can be recognized by NLRs and/or other cytosolic surveillance systems.

Similarly, we found that the ESX1 specialized protein secretion system of Mtb is required for Nod2 recognition. While it has been suggested that type I IFN induction via ESX1 might represent a specific immunomodulatory virulence strategy [169], analogies to these other pathogens suggests that perhaps NLR recognition is simply a byproduct of a membrane damaging function that allows bacterial products to enter the cytosol. This model is supported by our observations that other membrane perturbing agents, such as SLO and PANX1 can substitute for ESX1 function and allow cytosolic recognition. Thus, in a number of cases it appears that NLRs can be considered as sentinels for pathogens that rely on membrane damage as a pathogenic strategy.

Based on their common role in protein secretion and in facilitating cytosolic recognition, it is tempting to speculate that ESX1 and Gram-negative T3SS and T4SS function analogously to deliver effector proteins into the host cytosol. Despite these similarities, the role played by ESX1 during infection remains unclear, since no translocated effectors have been identified to date. In both Mtb and *M. marinum*, a related pathogen of ectotherms, ESX1 has been implicated in host membrane disruption and one of the major substrates of this system, EsxA, has been proposed to possess a membrane-lytic activity [181,188]. This single activity could be sufficient to account for the delivery of MDP and other PAMPs to the cytosol. It remains to be determined whether perturbing host membranes is the only role played by ESX1 during infection, or if this system also serves additional functions analogous to the specialized secretion systems of other pathogens.

A major consequence of the cytosolic recognition of Mtb is the induction of type I IFN. While the importance of this response in viral defense is clear and virtually universal, its role in antibacterial immunity appears to vary. Mice deficient in the type I IFN receptor, *Ifnar1*, are significantly more susceptible to several Gram-positive and -negative bacterial infections [202,203,204,205], indicating that IFN α/β are important for immunity to many bacteria. However, *Ifnar1* mutation has the opposite effect on the outcome of *L. monocytogenes* infection [206], suggesting that IFN α/β can also exacerbate disease. The role played by IFN α/β in Mtb infection remains somewhat uncertain. The induction of several immunologically important genes, including NOS2, depend on IFN α/β , suggesting a protective role. Initial studies of mouse and human infections appeared to support this view [207,208]. However, like the *L. monocytogenes* system, mutation of the IFN α/β receptor has in most cases been associated with decreased bacterial burden in mouse models of tuberculosis [169,208,209,210,211]. IFN α/β may fail to protect against disease because Mtb inhibits the response to these cytokines in infected macrophages [212]. Despite these differences however, some important themes emerge from these studies. Most importantly, the effect of IFN α/β is most apparent after the onset of adaptive immunity and not before, which we will discuss in detail in chapter IV.

A variety of bacterial pathogens trigger the type I IFN response, and a paradigm has begun to emerge regarding the induction of this response by bacteria. The current model suggests that bacterial DNA translocated into the host cytosol is the major eliciting

agent. Other PAMPs, such as MDP, can provide a synergistic IFN-inducing stimulus, but have not appeared to be sufficient for induction of IFN β in the absence of other triggers [143]. In contrast, our data support a model whereby Nod2 stimulation by Mtb infection induces the polyubiquitination of Rip2, which acts via the Tbk1 kinase to stimulate the activity of Irf5 and induce transcription of IFN α/β . The detailed discussion of this difference will be presented in chapter IV. Furthermore, we found that unlike the *N*-acetylated MDP found in many bacteria, stimulation with the *N*-glycolylated MDP derivative found in mycobacteria was sufficient to stimulate the IFN response in the absence of other stimuli.

A significant component of IFN β induction remains intact upon Mtb infection of Rip2-deficient macrophages (Figures 17 and 19), indicating that additional pathways are also involved. Since virtually all IFN β expression is ESX1-dependent, it appears that the residual induction observed in *rip2*^{-/-} macrophages also depends on cytosolic recognition pathways. These pathways could certainly include a DNA sensor that acts via Irf3, as proposed for other infections, since Irf3 deficiency had a moderate effect on IFN β expression in our experiments (Figure 21A and 22). Thus, our data do not imply that Mtb is stimulating IFN α/β in a fundamentally different manner from other bacteria. Instead, it is likely that bacterial pathogens stimulate the IFN response via multiple, partially redundant pathways, and that the relative importance of each is determined by the unique biology of the infection. In the case of Mtb, we speculate that the *N*-glycolylation of its peptidoglycan, and perhaps a paucity of other stimulants such as

DNA, favor recognition via Nod2. It is also possible that the balance of these pathways might be affected by the activation state of the macrophage. When resting macrophages are infected with *L. monocytogenes*, the IFN response requires LLO and is completely Irf3 dependent. In contrast, IFN γ -stimulated cells are able to deliver this bacterium to the lysosome, where the cell wall is degraded to produce abundant peptidoglycan fragments. In this situation, a significant component of the IFN β induction depends on Nod2 and not Irf3 [213]. While Irf5 was not investigated in this study, it is possible that this represents another situation in which robust Nod2 signaling promotes a Nod2- and Irf5- dependent type I IFN response.

While we found that loss of Nod2-Rip2 signaling only partially reduces the induction of IFN β , Rip2 deletion completely abrogated IFN α and RANTES expression. These results can be explained by the structure of the IFN regulatory circuit. Initially, only IFN β is expressed, and subsequently IFN α and other interferon regulated genes (IRGs), such as RANTES, are induced via an Ifnar1 and Irf7-dependent autocrine/paracrine signaling pathway [214]. Thus, it appears that the decrease in IFN β expression that we observe is sufficient to severely impair downstream IRG induction, at least in this cell culture model.

Multiple steps of this pathway are likely to depend on stable ubiquitin modifications. Not only did we observe that Rip2 is polyubiquitinated upon infection, but we also found that a Rip2 point mutant that cannot be stably ubiquitin modified is unable to mediate IFN α/β induction in response to Mtb infection (Figure 26).

Collectively, these data suggest that polyubiquitinated Rip2 is required for Mtb-induced type I IFN expression via Irf5. Interestingly, MyD88-dependent activation of Irf5 involves formation of a tertiary complex that includes the E3 ubiquitin ligase, Traf6 [164,215]. This E3 ubiquitin ligase associates with Rip2 upon MDP stimulation, raising the possibility that a Rip2-Traf6-Irf5 complex might exist and that the activity of Irf5 might also be regulated by ubiquitin.

The specificity of the innate immune system has been shaped by the very powerful natural selection imposed by microbial pathogens. Our work suggests that upon infection with Mtb, a particularly potent form of MDP is translocated into the host cell cytosol where it triggers a novel signaling pathway leading to the robust induction of the type I IFN response. It is unlikely to be coincidental that the active component of our most potent adjuvant, complete Freund's adjuvant (CFA), consists of mycobacterial cell fragments. The specific pathway described in this work might play a major role in this adjuvant's effectiveness, since IFN α/β production is required for CFA to promote antigen-specific immune responses (55). Thus, while PAMPs are often regarded as invariant microbial components, it is clear that functionally important pathogen-specific differences exist in the composition of these molecules, and that the immune system can differentiate these subtly distinct structures.

Given the potent adjuvant activity of mycobacterial components, it is somewhat surprising that the attenuated vaccine strain *M. bovis* BCG, which produces the same PAMPs present in CFA, provides poor protection against pulmonary TB in adults

[216,217]. The lack of ESX1 function in this strain appears to be at least partially responsible, since the reconstitution of ESX1 improves the efficacy of this vaccine [218,219]. While this effect has previously been attributed to either the secretion of additional antigens or altered antigen presentation, it is also possible that ESX1 activity improves immunity by delivering crucial PAMPs into the cytosol where they are optimally recognized. Understanding both the details of PAMP trafficking, as well as the precise specificity of PAMP recognition, promises to aid in both the design of improved adjuvants and more effective tuberculosis vaccines.

Materials and Methods

Mice

C57BL/6 mice ages 8-12 weeks were obtained from the Jackson Laboratory. *rip2*^{-/-} mice were a kind gift from Dr. Vishva M. Dixit (Genentech, Inc. South San Francisco, CA). *nod2*^{-/-} mice were provided by Dr. Peter J. Murray (Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN). *Nod1* and *Nod2* double knockout macrophages were isolated from *nod1*^{-/-}*nod2*^{-/-} mice provided by Dr. Gabriel Nunez (University of Michigan Medical School, Ann Arbor, MI). *irf3*^{-/-}, *irf5*^{-/-}, *tbk1*^{+/+}*tnfr1*^{-/-} and *tbk1*^{-/-}*tnfr1*^{-/-} mice and their littermate controls were provided by Dr. Kate A. Fitzgerald (University of Massachusetts Medical School, Worcester, MA). Mice were housed under specific pathogen-free conditions, and in accordance with the University of Massachusetts Medical School, IACUC guidelines.

Bacteria

The WT strain of *M. tuberculosis* used in these studies was the H37Rv strain. All the mutants were derived from the wild type strain. Δ ESX-1 was obtained from D. Sherman (SBRI, Seattle, WA) [190]. Δ BioF, Δ Rv3616 and Δ Rv3616-complemented strains have been described previously [191,192]. TN::Rv1410 contains a *himar-1* transposon inserted at nucleotide #688 of the 1557bp predicted open reading frame [220]. All strains were cultured in 7H9 medium containing 0.05% Tween 80 and OADC enrichment (Becton Dickinson). Pre-titered stocks of *Listeria monocytogenes* strain

10403 stored at -80°C (kindly provided by Victor Boyartchuk) were recovered for 1hr at 37°C in 9ml of Tryptic Soy Broth (BD Biosciences). Bacteria were then washed and resuspended in PBS prior to infection.

Antibodies and Reagents

Anti-Rip2 (Rabbit) and anti-ubiquitin (Mouse) antibodies were obtained from Santa Cruz Biotechnology. Anti-Irf3 antibody was obtained from Zymed. Anti-Irf5 antibody was obtained from Abcam. Anti- β -actin antibody was obtained from Sigma. MDP was obtained from InvivoGen. Mouse TNF- α was obtained from Sigma. LPS derived from *Escherichia coli* strain 0111.B4 was purchased from Sigma, dissolved, treated with deoxycholate, and re-extracted with phenol/chloroform as described in [175]. The pannexin-1 mimetic blocking peptides panx1 (WRQAAFVDSY) and the scrambled peptide control were synthesized by GeneScript Corporation (Piscataway, NJ) and have been described previously [221]. Streptolysin O (SLO) a pore forming protein derived from *Streptococcus* and Adenosine 5'- triphosphate (ATP) were purchased from Sigma. *N*-glycolyl muramyl dipeptide (*N*-glycolyl MDP) was custom synthesized (Carbohydrate Synthesis, Oxford, UK) and shown to be more than 95% pure by NMR spectrometry. This preparation was found to be free of endotoxin contamination using the Limulus amoebocyte lysate assay (Pyrotell, Cape Cod Inc., MA).

Macrophage infections

Bone marrow from 8- to 10-week-old mice was harvested from femurs and differentiated into macrophages for 7 days in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% L929-cell conditioned medium, 10% fetal bovine serum, 2 mM L-glutamine and 1 mM sodium pyruvate. After 7 days in culture, bone marrow derived macrophages (BMDMs) were washed with phosphate-buffered saline (PBS) and seeded into tissue culture plates for infection. RAW 264.7 macrophage cell line was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. All Mtb strains were cultivated in 7H9 broth, grown to exponential phase and washed thoroughly in DMEM media prior to infection. Bacterial clumps were removed by passing the washed suspension through a 5 μ m syringe filter. For the peptide blocking studies, the cells were pre incubated with the desired peptides for 30 minutes followed by ATP or SLO for additional 15 minutes. Macrophages were infected at an MOI of 10 for 1 or 2 hours after which filtered cell lysates were immunoprecipitated with anti-Rip2 antibody (Santa Cruz). Heat inactivation was achieved by incubating the bacteria at 80°C for 30 minutes. Immortalized macrophage cell lines from wild type, *rip2*^{-/-}, *nod2*^{-/-} and *nod1*^{-/-}*nod2*^{-/-} mice were established by infecting bone marrow cells with a *v-raf/mil* and *v-myc* retrovirus in the presence of GM-CSF and polybrene [222,223]. These *rip2*^{-/-}, *nod2*^{-/-} and *nod1*^{-/-}*nod2*^{-/-} macrophage cell lines express CD11b and Gr-1 and are capable of phagocytosing antibody coated beads. To determine the effect of cytochalasin D on the phagocytic function of the

macrophages, we used the Vybrant phagocytosis assay kit to quantify the uptake of fluorescent *E. coli*. This assay was performed according to the protocol provided by the manufacturer.

Immunoprecipitation and Western Blot Analysis

For the immunoprecipitation and ubiquitination assays, cell lysates were prepared in radioimmune precipitation assay (RIPA) buffer (150mM NaCl, 50mM Tris-HCl (pH 7.5), 1% NP40, 0.25% deoxycholate, 0.1% SDS, 1mM EDTA), supplemented with protease inhibitors (Roche Applied Science) and 5mM N-Ethylmaleimide (Sigma), immunoprecipitated with anti-Rip2 antibody (Santa Cruz). Polyubiquitinated Rip2 proteins were detected by immunoblotting with an anti-ubiquitin antibody (Santa Cruz). Total immunoprecipitated Rip2 protein was measured by immunoblotting with anti-Rip2 antibodies (Santa Cruz).

Luciferase reporter assay

HEK293 cells (2×10^4) seeded in 96 well plates were transfected with 40ng of the IFN β luciferase reporter plasmid together with a total of 100 ng of various expression plasmids using GeneJuice (Novagen). The total amounts of transfected DNA were kept constant in all experiments by adjustment with empty vector. Luciferase activity was measured 24h later using Dual Luciferase reporter assay system (Promega). The *Renilla* luciferase gene (40ng) was co-transfected and used as an internal control plasmid. IFN β

luciferase reporter activity was normalized to *Renilla* luciferase reporter activity. Each experiment was repeated three times. Data are expressed as mean \pm s.d. of three replicates.

Real time quantitative PCR analysis

To measure IFN α/β mRNA levels upon MDP treatment or Mtb infection, total RNA was extracted from the macrophage cultures using Trizol reagent (Invitrogen) according to the manufacturer's directions. cDNA was prepared from 2 μ g of total RNA and quantitative real-time PCR performed using SYBR green as a label with the following primers: mIFN α -F, 5'-AAGATGCCCTGCTGGCTG; mIFN β -R, 5'-TTCTGCTCTGACCACCTCCC; mIFN β -F, 5'-CGTCTCCTGGATGAACTCCAC; mIFN β -R, TGAGGACATCTCCCACGTCA; β -actin-F, 5'-CGAGGCCAGAGCAAGAGAG; β -actin-R, 5'-CGGTTGGCCTTAGGGTTCAG; mTNF α -F, CAGTTCTATGGCCCAGACCCT; mTNF α -R, CGGACTCCGCAAAGTCTAAG; mRANTES-F, GCCCACGTCAAGGAGTATTTCTA; mRANTES-R, ACACACTTGGCGGTTTCCTTC. Results shown are representative of more than three separate infection experiments, with each PCR performed in triplicate. All values reported were in the linear range of the experiment and were normalized to β -actin values. Standard curves were generated by linear dilution of a cDNA sample generated from poly I:C-stimulated macrophages.

ELISA

IFN α protein in cell culture supernatants was performed using a custom ELISA as described previously [224]. IFN α concentrations were calculated using a recombinant IFN α (HyCult, Biotechnology, Uden, Netherlands) standard curve performed in quadruplicate using linear regression, and expressed in units per ml. IFN β protein in cell culture supernatants was measured similarly using a custom ELISA as described in [225].

Figures and Legends

Figure 14. Live, intracellular *Mycobacterium tuberculosis* stimulates Rip2 polyubiquitination. **A. Live, but not heat killed Mtb (Rv) stimulates Rip2 polyubiquitination.** Bone marrow-derived macrophages (BMDM) were infected with live wt (Rv) or heat killed (HK-Rv) strains of *M. tuberculosis* (strain H37Rv) for the indicated times. Polyubiquitinated Rip2 protein was detected by immunoprecipitating the cell lysates with an anti-Rip2 antibody followed by immunoblotting with an anti-ubiquitin antibody. Immunoprecipitates were also immunoblotted with a Rip2 antibody (lower panel) to insure that equal amounts of protein were immunoprecipitated. **B. The cell permeable mycotoxin Cytochalasin D inhibits Rip2 polyubiquitination upon Mtb infection.** The murine RAW 264.7 macrophage cell line was pretreated with Cytochalasin D for 1 hour at the indicated concentrations before being infected with wt Mtb H37Rv (Rv) for 1 hour. Polyubiquitinated Rip2 proteins were detected as described above. 1 μ M cytD caused a 95% decrease in phagocytosis, as described in the Materials and Methods section. **C. *Mycobacterium tuberculosis*-induced Rip2 polyubiquitination is Nod2-dependent.** Wildtype, *nod1/nod2*^{-/-} or *nod2*^{-/-} bone marrow-derived macrophages cell lines were generated (see material and methods) and either left uninfected (-) or infected with live wt *M. tuberculosis* (H37Rv) for the indicated times. Polyubiquitinated Rip2 proteins were detected as described above.

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Figure 15. Rip2 polyubiquitination upon Mtb infection requires ESX-1. The murine RAW 264.7 macrophage cell line was left uninfected (UI), infected with Mtb H37Rv (Rv), with ESX1 mutant strains of Mtb (Δ ESX1, Δ espA), with a complemented Δ espA-C strain, with the attenuated vaccine strain *Mycobacterium bovis* BCG or with the unrelated attenuated mutants Tn::rv1410 and Δ bioF. Cell lysates were immunoprecipitated with an anti-Rip2 antibody followed by immunoblotting with an anti-ubiquitin antibody. Immunoprecipitates were also immunoblotted with a Rip2 antibody to insure that equal amounts of protein were immunoprecipitated.



Figure 16. Membrane damage allows Nod2-mediated recognition of ESX1 mutants.

A. The mouse RAW 264.7 macrophage cell line was left untreated or pre-treated with either blocking (P) or control (scramble) peptide (S) for 30 minutes followed by ATP(5mM) and SLO(5 g/ml) for 15 minutes before infecting with ESX1 wt Mtb H37Rv (Rv), ESX1 mutant strains of Mtb ($\Delta espA$) or with a complemented $\Delta espA$ -C strain for 1 hour. Cell lysates were immunoprecipitated with an anti-Rip2 antibody followed by immunoblotting with an anti-ubiquitin antibody. Immunoprecipitates were immunoblotted with a Rip2 antibody to insure that equal amounts of protein were immunoprecipitated. **B. Relative abundance of Rip2 polyubiquitination in each sample as determined by densitometry of the results in panel A.** All values were calculated relative to the uninfected sample (UI).

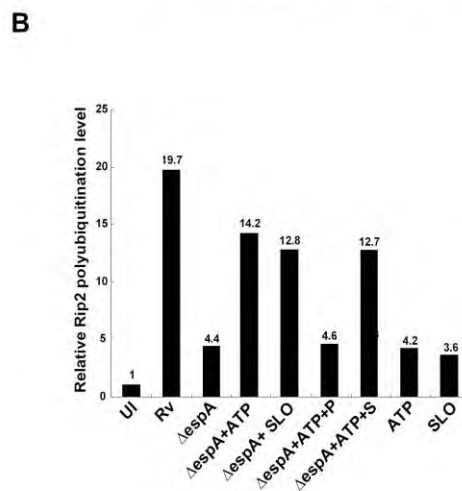
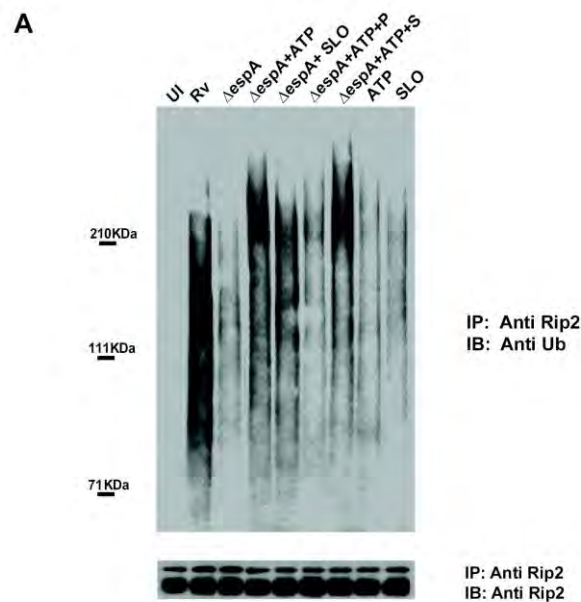


Figure 17. Type I Interferon production upon Mtb infection is reduced in Rip2- and Nod2-deficient macrophages. **A, B, E, and F.** BMDM derived from *wt*, *rip2*^{-/-} and *nod2*^{-/-} mice were left uninfected (UI) or infected with Mtb (MOI 10) for 4h. RNA was harvested, and IFN α , IFN β , RANTES and TNF α mRNA levels were quantified using real time PCR. Gene expression is reported as copy number per 1,000 copies of β -actin. Samples were assayed in triplicate; error bars represent the standard deviation. The experiment shown is representative of at least three. **C and D.** BMDM derived from *wt*, *rip2*^{-/-} and *nod2*^{-/-} mice were infected with Mtb (MOI 10) for 18h, the amount of IFN α and IFN β released in the supernatant was quantified by ELISA. Samples were assayed in triplicate; error bars represent the standard deviation. N.D. represents not detected, the actual value is below zero in standard curve.

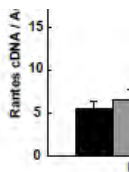


Figure 18. Type I Interferon production upon Mtb infection is reduced in Rip2- and Nod2-deficient macrophages but not in Nod1-deficient macrophages. BMDM derived from *wt*, *nod1*^{-/-}, *rip2*^{-/-} and *nod2*^{-/-} mice were left uninfected (UI) or infected with Mtb (MOI 10) for 4h. RNA was harvested, and IFN β mRNA levels were quantified using real time PCR. Gene expression is reported as copy number per 1,000 copies of β -actin. Samples were assayed in triplicate; error bars represent the standard deviation.

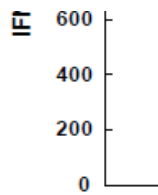


Figure 19. Multiple cytosolic pathways lead to IFN β induction. **A.** BMDM derived from *wt* and *rip2*^{-/-} mice were left uninfected (UI) or infected with ESX1 wt Mtb H37Rv (Rv) or with an ESX1 mutant (Δ ESX1) at an MOI of 10 for 4h. RNA was harvested, and IFN β mRNA levels were quantified using real time PCR. Gene expression of IFN β is reported as copy number per 1,000 copies of β -actin. Samples were assayed in triplicate; error bars represent the standard deviation. The experiment shown is representative of at least three. **B.** BMDM derived from *wt* and *rip2*^{-/-} mice were infected with ESX1 wt Mtb H37Rv (Rv) or with an ESX1 mutant (Δ ESX1) at an MOI of 10 for 4h. RNA was harvested, and RANTES mRNA levels were quantified using real time PCR. Gene expression of RANTES is reported as copy number per 1,000 copies of β -actin. Samples were assayed in triplicate; error bars represent the standard deviation. The experiment shown is representative of at least three.

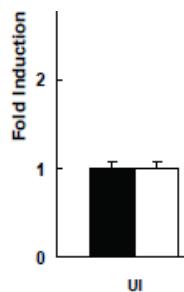


Figure 20. Nod2 stimulation is sufficient for type I IFN induction. A and B. N-Glycolyl MDP is more potent than the more common N-Acetylated derivative. The macrophage cell line RAW 264.7 was treated with the indicated concentrations of N-Glycolyl-MDP or N-Acetyl-MDP for 4h. RNA was harvested, and IFN β and RANTES mRNA levels were quantified using real time PCR. Gene expression of IFN β (A) and RANTES (B) were normalized to actin then normalized to untreated control for fold induction. Samples were assayed in triplicate; error bars represent the standard deviation. The experiment shown is representative of at least three. **C and D. The N-Glycolyl-MDP-induced type I IFN response is Rip2- and Nod2- dependent.** *Wt*, *rip2*^{-/-} and *nod2*^{-/-} transformed macrophage stable cell lines were treated for 4 hours with increasing concentrations of N-Glycolyl-MDP. RNA was harvested, and IFN β and RANTES mRNA levels were quantified using real time PCR. Gene expression of IFN β (C) and RANTES (D) were normalized to β -actin then normalized to untreated control to establish fold induction. Samples were assayed in triplicate; error bars represent the standard deviation. The experiment shown is representative of at least three.

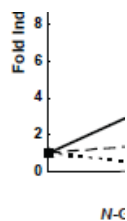


Figure 21. Mtb-induced type I IFN response is Tbk1-dependent and mediated through both Irf3 and Irf5. **A. *M. tuberculosis*-induced type I IFN response is Tbk1-dependent and only partially mediated through Irf3.** BMDM derived from *wt*, *irf3*^{-/-} and *tbk1*^{-/-}*tnfr1*^{-/-} mice and littermate controls were infected with ESX1 wt Mtb H37Rv (Rv) at an MOI of 10 for 4h. RNA was harvested, and IFN β mRNA level was quantified using real time PCR. Gene expression is reported as copy number per 1,000 copies of β -actin. Samples were assayed in triplicate; error bars represent the standard deviation. The experiment shown is representative of at least three. **B. Co-expression of RIP2 and IRF5 stimulate IFN β luciferase reporter activity.** HEK293T cells were co-transfected with IFN β -luciferase reporter plasmid (40ng) together with the indicated concentrations of MyD88, IRF5 and RIP2 expression plasmids. Luciferase activity was measured 24h later using Dual Luciferase reporter assay system (Promega). Renilla luciferase gene (40ng) was co-transfected and used as an internal control. Each experiment was repeated three times. Data are expressed as mean \pm s.d. of three replicates. **C. Co-expression of RIP2 and IRF3 does not stimulate IFN β luciferase reporter activity.** HEK293T cells were co-transfected with IFN β -luciferase reporter plasmid (40ng) together with the indicated concentrations of IRF5, IRF3 and RIP2 expression plasmids. Luciferase activity was measured 24h later using Dual Luciferase reporter assay system (Promega). Renilla luciferase gene (40ng) was co-transfected and used as an internal control. Each experiment was repeated three times. Data are expressed as mean \pm s.d. of three replicates. **D. Irf5 is required for an optimal type I IFN response upon Mtb infection.** BMDM from *irf5*^{-/-} or control littermates were infected with ESX1 wt Mtb H37Rv (Rv) at an MOI of 10, or with *Listeria monocytogenes* (Lm) strain 10403S (MOI 10) for 4 hours. RNA was harvested, and IFN β mRNA level was quantified by real time-PCR. Gene expression of IFN β is reported as copy number per 1,000 copies of β -actin. Samples were assayed in triplicate; error bars represent standard deviation. Data shown is representative of at least three independent experiments.

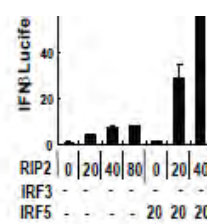


Figure 22. Mtb-induced type I IFN response is only partially mediated through Irf3. BMDM derived from *wt* and *irf3*^{-/-} mice were infected with ESX1 wt Mtb H37Rv (Rv) at an MOI of 1, 3 and 10 for 4h. RNA was harvested, and IFN β mRNA level was quantified using real time PCR. Gene expression is reported as copy number per 10,000 copies of β -actin. Samples were assayed in triplicate; error bars represent the standard deviation.

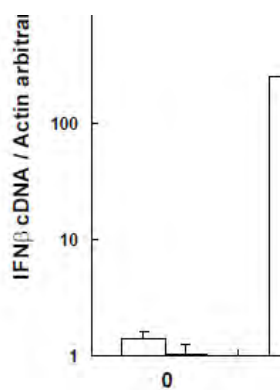


Figure 23. Irf5 is required for an optimal type I IFN α response upon Mtb infection. BMDM from *irf5*^{-/-} mice or control littermates were left uninfected (UI) or infected with ESX1 wt Mtb H37Rv (Rv) at an MOI of 10, or with *Listeria monocytogenes* (Lm) strain 10403S (MOI 10) for 4 hours. RNA was harvested, and IFN α mRNA level was quantified by real time-PCR. Gene expression of IFN α is reported as copy number per 1,000 copies of β -actin. Samples were assayed in triplicate; error bars represent standard deviation.

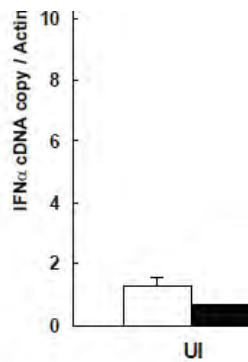


Figure 24. Irf3 and Irf5 expression levels in *irf3*^{-/-} and *irf5*^{-/-} macrophages. BMDM derived from *irf3*^{-/-} and *irf5*^{-/-} mice and their littermate controls were lysed in RIPA buffer and the Irf3 and Irf5 expression levels was determined by immunoblotting of anti Irf3 (Zymed) and Irf5 (Abcam) antibodies. Protein loading level was measured by β -actin antibody (Sigma).



Figure 25. The *N*-Glycolyl-MDP-induced type I IFN response is *Irf5*-dependent and *Irf3* independent. BMDM derived from *irf3*^{-/-} and *irf5*^{-/-} mice and their littermate controls were left untreated or treated for 6 hours with 10 μ g/ml of *N*-Glycolyl-MDP. RNA was harvested, and IFN β mRNA levels were quantified using real time PCR. Gene expression of IFN β is reported as copy number per 1,000 copies of β -actin. Samples were assayed in triplicate; error bars represent the standard deviation.

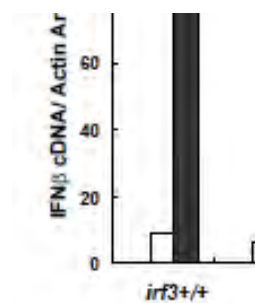


Figure 26. Rip2 polyubiquitination may be required for the Mtb-induced Type I IFN response. The *rip2*^{-/-} transformed macrophage cell line was infected with the retroviral vector alone or with retroviruses expressing wild type Rip2 or a form of Rip2 (K209R) that cannot be ubiquitin modified [104]. The *rip2*^{-/-} reconstituted macrophage cell lines were then infected with Mtb (MOI 10) for 4h. RNA was harvested, and IFN β mRNA levels were quantified using real time PCR. Gene expression is reported as copy number per 1,000 copies of β -actin. Samples were assayed in triplicate; error bars represent the standard deviation. Rip2 expression levels in each of the *rip2*^{-/-} reconstituted macrophage cell lines were examined by immunoblotting to insure that equivalent expression levels of Rip2 were achieved.

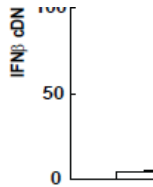
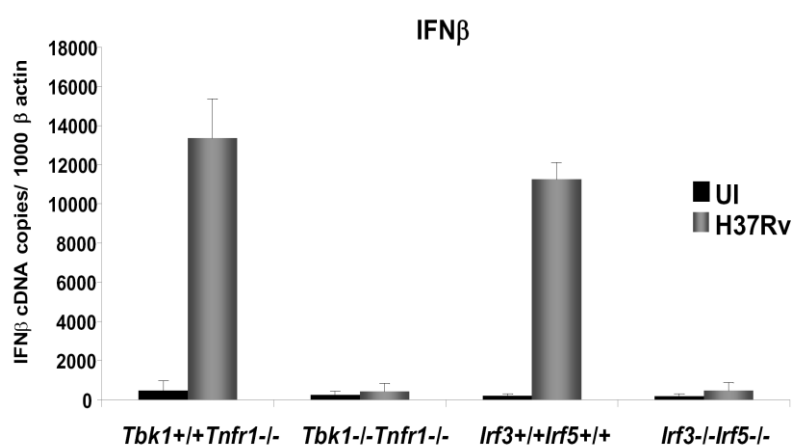


Figure 27. *M. tuberculosis* infection fails to elicit type I IFN production in the absence of IRF3 and IRF5. BMDM derived from *Irf3*^{+/+}*Irf5*^{+/+}, *Irf3*^{-/-}*Irf5*^{-/-}, *Tbk1*^{+/+}*Tnfr1*^{-/-} and *Tbk1*^{-/-}*Tnfr1*^{-/-} mice were left uninfected (UI) or infected with ESX1 wt Mtb H37Rv (Rv) at an MOI of 10 for 4h. RNA was harvested, and IFN β mRNA level was quantified using real time PCR. Gene expression is reported as copy number per 1,000 copies of β -actin. Samples were assayed in triplicate; error bars represent the standard deviation. The experiment shown is representative of at least three.



Chapter IV

Discussion

First discovered as the second member of the RIP protein family, Rip2 has critical functions in NF- κ B activation and cell death regulation. The original mouse knockout study implicated Rip2 in signaling from multiple TLRs including TLR 2, 3 and 4, the T cell receptor, as well as the cytosolic Nod1/2 pathways [64,102]. Our work in this thesis, as well as the work from other groups, demonstrates that Rip2 participates in the Nod-mediated innate immune response to pathogens, but does not mediate TLR or TCR signaling ([103], Figure 6). However, how Rip2 mediates these innate immune responses remains largely unclear.

The work described in this thesis demonstrates a crucial role for Rip2 in Nod2 mediated NF- κ B activation and induction of the Type I interferon response. We provide evidence that the Nod2 pathway is ubiquitin-regulated and that the K63-linked ubiquitin modification of Rip2 is required for cytosolic recognition of bacterial products. IKK activation in the Nod2 pathway is mediated by a complex consisting of Rip2, Ubc13/Uer1a, Traf6 and Tak1; whereas, the Rip2-mediated Type I interferon response depends on TBK1, and involves the stable ubiquitin modification of Rip2. We provide additional evidence for the expanding role of ubiquitin in the regulation of innate immune pathways. Polyubiquitination is not only important for terminating immune responses through the destruction of immune regulatory proteins, but also for initiating and amplifying immune responses, as exemplified by the role of K63-linked polyubiquitination in the activation of protein kinases and immune signaling in the NF- κ B and IRF pathway [14,226].

However, the precise mechanisms whereby polyubiquitinated Rip2 mediates Tak1 or IKK activation remain unclear. Our data suggests that Tak1 and IKK are activated by K63 specific ubiquitin chain attached in Rip2 protein in the Nod2-Rip2 pathway, consistent with a well established model that preferential binding to K63 polyubiquitin chains leads to the activation of Tak1, perhaps by triggering Tak1 autophosphorylation [14,226]. However, the role of K63-linked polyubiquitination chains in Tak1/IKK activation has been challenged recently. Several different groups show that the ubiquitin ligase complex LUBAC (linear ubiquitin chain assembly complex) composed of two RING domain proteins, RNF31 (also known as HOIP) and RBCK1 (also known as HOIL-1L); along with UbcH5C catalyze the synthesis of linear ubiquitin chains on the substrate. LUBAC has been shown to bind to NEMO in the IKK complex after TNF treatment and to catalyze the addition of linear ubiquitin chains onto lysine 285 and/or 309 of NEMO. Importantly, the involvement of LUBAC in NF- κ B activation was confirmed in genetically modified mice lacking the regulatory subunit HOIL-1L. TNF induced NF- κ B activation was impaired in primary hepatocytes and MEFs from HOIL-1L null mice [19]. Additional support for the linear ubiquitination model of IKK activation comes from work demonstrating that the UBAN (ubiquitin binding in ABIN and NEMO) motif of NEMO selectively binds linear ubiquitin chains, but not K63 ubiquitin chains. Binding of these linear ubiquitin chains to NEMO is hypothesized to trigger a conformational change in the IKK complex, resulting in IKK activation [20]. These studies suggest that in addition to K63-linked ubiquitin chains, linear ubiquitin

chains may also have specific signaling functions.

In contrast, work by Xia *et al* indicated that the RNF31-RBCK1 synthesized linear polyubiquitin chains failed to activate IKK or Tak1, and that only K63-linked polyubiquitin chains result in IKK and Tak1 activation [21]. Moreover, RNF31 or RBCK1 silencing in human cells had no effect on TNF induced IKK activation, and some studies found that under physiological conditions, NEMO preferentially binds K63 linked ubiquitin chains rather than linear ubiquitin chains [227]. Using an ubiquitin replacement strategy in human cells, only K63 linked ubiquitin chains, but not linear ubiquitin chains, appear capable of activating the IKK in the TNF pathway [227]. Crystal structure studies support a K63 polyubiquitination activation model, as they show that the Tab2 NZF domain specifically binds K63-linked di- and tri-ubiquitin [228]. Collectively, these published data support the K63 ubiquitination model of IKK activation.

In our studies, we demonstrated that the Rip2 protein is K63 ubiquitin modified in MDP treated cells (Figure 7). However, the contribution of the LUBAC system to Rip2 and NEMO ubiquitination has not been tested. The recent work done by Xu *et al* indicates that Rip1 was not modified by linear ubiquitin chains [227]. Using siRNA to knock down the expression of HOIP-1L, Xu *et al* failed to observe a change in Rip1 polyubiquitination. These data are consistent with the previous finding that the HOIP-HOIL-1L E3 complex does not ubiquitinate Rip1 in vitro [19]. However, it remains to be tested whether the HOIP-HOIL-1L E3 complex contributes to Nod-mediated NF- κ B or IRF5 activation and Rip2/NEMO ubiquitination.

Another unanswered question in Nod2 mediated signaling, is the precise role of the E3 ligase Traf6. We and others found that Traf6 contributes to Nod2 induced NF- κ B activation, and that MDP treatment stimulates formation of a complex consisting of Traf6, Rip2 and Tak1 (Figure 9 and 10, [113,172]). In Hek293 cells, Traf6 silencing impairs Rip2 polyubiquitination upon MDP treatment. But currently there is no evidence that Traf6 is the direct E3 ligase for Rip2. Additionally, the effect of Traf6 on Rip2 polyubiquitination has not been examined *in vivo* or in immune related cell types.

Two published studies have implicated other E3 ligases including cIAP1/cIAP2 and ITCH in Rip2 polyubiquitination [229,230]. It is possible that Rip2 may have multiple E3 ligases in the Nod pathway potentially for activation of different signaling pathways. Indeed, the E3 ligase ITCH was proposed to modify Rip2 and promote p38 MAPK and JNK activation, and to inhibit NF- κ B activation [230]. It is also possible that multiple E3s work together to achieve optimal ligase activity, which is the case for the Rip1 protein modification. Traf6 and the IAP family protein XIAP function collaboratively in the TGF β pathway [231,232]. Thus, Traf6, cIAP1/cIAP2 and ITCH may all contribute to Nod pathway regulation.

Similarly, multiple E3 ligases have been shown to regulate Rip1 polyubiquitination in the TNF and TLR pathways. The Kelliher lab, together with Vishva Dixit and colleagues, demonstrated that TNF induced Rip1 polyubiquitination is impaired in Traf2/5 deficient MEFs and in Traf2 silenced Hek293 cells [34,36]. However, several groups have since reported that cIAP1/2 is the physiological E3 ligase that promotes Rip1

ubiquitination [38,233,234]. Traf2 does not appear to be directly involved in the ubiquitin modification of Rip1, but may be required for the stable recruitment of cIAP1/2 to the TNFR1 complex. In this case, Traf2 mainly serves as a critical adapter protein recruiting cIAPs to Rip1 to promote Rip1 polyubiquitination. Recent structural studies have provided evidence that the ring domain of Traf2 lacks the interacting interfaces required for interaction with Ubc13 or UbcH5 yet retains the ability to bind cIAPs and Rip1[235]. Thus, Traf2 and Traf6 may function as scaffold proteins to bring cIAPs to Rip substrates.

In addition to Ubc13, another E2 enzyme Ubc5HA has been shown to promote K63-specific ubiquitination [227,236]. TNF-induced IKK activation requires Ubc5HA, which functions with the E3 cIAP1 to catalyze the polyubiquitination of Rip1 [227]. Ubc13, previously thought to be required for Rip1 polyubiquitination, does not appear to be required for NF- κ B activation in TNF pathway [227]. Although we did not address the contribution of Ubc5HA in our Nod2-Rip2 studies, it is possible that Ubc5HA could also contribute to Rip2 polyubiquitination and NF- κ B activation in the Nod pathway.

Although our initial studies revealed that the Nod2 pathway is ubiquitin regulated, whether this modification occurs *in vivo* under conditions of pathogen infection was unclear. Our studies using the bacterial pathogen *Mycobacterium tuberculosis* (Mtb) demonstrated that Rip2 is ubiquitin modified in Mtb infected cells and that Rip2 polyubiquitination is required for the host type I interferon response to Mtb. We showed that Mtb infection of macrophages triggers Rip2 polyubiquitination in a TLR and MyD88

independent manner (Figure 11 and 14), and that this stimulation is due to the ESX1-dependent entry of bacterial products into the cytosol where they are recognized by Nod2, implicating MDP as the relevant PAMP (Figure 15 and 16). Unexpectedly, Mtb infection stimulates IFN α/β production that is dependent on a novel pathway consisting of Nod2, Rip2, TBK1, and IRF5 (Figure 17 and 21). This work is the first to implicate NLRs in IRF activation and to suggest a role for Irf5 in anti-bacterial innate immune responses. Thus, the mammalian Nod2 pathway appears to be remarkably sensitive to mycobacterial MDP and responds to infection by triggering the production of type I interferon, which is responsible for a significant component of the transcriptional response to Mtb infection.

Infection with a variety of bacterial pathogens induces a type I IFN response, yet how this pathway is activated remains unknown. One current model suggests that bacterial DNA that translocates into the host cytosol is the major eliciting agent. This model is based largely on the observations that infection with *L. monocytogenes* or *L. pneumophilla*, or transfection of DNA into the cytosol induces a IFN β response that is TBK1- and IRF3-dependent [183]. Our results showed that both IRF3 and the related IRF5 transcription factor, previously shown to be activated by the MyD88-dependent TLR7 and TLR9 pathways [237], contribute to IFN β expression in Mtb infected cells (Figure 21 and 23). We provide genetic evidence that the Nod2-, Rip2- dependent type I interferon response is IRF5 -dependent and IRF3 independent (Figure 25). Although the majority of the IFN response may be through IRF3, a significant amount of the type I

interferon response to Mtb occurs via Rip2 and IRF5.

The Nod2-Rip2-IRF5 pathway differs from the response triggered by other bacteria such as *L. monocytogenes*, which depend entirely on IRF3. Thus, our data do not imply that Mtb is stimulating type I interferon in a fundamentally different manner from other bacteria. Instead, it is possible that the balance of these pathways might be affected by the activation state of the macrophage. Also, it is likely that bacterial pathogens stimulate the IFN response via multiple, partially redundant pathways, and that the relative importance of each is determined by the unique biology of the infection. In the case of Mtb, we speculate that the *N*-glycolylation of its peptidoglycan, and perhaps a paucity of other ligands such as DNA, favor recognition via Nod2. We demonstrated that the *N*-Glycolylated form of MDP is a more potent stimulator for Rip2 polyubiquitination and type I IFN response than the more common *N*-Acetylated derivative (Figure 20).

Are IRF3 and IRF5 the only IRFs required for Mtb induced type I IFN response? Our studies in IRF3/IRF5 double knockout macrophages demonstrated that Mtb infection fails to result in any type I interferon response in the absence of IRF3 and IRF5 (Figure 27). However, the nature of the Mtb ligand responsible for IRF3 activation in Mtb infected cells is unknown. We proposed that bacterial DNA translocated into the host cytosol might be the ligand that stimulates IRF3 activation; based on studies that transfection of *L. monocytogenes* DNA into the cytosol induces IRF3 activation [183]. If true, a specific cytosolic DNA sensor may contribute to type I interferon response.

A Rip2 point mutant that cannot be stably ubiquitin modified is unable to mediate IFN α/β induction in response to Mtb infection (Figure 26), suggesting that polyubiquitinated Rip2 is required for Mtb-induced type I IFN production. One possible mechanism is that Rip2 polyubiquitination may regulate TBK1 activity. Indeed, other studies have indicated that the TBK1 mediated type I IFN response is K63-ubiquitination regulated [238]. Traf3, a ring domain containing E3 ligase, is K63 specific and undergoes K63-linked auto-ubiquitination upon viral infection. K63 ubiquitin modified Traf3 recruits TBK1 in a ubiquitin dependent manner and activates TBK1. The deubiquitinase DUBA cleaves the K63 polyubiquitin chains on TRAF3, resulting in its dissociation from the downstream signaling complex containing TBK1 and terminating the type I IFN response upon viral infection. Thus K63 ubiquitin may be a general mechanism of kinase activation in the innate immune signaling pathway.

A ubiquitin-like domain (UBL) was identified in TBK1 and IKK ϵ . The TBK1 UBL domain binds to its kinase domain and is required for kinase activation. Deletion or mutations of the UBL in TBK1 impairs kinase activation, and results in an inability to phosphorylate IRF3 [239]. In our studies, we observed an interaction between TBK1 and polyubiquitinated Rip2 (data not shown), raising the possibility that Rip2 polyubiquitination triggers TBK1 recruitment and activation upon Mtb infection.

What is the biological role of Nod2-Rip2 induced Type I interferon response in Mtb pathogenesis? While the importance of IFN α/β in viral defense is clear and virtually universal, the role of type I interferon in antibacterial immunity appears to vary. Mice

deficient in the type I IFN receptor, *Ifnar1*, are significantly more susceptible to several Gram-positive and -negative bacterial infections [202,203,204,205], indicating that IFN α/β are important for immunity to many bacteria. However, an *Ifnar1*-deficiency has the opposite effect on the outcome of *L. monocytogenes* infection [206], suggesting that IFN α/β can also exacerbate disease.

The role played by IFN α/β in *Mtb* infection remains somewhat uncertain and there is conflicting evidence regarding the role of type I IFN in the infection. Initially, *M. tuberculosis* was shown to have a slight growth advantage in the lungs of *Ifnar1*^{-/-} mice following aerosol infection, although growth in other organs was not examined [240]. This study suggests a protective role for type I IFNs during infection with *M. tuberculosis*. Consistent with this, it was shown that signaling through the type I IFN receptor during *M. tuberculosis* infection of macrophages was required for the production of a number of immunologically important products, including inducible NO synthase, IFN- γ -inducible protein-10 (IP-10), RANTES, and IRG1 [139]. It has also been shown that *M. tuberculosis* may actively inhibit type I IFN signaling related to pathogenicity by increasing the association of protein tyrosine phosphatase 1c with the IFNAR1 [212]. However, other *Mtb* infection studies in *Ifnar1*^{-/-} mice showed increased splenic resistance to infection with decreased bacterial burden in the spleen [169]. Consistent with this, a hypervirulent clinical isolate of *M. tuberculosis* was found to induce significantly higher levels of type I IFN [210]. Moreover, exogenous addition of type I IFNs to macrophages infected with *Mtb*, enhanced bacterial growth, providing further

evidence for a detrimental role of type I IFNs during Mtb infection [209].

The ultimate influence of IFN α/β on Mtb infection appears to depend on a number of experimental factors, including host genetic background, bacterial strain, route of infection and dose. Most importantly, the effect of IFN α/β is most apparent after the onset of adaptive immunity and not before, suggesting that type I IFNs may instruct the priming and /or maintenance of the adaptive immune response, and perhaps control the differentiation of regulatory T cells [208].

Mtb infection of Nod2-deficient mice revealed similar bacterial counts but reduced inflammatory responses during the early phase of disease. However, Nod2-deficient mice succumbed to the disease at 6 months post infection with a half-log increase in bacterial load compared to wild-type controls, potentially due to an impaired adaptive response to Mtb [241]. This result, suggests that the Nod2 pathway has a protective role in Mtb pathogenesis and may promote the adaptive immune response. However, this delayed susceptibility phenotype could result from impaired cytokine production in Nod2 deficient mice upon Mtb infection, such as IL-12 and IL-6. The Rip2 deficient mouse susceptibility studies upon Mtb infection are ongoing in our lab.

The Nod2 pathway described in this work might play a major role in determining the effectiveness of tuberculosis vaccines, since Type I IFN production is required for complete Freund's adjuvant (CFA) to promote antigen-specific immune responses [55]. Our work suggests that a particularly potent form of MDP is translocated into the host cell cytosol upon Mtb infection, leading to robust induction of the type I IFN response.

This may at least be partially responsible for the poor protection against pulmonary TB in adults provided by the attenuated vaccine strain *M. bovis* BCG, which produces the same PAMPs present in CFA but lack ESX1 function, and is therefore unable to activate the Nod2 pathway or induce Rip2 polyubiquitination [216,217]. Understanding both the details of PAMP trafficking, as well as the precise specificity of PAMP recognition, promises to aid in both the design of improved adjuvants and more effective tuberculosis vaccines.

In addition, Rip2 polyubiquitination might serve as a sign of cytosolic Nod2 pathway activation. In vitro studies have identified a variety of pathogens that activate Nod2, including *S. pneumoniae*, *S. aureus*, *Salmonella typhimurium*, *L. monocytogenes*, *S. flexneri* and *Mycobacterium tuberculosis* [120,121,122,123]. Thus, it is reasonable to think other pathogens may also activate Nod2 *in vivo* and induce Rip2 polyubiquitination. A recent study has revealed that infection of HT-29 cells with *L. monocytogenes* also triggers Rip2 polyubiquitination [241]. This result correlates with the previous work that *L. monocytogenes* infection activates the Nod2 pathway *in vivo* and that Nod2^{-/-} and Rip2^{-/-} mice are more resistance to *L. monocytogenes* infection [64,100]. Indeed, our recently unpublished studies also suggest that *Neisseria Gonorrhoea* induces Nod2 activation and Rip2 polyubiquitination *in vivo*.

In the past few years, research has revealed that the Rip protein family integrates many diverse upstream signals and mediates MAPK, IKK or IRF activation. Collectively, they share the capacities to transmit intracellular responses to various cellular stresses.

Our work in this thesis presents a new regulatory mechanism where the Rip2 protein, similar to Rip1, is K63-linked polyubiquitination modified, and this modification is essential for a Rip2 mediated innate immune response. This study reveals critical roles for polyubiquitinated Rip proteins in innate immune recognition of bacterial and potentially viral pathogens. The E3 ubiquitin ligases or deubiquitinases that regulate innate immune responses may therefore serve as a new drug targets for human inflammatory disease.

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Appendix I

Additional data (*not shown* in Chaper II and III)

Figure 1. MDP induced NF- κ B activation requires the expression of Ubc-13. The mouse macrophage cell line RAW 264.7 was infected with lentiviruses containing shRNA sequences specific for Ubc13 or GFP as a control and stable puromycin resistant pools selected. These lines were left untreated or stimulated with MDP (10 μ g/ml) for the time periods indicated. Cells were lysed and NF- κ B activation was measured by immunoblotting with a phospho-I κ B α antibody (Cell Signaling Technology). Ubc-13 and β -actin levels were determined by immunoblotting with the indicated antibodies.

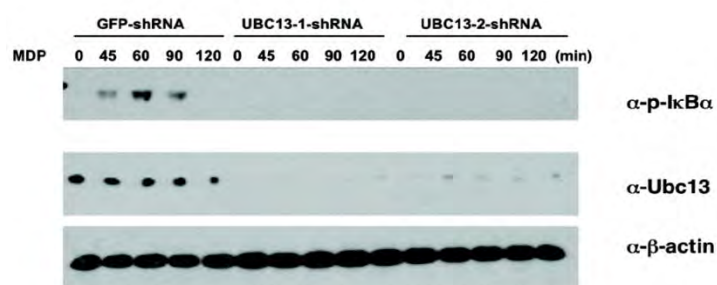


Figure 2. Expression of wild type NOD2, but not the Crohn's Disease associated alleles, stimulates the stable polyubiquitination of Rip2. A. NOD2 mutants associated with Crohn's Disease fail to stimulate Rip2 polyubiquitination. Omni-tagged wild type NOD2 or the Crohn's disease-associated NOD2 mutants L1007insC and D291N were expressed with FLAG-tagged Rip2 in the presence of HA-tagged ubiquitin in HEK293T cells. Rip2 was immunoprecipitated with an anti-Rip2 antibody (Santa Cruz), and western blots were performed with the indicated antibody. Expression levels of Rip2 and wild type NOD2 and mutants were measured by immunoblotting the total cell lysate with anti-FLAG or anti-Omni antibodies. **B. L1007insC interacts with Rip2 whereas D291N is impaired in its ability to stably interact with Rip2.** Omni-tagged wild type NOD2 and the CD-associated mutants L1007insC or D291N were expressed with Flag-tagged Rip2 in HEK293 cells. Wild type or CD-associated NOD2 alleles were immunoprecipitated with anti-Omni antibody followed by immunoblotting with an anti-Flag antibody. Expression levels of Rip2, wild type and mutant forms of NOD2 were monitored by immunoblotting the total cell lysate with anti-Flag or anti-Omni antibodies, respectively.

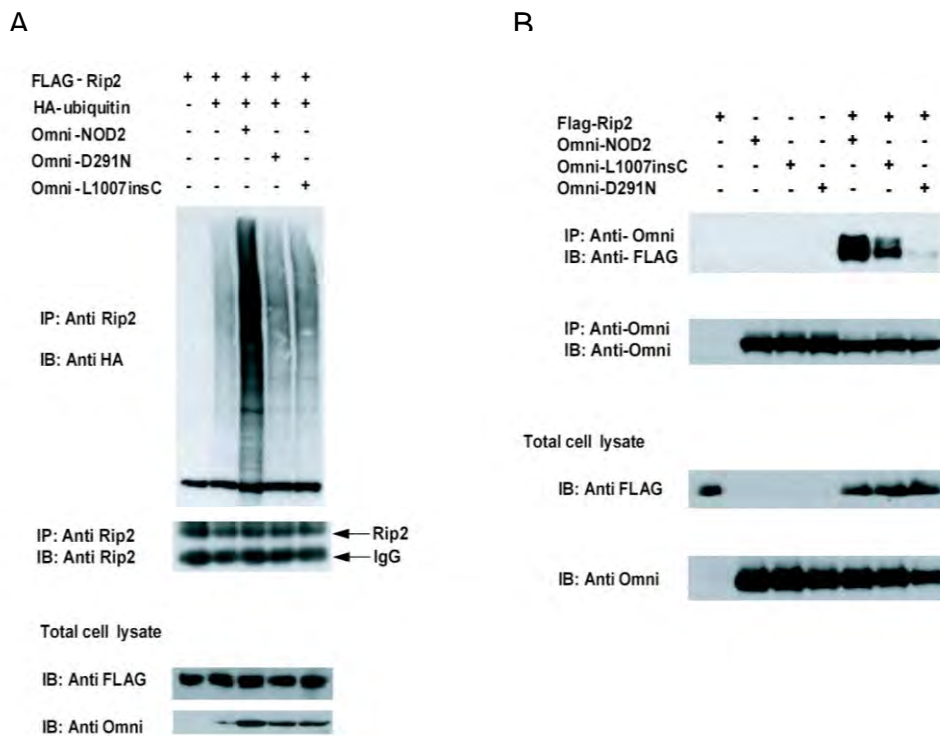


Figure 3. Co-expression of RIP2 and IRF5 stimulate ISRE luciferase reporter activity. HEK293T cells were co-transfected with ISRE-luciferase reporter plasmid (40ng) together with the indicated concentrations of IRF5 and RIP2 expression plasmids. Luciferase activity was measured 24h later using Dual Luciferase reporter assay system (Promega). Renilla luciferase gene (40ng) was co-transfected and used as an internal control. Each experiment was repeated three times. Data are expressed as mean \pm s.d. of three replicates.

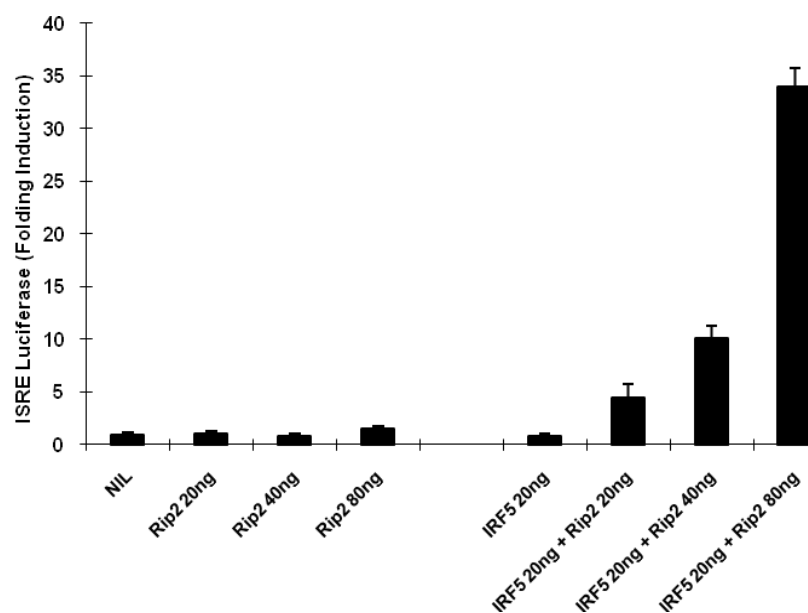


Figure 4. *Irf7* is not required for an optimal type I IFN response upon *Mtb* infection. BMDM from *Irf7*^{-/-} or control littermates were infected with ESX1 wt *Mtb* H37Rv (Rv) at an MOI of 10, or left uninfected for 4 hours. RNA was harvested, and IFN β mRNA level was quantified by real time-PCR. Gene expression of IFN β is reported as copy number per 1,000 copies of β -actin. Samples were assayed in triplicate; error bars represent standard deviation. Data shown is representative of at least three independent experiments.

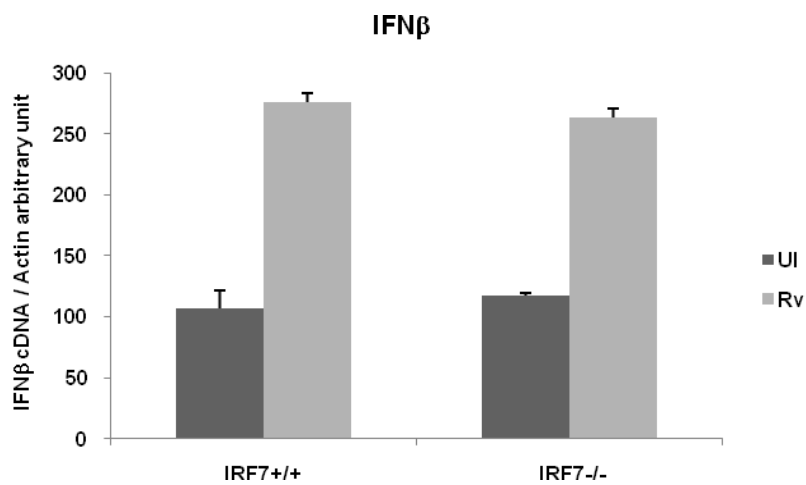
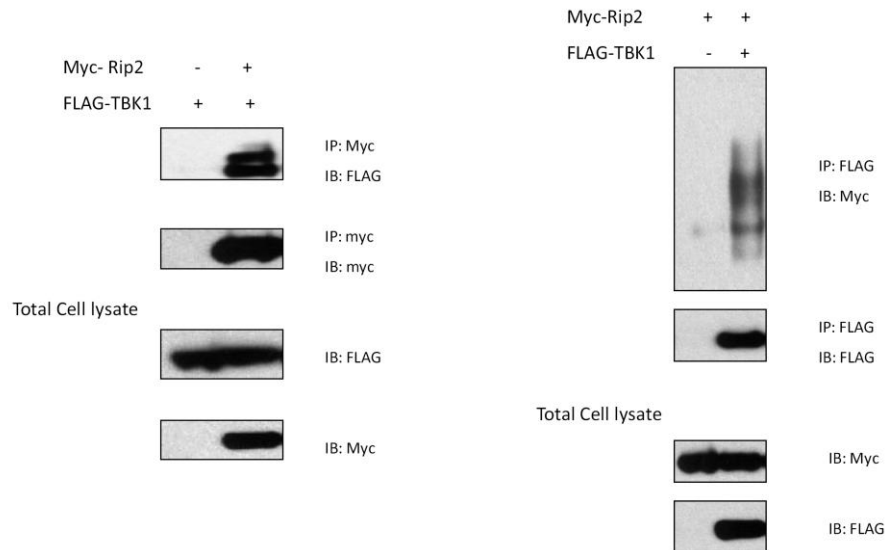


Figure 5. Rip2 and TBK1 interact in transfected cells. Flag-tagged TBK1 and Myc-tagged Rip2 were either co-expressed or expressed alone in HEK293T cells. TBK1 was immunoprecipitated with an anti-Flag antibody and Rip2 detected by immunoblotting with anti-Myc antibody or Rip2 was immunoprecipitated with an anti-Myc antibody followed by immunoblotting with anti-Flag antibody to detect associated TBK1 proteins. Relative expression of each construct was measured by immunoblotting the total cell lysate with anti-Myc or -Flag antibodies.



Appendix II

**A cytosolic NEMO/RIP1 complex recruits TAK1
to mediate the NF- κ B and p38 MAP
kinase/MAPKAP-2 responses to DNA damage**

Summary

Genotoxic stress triggers translocation of the regulatory subunit of the IKK complex NEMO I γ from the nucleus to the cytosol, following a series of post-translational modifications that lead to I κ B kinase activation. RIP1 has been implicated in this response and found modified in DNA damaged cells; however, the nature of the RIP1 modification and its precise role in the pathway remain unclear. Here we demonstrate that RIP1 and the ubiquitin-activated kinase TAK1 are principal mediators of the genotoxic stress response. We find RIP1 SUMO- and ubiquitin-modified in response to DNA damage and show that a RIP1 deficiency sensitizes cells to DNA damage due to an inability to induce survival gene expression. DNA damage stimulates TAK1 kinase activity and formation of a cytosolic complex containing ATM, NEMO I γ , RIP1 and TA 1. We demonstrate that a TA 1 deficiency or inhibition sensitizes tumor cells to DNA damage due to an inability to activate NF- κ B and the alternative DNA damage response pathway mediated by p38 MAPK-MAPKAP-2 kinases. This study has translational implications and reveals TAK1 as a potential therapeutic target in chemoresistance.

Word count: 180

Highlights

DNA damage stimulates formation of a cytosolic NEMO, RIP1, TAK1 and IKK β complex

RIP1 is SUMO-and ubiquitin-modified and TAK1 binds ubiquitinated RIP1

A TAK1 deficiency impairs the NF- κ B response and sensitizes tumor cells to DNA damage

TAK1 mediates the p38 MAPK/MK2 checkpoint in DNA damaged, p53-deficient tumor cells

Introduction

The DNA damage response activates cell cycle checkpoint and survival pathways that function to prevent DNA replication until damaged DNA is repaired. These pathways include the well-characterized ATM (Ataxia telangiectasia mutated)/CHK2 and ATR (Ataxia telangiectasia and Rad-3-related)/CHK1 pathways and the more recently identified ATM/ATR/p38MAPK/MAPKAP-2 checkpoint active in p53-deficient tumor cells (Lavin, 2008; Reinhardt et al., 2007). The transcription factor NF- κ B regulates apoptosis induced by genotoxic stress and is an attractive therapeutic target in tumor cells whose response to DNA damaging agents is impaired due to compromised p53 function. Moreover, constitutive NF- κ B activity is a hallmark of several cancers and mutations in NF- κ B pathway components have been associated with the activated B cell (ABC) subtype of diffuse large B cell lymphoma (DLBCL), breast cancer and multiple myeloma (Annunziata et al., 2007; Boehm et al., 2007; Demchenko et al., 2010; Hideshima et al., 2009). Thus the inclusion of NF- κ B inhibitors in cancer therapy could have anti-oncogenic activities as well as augment the tumor chemotherapeutic response.

NF- κ B is normally held in the cytoplasm in an inactive form bound to inhibitor proteins such as I κ B α . Diverse stimuli such as infection, pro-inflammatory cytokine production or treatment with agents that induce DNA damage illicit NF- κ B mediated transcriptional activity by activating the cytosolic IKK complex, consisting of IKK α and IKK β and a regulatory subunit designated IKK γ or NEMO (hereafter referred to as NEMO). IKK activation results in I κ B α phosphorylation, ubiquitination and subsequent degradation. The NF- κ B (p65/p50) heterodimer is then free to enter the nucleus and stimulate gene expression (Hayden and Ghosh, 2008).

DNA double strand breaks are also sensed by the poly (ADP-ribose)lating enzyme PARP-1. Upon recognition of DNA double strand breaks, PARP-1 catalyzes the attachment of poly(ADP-ribose) (PAR) chains to glutamic acid residues on PARP-1 itself, as well as other substrates. PAR-modified PARP-1 recruits the ATM kinase and the inhibitor of activated STATy (PIASy) which sumoylates the IKK regulatory subunit, NEMO at lysines K277 and K309 (Mabb et al., 2006; Stilmann et al., 2009; Wu et al., 2006b). DNA damage also stimulates interactions in the nucleus between NEMO, the receptor-interacting protein (RIP1) and the p53-induced protein with death domain (PIDD) (Janssens et al., 2005). PIDD has been shown to translocate to the

nucleus in response to genotoxic stress and either PIDD or RIP1 depletion abolishes DNA damage-induced NF- κ B activation and NEMO sumoylation (Janssens et al., 2005). Once sumoylated, NEMO is phosphorylated by ATM and mono-ubiquitinated potentially by the inhibitor of apoptosis protein, cIAP1 in the nucleus (Huang et al., 2003; Jin et al., 2009). These events trigger the translocation of ATM and NEMO into the cytosol, however the mechanism by which NEMO mono-ubiquitination leads to IKK activation is unknown.

Ubiquitin modification has been linked to cytosolic IKK activation by the pro-inflammatory cytokines IL-1 β and TNF- α . In the IL-1 β pathway, the E3 ubiquitin ligase TRAF6 and the UBC13/UEV1A E2 complex catalyze the K63-linked polyubiquitination of TRAF6, IRAK1 and NEMO and stimulate the recruitment of the ubiquitin-activated TAK1 kinase complex. The TAK1 kinase complex consists of TAB2 and TAB3 proteins that contain NZF-type ubiquitin binding domains that bind polyubiquitinated TRAF6 and NEMO (Ea et al., 2006; Kanayama et al., 2004). In the TNF pathway, the E3 ubiquitin ligases cIAP1/2 and TRAF2/5 mediate the K63-polyubiquitination of RIP1, TRAF2 and NEMO, which in turn recruits TAK1 and IKK complexes to the TNF receptor through the ubiquitin binding domains present in TAB2/3 and NEMO (Ea et al., 2006; Lee et al., 2004; Mahoney et al., 2008). Thus, in cytokine signaling, the polyubiquitin chains serve as a scaffold to recruit the IKK complex, allowing TAK1 to phosphorylate and activate IKK β . Deubiquitinases such as CYLD and A20 disassemble K63 polyubiquitin chains from the signal transducers to abrogate these inflammatory responses (Trompouki et al., 2003; Wertz et al., 2004). K63-polyubiquitin chains contribute to IKK and TBK-1 kinase activation in additional pathways including the T cell receptor, NOD-like receptors and RIG-I cytosolic receptors (Bhoj and Chen, 2009).

In the present study, we demonstrate that the NF- κ B response to DNA damage may also be ubiquitin-regulated and mediated by the ubiquitin-activated kinase TAK1. Our studies reveal that DNA damage stimulates TAK1 kinase activity and formation of a large cytosolic complex that includes NEMO, RIP1, TAK1 and IKK β . We reveal that RIP1, like NEMO, is SUMO-1 modified by the SUMO E3 ligase PIASy and that modified RIP1 forms a complex with NEMO, TAK1 and IKK β . RIP1 is also K63-ubiquitin modified in response to DNA damage and TAK1 binds polyubiquitinated RIP1 in DNA damaged cells. We provide evidence that a RIP1 or TAK1 deficiency abrogates NF- κ B activity and sensitizes mouse fibroblasts and multiple human tumor cell lines to DNA damaging agents. This study also reveals novel roles for TAK1 as a critical

mediator of the p38 MAP kinase/MAPKAP-2(MK-2) checkpoint in p53-deficient human tumor cells. Thus, TAK1 silencing or inhibition in human tumor cells interferes with both the NF- κ B- and p38 MAP kinase/MK2-mediated survival pathways, raising TAK1 as a potential therapeutic target in chemotherapeutic resistance.

Results

A Rip1- or Nemo-deficiency impairs the NF- κ B response and sensitizes MEFs to genotoxic stress

The RIP1 protein has been implicated in the NF- κ B response to DNA damage and its role in the DNA damage response has been shown to be independent of its kinase activity and effects on the TNF pathway (Hur et al., 2003; Janssens et al., 2005). Although implicated in DNA damage-induced NF- κ B activity, the exact function and relative position(s) of RIP1 in this NF- κ B pathway remain unclear. Previous studies suggested a nuclear role for RIP1, demonstrating that RIP1 is required for formation of the PIDDosome and the SUMO-modification of NEMO in DNA damaged cells (Janssens et al., 2005). Although not tested, these findings predict that cells deficient for RIP1 or NEMO would be equivalently sensitive to DNA damage. To test this prediction, we compared the sensitivity of Rip1-deficient and Nemo-deficient murine embryonic fibroblasts to doxorubicin treatment. We found Rip1-deficient and Nemo-deficient cells equivalently hypersensitive to doxorubicin treatment (Figure 1A), supporting the idea that both RIP1 and NEMO contribute to the DNA damage response. We also found I κ B α degradation impaired in both Rip1- and Nemo-deficient MEFs treated with doxorubicin (Figure 1B), consistent with published studies (Huang et al., 2002; Hur et al., 2003; Stilmann et al., 2009).

To determine why a Rip1 deficiency results in sensitivity to DNA damaging agents, we used gene expression profiling to compare the responses of wild type and Rip1-deficient MEFs to etoposide treatment. Ingenuity pathway analysis revealed a failure to induce survival gene expression when cells lacking Rip1 were exposed to DNA damage. Specifically, induction of *Xiap*, *Bcl211* (Bcl-xL), and *Birc3* (clap2) mRNAs was impaired in Rip1- as well as in Nemo-deficient cells (Figure 1C). Thus, the absence of Rip1 or Nemo, sensitizes cells to DNA damage due to a failure to activate NF- κ B and induce survival gene expression.

DNA damage stimulates the SUMO- and K63-ubiquitin modification of RIP1

Previous studies detected a modified form of RIP1 in etoposide-treated cells (Janssens et al., 2005). However, the precise nature and functional significance of the RIP1 modification(s) remain unclear. We detect a modified form of RIP1 in etoposide-treated cells that migrates at approximately 95-100 kDa. The 20-25 kDa increase in the apparent molecular weight suggested that RIP1, like NEMO, might be SUMO-1 (small ubiquitin-like modifier) modified in DNA

damaged cells. To determine whether DNA damage leads to RIP1 sumoylation, we treated U2OS cells with etoposide, immunoprecipitated RIP1 and examined the immunoprecipitates for the presence of sumoylated forms. We detected sumoylated forms of RIP1 40 min following etoposide treatment (Figure 2A, upper panel). Consistently, the detection of sumoylated RIP1 appears co-incident with phospho-I κ B α reactivity detected at 30 min following etoposide exposure (Figure 2A, lower panel). The SUMO ligase PIASy mediates NEMO sumoylation in response to genotoxic stress and RIP1 and NEMO interact in the nucleus of DNA damaged cells (Janssens et al., 2005; Wu et al., 2006a). To examine whether PIASy can function as a direct SUMO E3 ligase for RIP1, we introduced 2 individual PIASy siRNA oligonucleotides into U2OS cells and tested whether the etoposide-induced modification of RIP1 was affected. We were unable to detect sumoylated RIP1 in the etoposide-treated, PIASy-depleted cells, indicating that in response to genotoxic stress PIASy may modify both NEMO and RIP1 (Figure 2B, Supplementary Figure 1A and B).

In addition to the SUMO-1 modification, RIP1 was also found stably modified with K63-linked polyubiquitin chains (Figure 2C and D). K63-ubiquitin modified RIP1 was detected at 30 min following etoposide treatment and steadily increased for 2 hours, consistent with the maintenance of NF- κ B activity in DNA damaged cells (Figure 2A, lower panel). However, the amount of polyubiquitinated RIP1 was significantly less in DNA damaged cells, than in those treated with TNF- α for 10 min (Figure 2C), suggesting that only a fraction of the total RIP1 is modified in DNA damaged cells. These findings indicate that in addition to its nuclear role(s), RIP1 and potentially K63-ubiquitin-modified RIP1 may function in the cytosol to assemble an IKK activating complex in response to genotoxic stress.

DNA damage induces the formation of a large cytosolic complex containing ATM, NEMO, RIP1, TAK1 and IKK β

DNA damage stimulates the formation of complexes in the nucleus consisting of ATM, PARP-1, PIASy and NEMO as well as a PIDD, RIP1 and NEMO complex (Janssens et al., 2005; Stilmann et al., 2009). However, cytosolic complexes induced by DNA damage have been less well studied and as a result, how a nuclear damage signal is conveyed to the cytosolic IKK and MAK kinases remains unresolved. We utilized size exclusion chromatography and co-immunoprecipitation to uncover the cytosolic complexes induced in DNA damaged cells. Size

exclusion chromatography of cytosolic lysates from untreated or etoposide-treated cells revealed that DNA damage causes a shift in the migration of both NEMO and RIP1 into overlapping fractions corresponding to a MW of ~500-700 kDa (Figure 3A, fractions 17-21). In contrast to NEMO, only a fraction of the total RIP1 protein appears to shift to the higher molecular weight complex. We then examined the complexes for the presence of TAK1. TAK1 migrates in three apparent complexes in DNA damaged cells. One complex (fractions 17-21) migrates with an apparent molecular weight that overlaps with the NEMO- and RIP1-containing complex. To determine whether this complex contains NEMO, RIP1 and TAK1 in one complex, we immunoprecipitated TAK1 from fractions 17-21 and then examined the immunoprecipitates for the presence of NEMO and RIP1. In the untreated cells, interactions between RIP1, NEMO and TAK1 were not observed (Figure 3B). However, when these column fractions were immunoprecipitated from etoposide-treated cells, RIP1 and TAK1 and TAK1 and NEMO associations were readily detected in the cytosol (Figure 3B). Interestingly, the DNA damage-induced cytosolic complex that contains NEMO and TAK1 appears to also contain a modified form of RIP1 (Figure 3A, marked with asterisk). Further analysis revealed that the RIP1 associated with this complex ran as a smear on a 3-8% gradient SDS-PAGE gel, suggesting that the complex contains ubiquitinated forms of RIP1 (Supplementary Figure 2). We also examined the column fractions for the presence of the catalytic IKK subunit, IKK β . DNA damage induced a shift in IKK β into a similar sized complex, suggesting that DNA double strand breaks stimulate formation of a cytosolic complex consisting of RIP1, NEMO, TAK1 and IKK β (Figure 3A). These data are consistent with the hypothesis that DNA damage stimulates the SUMO- and ubiquitin-modification of RIP1, which in turn recruits and activates TAK1 and IKK β to achieve IKK activation.

To investigate whether K63-polyubiquitinated RIP1 triggers the recruitment of the ubiquitin-activated TAK1 kinase, we asked whether the mobility of the TAK1-associated RIP1 protein was altered in DNA damaged cells, as it is in TNF- α -treated cells (Kanayama et al., 2004; Lee et al., 2004). We immunoprecipitated TAK1 from cells treated with TNF- α for 10 min or from cells exposed to etoposide for 30 min to 2 hours. We then analyzed the nature of the RIP1 protein found associated with TAK1 by immunoblotting. Etoposide or TNF- α treatment stimulated TAK1 interactions with higher molecular weight forms of RIP1 (Figure 3C), consistent with our model that ubiquitinated RIP1 stimulates TAK1 recruitment and activation in

response to DNA damage. Consistently, expression of a K63-only form of ubiquitin is sufficient to trigger the association of TAK1 with RIP1 in the absence of additional stimuli, indicating that ubiquitinated RIP1 is a preferred TAK1 binding partner (Supplementary Figure 3).

We also prepared nuclear and cytoplasmic fractions from cells treated with etoposide for various times and examined each for the presence of ATM, PARP1 and CASPASE 3. Although ATM is predominantly a nuclear kinase, a small quantity of ATM can be detected in the cytosol (Figure 3D and Wu et al., 2006b). When TAK1 was immunoprecipitated from the cytosolic fractions, we found that etoposide treatment induced interactions between TAK1 and cytosolic ATM (Figure 3E). We then examined the nuclear and cytoplasmic extracts for the presence of RIP1. A transient and weak RIP1/NEMO interaction was observed in the nucleus of cells treated with etoposide for 30 min, similar to previously published work (Janssens et al., 2005). In contrast, a more robust and sustained RIP1/NEMO interaction was readily detected in the cytosol of doxorubicin- or etoposide-treated cells (Figure 3E and Supplementary Figure 4). Moreover, RIP1 also associated with TAK1, by co-immunoprecipitation (Figure 3E). Thus, TAK1 associates with ATM and RIP1, while RIP1 also interacts with NEMO about 1 hour following DNA damage (Figure 3E). The cytosolic gel filtration experiment suggests that these proteins associate in one complex. These interactions are co-incident with the induction of NF- κ B activity as measured by phospho-I κ B α reactivity or I κ B α degradation (Figure 2A, lower panel), suggesting that a cytosolic ATM, RIP1, NEMO and TAK1 complex recruits and activates IKK β .

DNA double strand breaks stimulate TAK1 kinase activity and TAK1 inhibition impairs the NF- κ B response and sensitizes cells to genotoxic stress

Treatment with the pro-inflammatory cytokines TNF- α or IL-1 β has been shown to stimulate TAK1 kinase activity, which can be detected using an *in vitro* kinase assay or by probing cell lysates with an anti-phospho-TAK1 antibody to measure TAK1 autophosphorylation (Singhirunnusorn et al., 2005; Xia et al., 2009). To determine whether DNA damage stimulates TAK1 kinase activity, we treated the human tumor cell line U2OS with doxorubicin, etoposide or γ -irradiation for the time periods indicated and measured anti-phospho-TAK1 levels. We detected a modest increase in phospho-TAK1 reactivity in doxorubicin-treated cells (Figure 4A). Treatment of U2OS cells with etoposide or γ -irradiation, treatments known to induce numerous DNA double strand breaks, significantly increased and sustained phospho-TAK1 reactivity;

indicating that like TNF- α treatment, DNA double strand breaks are potent inducers of TAK1 kinase activity (Figure 4A).

To further test the contribution of TAK1 to the genotoxic stress response, we examined NF- κ B activity and sensitivity to doxorubicin in Tak1-deficient, SV40T antigen immortalized MEFs. A Tak1-deficiency results in an inability to activate NF- κ B in response to TNF- α , IL-1 β or TLR3/4 ligands (Shim et al., 2005). We found the NF- κ B response to TNF- α and doxorubicin treatment impaired in the absence of Tak1 (Figure 4B) and found Tak1-deficient cells hypersensitive to doxorubicin treatment compared to wild type controls (Figure 4C; $p < 0.01$).

We then tested whether TAK1 kinase activity was required for the NF- κ B response to DNA damage. To examine a requirement for the kinase activity of TAK1, we pretreated cells with vehicle or with 5Z-7-oxozeaenol, a potent and highly selective inhibitor of TAK1 (Bioaustralis) (Takaesu et al., 2003; Windheim et al., 2007). The cells were left untreated, exposed to the proinflammatory cytokine TNF- α for 10 min or to doxorubicin for the time periods indicated. We examined the cell lysates for evidence of I κ B α degradation and measured cell viability. As expected, TNF- α or doxorubicin treatment induced I κ B α degradation in the vehicle treated cells. Pretreatment with the TAK1 kinase selective inhibitor 5Z7 reduced the I κ B α degradation observed in cells treated with TNF- α or doxorubicin (Figure 4D). Consistent with the impaired NF- κ B response, TAK1 kinase inhibition additionally sensitized the cells to doxorubicin-induced cell death (Figure 4E; $p < 0.05$), indicating that the kinase activity of TAK1 is required for NF- κ B activation and survival in response to DNA damage.

DNA damage fails to activate TAK1 or NF- κ B in the absence of ATM, NEMO or RIP1

The formation of a large cytosolic complex containing modified RIP1, NEMO, TAK1 and IKK β in DNA damaged cells reveals ubiquitin-modified RIP1 and the ubiquitin-activated kinase TAK1 as critical links between nuclear damage and cytosolic IKK activation. Our biochemical studies suggest that ATM, NEMO and RIP1 are sensors and transducers of the DNA damage signal and lie upstream of TAK1 and IKK- β . To genetically test this model, we asked whether *Atm*, *Nemo* or *Rip1* are each required for DNA damage-induced Tak1 activation. Wild type, *Atm*^{-/-}, *Nemo*^{-/-} and *Rip1*^{-/-} mouse embryonic fibroblasts were treated with the pro-

inflammatory cytokines IL-1 β or TNF- α for 10-15 min or exposed to etoposide for the time periods indicated. We measured Tak1 kinase activity using an *in vitro* kinase assay with a His-tagged, kinase-inactive MKK6 as a substrate. Additionally, the cell lysates were examined for phospho-Tak1 and phospho-I κ B α levels.

As expected, the IL-1 β or TNF- α -induced Tak1 kinase activity appeared unaffected by an Atm deficiency (Figure 5A and B) and consistent with the role of Nemo downstream of Tak1 in the IL-1 β pathway, similar phospho-Tak1 levels were detected in the IL-1 β -treated Nemo-deficient cells (Figure 5D). However, Tak1 kinase and NF- κ B activity was significantly reduced in the etoposide-treated fibroblasts lacking Atm, Nemo or Rip1 (Figure 5A-F). A Rip1 deficiency impaired the NF- κ B response to both TNF- α and etoposide, as reduced I κ B α and Tak1 phosphorylation levels were observed when Rip1-deficient MEFs or RIP1-silenced human tumor cells were treated with TNF- α or etoposide (Figure 5E, F). Collectively, these data support a model whereby Atm/Nemo and Rip1 are each required for Tak1 kinase activation and for an optimal NF- κ B response to DNA damage.

TAK1 silencing impairs the tumor NF- κ B response to multiple DNA damaging agents

Our studies in mouse fibroblasts implicate Tak1 in the NF- κ B response to genotoxic stress and raise the possibility that TAK1 may protect human tumor cell lines from the effects of DNA damaging agents and thereby contribute to chemotherapeutic resistance. To determine whether TAK1 silencing sensitizes human tumor cells to DNA damage, we transfected human U2OS cells with control or TAK1-specific siRNAs and then exposed the cells to the pro-inflammatory cytokine TNF- α or to the DNA damaging agents doxorubicin, etoposide or γ -irradiation. In the U2OS cells transfected with the control siRNA, TNF- α treatment or exposure to DNA damaging agents induced I κ B α phosphorylation and I κ B α degradation whereas, TAK1 depletion impaired the tumor NF- κ B response to TNF- α and to DNA damage-induced by doxorubicin, etoposide or γ -irradiation (Figure 6 A-C). We examined multiple human tumor cell lines including the human cervical adenocarcinoma cell line HeLa, the human breast adenocarcinoma cell line MCF7 and the human lung adenocarcinoma cell line H1299. We found that TAK1 silencing impaired the NF- κ B response to DNA damage in all the human tumor cell lines examined (Figure 6 and data not shown).

Failure to induce NF- κ B-dependent survival gene expression and sensitivity to DNA damage in RIP1- or TAK1-depleted human tumor cells

The impaired NF- κ B response to DNA damage predicts that TAK1 silencing should sensitize human tumor cells to DNA damaging agents. To test this prediction, we knocked down TAK1 expression in human U2OS cells and exposed the cells to DNA damaging agents. The TAK1-depleted tumor cells exhibited significantly more cell death than control cells treated with etoposide or exposed to γ -irradiation (Figure 7A and B; $p < 0.05$). Collectively, these data indicate that TAK1 contributes to the NF- κ B response to DNA damage and TAK1 depletion increases tumor sensitivity to treatment with chemotherapeutic agents or radiation.

We demonstrated that Rip1-, Nemo- and Tak1-deficient MEFs are sensitive to DNA damage due to an inability to induce NF- κ B-dependent gene expression (Figures 1 and 4). To determine whether TAK1- or RIP1-silencing in human tumor cells affects NF- κ B-dependent, survival gene expression, we quantified *XIAP* and *BCL2L1(BCL-xL)* mRNA levels in human tumor cells transfected with control, TAK1- or RIP1-specific siRNA oligonucleotides. Etoposide treatment of cells expressing the control siRNA stimulated a 2.5-fold increase in *XIAP* and *BCL2L1(BCL-xL)* mRNA levels at 30 min, followed by a 5-fold increase in mRNA levels at 1 and 2 hours. However, we observed little to no induction of *XIAP* or *BCL2L1(BCL-xL)* expression in the etoposide-treated RIP1- or TAK1-silenced cultures (Figure 7C and D), revealing that both RIP1 and TAK1 contribute to NF- κ B-mediated survival gene expression in tumor cells exposed to DNA damage.

Although our biochemical studies support a model whereby TAK1 lies downstream of the ATM/NEMO/RIP1 complex in this NF- κ B pathway, it remains plausible that RIP1 and TAK1 mediate independent survival pathways. To perform an epistatic analysis, we silenced RIP1 and/or TAK1 expression in HeLa cells and then measured sensitivity to DNA damaging agents. As in mouse fibroblasts and in the human U2OS tumor cell line, TAK1- or RIP1 depletion sensitized HeLa cells to doxorubicin treatment with an average of 47% viable cells (Figure 7E; TAK1-knockdown: $50.9 \pm 2.3\%$ RIP1-knockdown: $42.7 \pm 3.0\%$) recovered 12 hours following treatment, compared to $83.2 \pm 3.5\%$ viability in the control cultures. Silencing both TAK1 and RIP1 had no added effect on tumor cell viability following doxorubicin treatment (Figure 7E). Consistent with induction of apoptosis, PARP1 and CASPASE 3 cleavage was

detected in the RIP1-, TAK1- and in the RIP1- and TAK1-silenced cells at 12 hours, but not in doxorubicin treated control cells (not shown). The fact that RIP1 or TAK1 silencing had similar effects on cell viability and that no increase or acceleration in cell death was observed when both TAK1 and RIP1 were silenced supports a model where RIP1 and TAK1 function in the same pathway to mediate tumor cell survival.

TAK1 mediates the p38 MAP kinase-MK2 checkpoint in p53-deficient human tumor cells

The DNA damage response is primarily mediated by the kinases ATR and ATM, which signal to the checkpoint kinases CHK1 and CHK2. Yet in the absence of a functional ATM-CHK2 or an ATR-CHK1-TSP53 pathway, DNA damage activates a third pathway that involves ATM/ATR signaling to p38 MAP kinase which activates a newly identified checkpoint mediated by MAPKAP Kinase 2 (MK2) (Reinhardt et al., 2007). Tumor cells that inactivate p53 depend on this alternative pathway to mediate cell cycle arrest and survival following treatment with chemotherapeutic drugs that cause DNA damage. Pre-treatment with the p38 MAPK selective inhibitor SB203580 prior to the administration of DNA damaging agents abolishes MK2 activation and fails to result in cell cycle arrest (Reinhardt et al., 2007). Although this alternative DNA damage pathway is active in most p53-deficient human tumor cells, how DNA damage results in p38 MAP kinase activation was unknown. To investigate whether this alternative survival and repair pathway may be mediated by TAK1, we infected the p53-deficient, human lung adenocarcinoma cell line H1299 with a control GFP or a TAK1-specific shRNA lentivirus. Alternatively, we pre-treated H1299 tumor cells with the TAK1 selective kinase inhibitor prior to etoposide exposure. Both TAK1 depletion and TAK1 kinase inhibition impaired the p38 MAP kinase and JNK responses to DNA damage (Figure 8A and B and not shown). We then directly examined the cell lysates for evidence of MK2 activation. DNA damage-induced MK2 phosphorylation was significantly impaired in the TAK1-silenced cells, but not in the control cultures (Figure 8C), suggesting that TAK1 contributes to the p38 MAPK/MK2-mediated DNA damage response pathway.

To determine whether a TAK1-deficiency interferes with p38 MAPK/MK2 checkpoint signaling, we compared the cell cycle profiles of control or TAK1-silenced cells left untreated or treated with etoposide for 24 or 48 hours. Treatment of the control infected cells with etoposide for 24h led to the accumulation of cells with 4N DNA content, indicative of an intact checkpoint.

In contrast, when TAK1 expression is limiting, etoposide-induced cell cycle arrest is impaired with half as many cells detected with 4N DNA content (Figure 8D; Control: $70.3 \pm 3.7\%$ vs. TAK1-knockdown: $42.3 \pm 5.4\%$ in three independent experiments; $p < 0.05$). Consistently, an impaired p38 MAPK/MK2 response to DNA damage reduced phospho-CDC2 levels detected in the TAK1-depleted tumor cells compared to cells expressing the control GFP shRNA. These data demonstrate that TAK1 mediates the NF- κ B genotoxic stress response and the p38 MAPK-MK2 checkpoint. These findings have translational implications and raise the TAK1 kinase as a potential therapeutic target for tumors refractory to conventional chemo/radiation therapy.

Discussion

In the present study, we reveal how nuclear DNA damage signals are conveyed to the cytosolic IKK and p38 MAPK/MK2 kinases to mediate cell survival. We demonstrate crucial cytosolic roles for RIP1 and TAK1 in the NF- κ B response, demonstrating that ubiquitin-modified RIP1 triggers recruitment of the ubiquitin-activated kinase TAK1. These findings indicate that in addition to its nuclear role(s), RIP1 has cytosolic functions and contributes downstream of the nuclear ATM/NEMO/RIP1 complex. Our studies also reveal a requirement for TAK1 in the genotoxic stress-induced NF- κ B pathway and the alternative survival pathway mediated by the p38 MAPK/MK2 kinases. We demonstrate that DNA damage stimulates TAK1 kinase activity and importantly, that TAK1 kinase inhibition impairs both NF- κ B and p38MAP/MK2 kinase activation and thereby sensitizes p53-deficient human tumor cells to DNA damaging agents. These findings have implications for translational cancer research and predict that TAK1 kinase inhibition may sensitize p53-deficient, chemoresistant tumors to DNA damaging agents.

Our co-immunoprecipitation studies reveal that DNA damage stimulates interactions between ATM, NEMO, RIP1 and TAK1 in the cytosol of etoposide-treated cells. These findings led us to interrogate the nature of the cytosolic complexes formed in response to DNA damage. Size exclusion chromatography revealed that DNA damage causes shifts in RIP1, NEMO, TAK1 and IKK β proteins into overlapping fractions corresponding to a MW range of 500-700 kDa. Co-immunoprecipitation of column fractions confirmed that DNA damage induces formation of a NEMO, RIP1 and TAK1 complex. Interestingly, these studies suggest that ubiquitinated RIP1 shifts into the NEMO/TAK1 complex in response to DNA damage. Surprisingly, only a fraction of the total RIP1 protein appears ubiquitin-modified and shifts into the 500-700 kDa complex in DNA damaged cells (Figures 2C; 3A and 3C and Supplementary Figure 2). This is in contrast to the amount of polyubiquitinated RIP1 detected when the same cells are treated with TNF- α for 10 min (Figure 2C). The reasons for these differences are unclear, but may reflect the nature of the NF- κ B responses. Although the TNF-induced NF- κ B response is rapid and robust, occurring within minutes of receptor activation; DNA damaging agents induce a weaker, more sustained NF- κ B response, detected at 30 minutes and lasting several hours. The delay observed in the damage response may reflect the time required to relay the nuclear damage signal to the cytosol.

Whereas the duration of the DNA damage NF- κ B response may reflect how the pathway is negatively regulated. Cytokine-induced NF- κ B responses are terminated in part by the rapid de-ubiquitination of cytosolic components such as RIP1, TRAF6 and NEMO. In contrast, the DNA damage-induced NF- κ B pathway may be regulated in the nucleus potentially at the level of the SUMO proteases that control NEMO and/or RIP1 sumoylation.

We provide evidence that NEMO, RIP1 and TAK1 mediate NF- κ B activation and function to protect normal cells against DNA damage-induced cell death. Consistently, Rip1- and Nemo-deficient mouse embryonic fibroblasts fail to activate NF- κ B and were equivalently sensitive to doxorubicin-induced apoptosis (Figure 1A and B). In the absence of Rip1 or Nemo, we found the induction of anti-apoptotic gene expression (*Xiap*, *Birc3* and *Bcl2l1*) significantly impaired (Figure 1C). Similarly, in the absence of Tak1 or upon treatment with a Tak1-selective kinase inhibitor, DNA damage fails to activate NF- κ B and sensitizes SV40-immortalized, Tak1-deficient cells to DNA damage (Figure 4). The NF- κ B and cell death studies in genetically modified mouse fibroblasts support a model whereby cytosolic RIP1, NEMO and TAK1 function in the same pathway to mediate the NF- κ B response to DNA damage.

In addition to RIP1 and NEMO, the p53-induced protein with death domain (PIDD) was also shown to interact with RIP1 to facilitate NEMO sumoylation (Janssens et al., 2005). Yet in contrast to a Rip1-, Nemo- or Tak1-deficiency, Pidd-deficient fibroblasts appear to respond normally to DNA damage (Manzl et al., 2009). These findings indicate that either PIDD is not absolutely required for the NF- κ B response or that redundant pathways exist in certain cell types. Consistent with this idea, the requirement for PARylation in DNA damage-induced NF- κ B activation appears cell type-dependent (Stilman et al., 2009). In contrast to the Janssens' study which identified PIDD, RIP1 and NEMO in the nucleus of DNA damaged HEK293 cells, Stilman et al., failed to detect PIDD or RIP1 as components of the nuclear PARP-1 complex induced in mouse embryonic fibroblasts following γ -irradiation (Stilman et al., 2009). These seemingly disparate findings could indicate that the composition of the DNA damage-induced nuclear complexes vary among cell types or that the PIDD/RIP1/NEMO nuclear complex functions downstream of the PARP-1 signalsome.

Consistent with this model, the PARP-1 signalsome is detected 10 minutes after DNA damage, prior to the formation of the cytosolic RIP1, NEMO and TAK1 complex which

becomes detectable between 30 min and 1 hour following etoposide treatment (Stilmann et al., 2009) and Figure 3). Hence, a delay exists between PARP-1 signalsome (10 min) formation and RIP1 and NEMO sumoylation (>30 min), suggesting that sumoylation is downstream of the PARP-1 signalsome. This study detects modified RIP1 in the cytosolic complex coincident with the detection of phospho-I κ B α reactivity, thereby temporally linking the post-translational modification(s) of RIP1 to IKK activation (Figures 2A and 3).

In the present study, we reveal the nature of the RIP1 modifications observed in DNA damaged cells. We find RIP1 SUMO- and ubiquitin-modified and demonstrate that PIASy silencing impairs our ability to detect both sumoylated RIP1 and NEMO in etoposide-treated cells (Figure 2B and Supplementary Figure 1B). Moreover, we detect sumoylated RIP1 and NEMO at similar time points following DNA damage (Figure 2A and Supplementary Figure 1A), suggesting that PIASy modifies both NEMO and RIP1.

DNA damage also stimulates the K63-linked ubiquitin modification of RIP1, which in turn signals the recruitment of the ubiquitin-activated kinase TAK1. Consistent with this model, expression of a K63-only form of ubiquitin stimulates a Rip1/Tak1 interaction in unstimulated cells and TAK1 binds a modified form of RIP1 in DNA damaged cells (Supplementary Figure 3 and Figure 3C). Moreover, silencing of the E2 conjugating enzyme UBC13 responsible for K63-linked polyubiquitination diminishes the NF- κ B response to doxorubicin (Supplementary Figure 5). Collectively, these data reinforce a critical role for K63-ubiquitin in the stabilization and assembly of the cytosolic DNA damage response complex and reveal that this NF- κ B response utilizes ubiquitin-dependent signaling mechanisms to mediate cell cycle arrest and survival.

The sensitivity to DNA damage observed in mouse embryonic fibroblasts deficient for Nemo, Rip1 or Tak1 prompted us to test whether RIP1 and TAK1 contribute to the DNA damage response in human tumor cells. We demonstrate that RIP1- and/or TAK1-silencing in multiple human tumor cell lines results in an impaired NF- κ B-dependent, anti-apoptotic response and sensitivity to DNA damage. In addition to the DNA damage-induced NF- κ B response, our studies reveal that TAK1 also mediates the alternative p38 MAP kinase/MK-2 checkpoint found active in human tumor cells that lack functional p53 (Reinhardt et al., 2007). We demonstrate that TAK1 silencing or TAK1 kinase inhibition impairs both the p38 MAP kinase/MK2 and JNK responses to DNA damage, interferes with cell cycle arrest and sensitizes tumor cells to etoposide treatment.

The present study reveals the RIP1 and TAK1 kinases as critical mediators of the genotoxic stress response. Although the kinase activity of RIP1 has been shown to be dispensable for TNF- and DNA damage-induced NF- κ B activation (Hur et al., 2003; Lee et al., 2004; Ting et al., 1996), we demonstrate that TAK1 kinase activity is required for optimal NF- κ B, p38 MAP kinase/MK2 and JNK responses to DNA damage. Importantly, these findings have translational implications for chemoresistance and predict that TAK1 kinase inhibition may sensitize resistant, mutant p53 human tumor cells to chemo/radiation therapies.

Experimental Procedures

Cell culture and Biological Reagents

Targeted disruption of the *rip1* loci and generation of *rip1*^{-/-} mouse embryonic fibroblasts has been described previously (Kelliher et al., 1998). Nemo- and Tak1-deficient fibroblasts and fibroblasts from wild type littermate controls were generously provided by Manolis Pasparakis (Institute for Genetics, University of Cologne) and Sankar Ghosh (Columbia University, NYC), respectively. Human U2OS, HeLa, MCF7 and H1299 cell lines were obtained from ATCC and grown in DMEM containing 10% FBS. For some experiments, U2OS and H1299 cells were infected with a lentivirus expressing a control GFP- or TAK1-specific shRNA (Open Biosystems), selected with 2.0 μ g/ml puromycin and TAK1 protein levels determined by immunoblotting. Doxorubicin was obtained from Sigma and etoposide purchased from Alexis. TAK1 kinase inhibitor 5Z-7-oxozeaenol was obtained from Bioaustralis. Recombinant TNF- α and IL1- β was obtained from Sigma and eBioscience, respectively.

Preparation and Transfection of siRNA

siRNA oligonucleotides against human RIP1 (CCGACATTTCTGGCATTGAA) and (CAGCTTGATTTACGTCAGCCA), PIASy (AACAAAGACAGGTGGAGTTGAT) and (AAAAGCTGCCGTTCTTTAATA), UBC13 (GATCCGCACAGTTCTGCTATC) or TAK1 (UGGCUUAUCUUACACUGGA) were purchased from QIAGEN and Dharmacon respectively. A scrambled siRNA was purchased from Dharmacon. U2OS or HeLa cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and as described previously (Yang et al., 2007).

Western Blotting, Coimmunoprecipitation and Kinase Assays

The RIP1 and NEMO antibodies were obtained from BD Bioscience. The ATM antibody was obtained from Calbiochem. The UBC13 and SUMO-1 antibodies were purchased from Zymed and the β -actin antibody from Sigma. The Tak1, PIASy, IKK β , PARP-1, ubiquitin, I κ B α , p38 MAP kinase and JNK antibodies were all obtained from Santa Cruz Biotechnology. The phospho-specific antibodies for TAK1, MKK6, MK2, CDC2, I κ B α , p38 MAP kinase and

JNK were obtained from Cell Signaling Technology. Anti total MK2, CDC2, CASPASE-3 and MKK6 antibodies were obtained from Cell Signaling Technology. The K63-ubiquitin antibody was obtained from Millipore.

For coimmunoprecipitation, cells were lysed in an endogenous lysis buffer (0.5M Tris-HCl pH 7.6, 0.5M NaCl, 0.1M EDTA, 1% Triton X-100, 0.5M NaF, and 0.5M sodium pyrophosphate) supplemented with complete protease inhibitor cocktail (Roche).

For ubiquitination and sumoylation assays, cells were boiled 10 mins in 1% SDS before immunoprecipitation. Boiled lysates were diluted to 0.1% SDS with a modified RIPA buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1% NP40, 0.25% deoxycholic acid, 1mM EDTA, supplemented with protease inhibitors and 5mM N-ethylmaleimide (Sigma)). Cleared lysates were incubated overnight with anti-RIP1, anti-NEMO or anti-K63 ubiquitin antibody. Immunoprecipitates were washed 3 times with lysis buffer, separated by SDS-PAGE, transferred to nitrocellulose and analyzed by immunoblotting with an anti-ubiquitin, anti-SUMO-1 or anti-RIP1 antibodies.

For western blot analysis to detect either total protein or phosphorylated protein, cells were lysed in kinase lysis buffer (10mM/10mM KPO₄/EDTA pH 7.05, 5mM EGTA pH 7.2, 10mM MgCl₂, 50mM β-glycerophosphate pH 7.2, 0.5% NP-40, 0.1% Brij-35) supplemented with a protease inhibitor mixture (Roche), 1mM DTT, 1mM Na₃VO₄, 1mM PMSF.

For the TAK1 *in vitro* kinase assays, cell lysates were immunoprecipitated with an anti-TAK1 antibody and then incubated with protein A-conjugated beads. Immunoprecipitates were washed 3 times with lysis buffer and kinase reactions were allowed to proceed for 60 min at 30°C in 20 μl kinase buffer (20 mM HEPES pH 7.2, 10 mM MgCl₂, 0.1mg/ml BSA, 3mM 2-mercaptoethanol) containing 1μg of His-tagged-kinase inactive MKK6(K82A). The reaction samples were separated by SDS-PAGE and MKK6 phosphorylation determined by immunoblotting with a phospho-specific MKK6 antibody (Cell Signaling).

Biochemical Fractionation

Cytoplasmic and nuclear fractions were prepared as described in (Dignam et al., 1983). Briefly, treated or untreated U2OS cells were lysed in a cytosolic lysis buffer containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 300 mM sucrose, 0.5% NP-40, 10 mM KCl supplemented with DTT and protease inhibitors. Nuclei were pelleted by a short centrifugation and lysed in a

nuclear buffer containing 20 mM HEPES (pH 7.9), 100 mM NaCl, 0.2 mM EDTA, 20% glycerol, 100 mM KCl supplemented with DTT and protease inhibitors. Nuclear pellets were subjected to a freeze-thaw cycle, sonicated, and centrifuged to obtain a solubilized nuclear fraction. In coimmunoprecipitation and gel filtration chromatography experiments, both cytosol and nuclear fraction were added or diluted to 150 mM NaCl.

For gel filtration chromatography, U2OS cytosolic extracts were fractionated using FPLC protein purification system (Pharmacia) on a Superose 6 column (Amersham Biosciences) at 4°C equilibrated with buffer A (20 mM Hepes-KOH, 10 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT pH 7.9, 150mM NaCl and 0.5% NP-40). Cytosolic (5mg) extract was loaded and eluted from the column in buffer A at a flow rate of 0.4 ml/min and 500 l fractions were collected. The column was calibrated with gel filtration standards (Bio-Rad Laboratories).

Flow Cytometry

For cell cycle analysis, human U2OS or H1299 cells infected with lentiviruses expressing a GFP or TAK1-specific shRNA were left untreated or treated with etoposide for 24 and 48 hours. The cells were then fixed in 70% ethanol, stained with propidium iodide (PI), and analyzed for DNA content.

Real time quantitative PCR analysis

Total RNA was isolated from untreated and etoposide treated mouse wild type, RIP1- or NEMO-deficient cells or U2OS cells using Trizol reagent (Invitrogen) and cDNA was generated with Mo-MLV RT (Invitrogen). Real time PCR primers used in this study are listed in Supplementary Table 1. Product accumulation was monitored by SYBR green fluorescence, with the relative expression levels determined from a standard curve of serial dilutions of cDNA. All samples were normalized to β -actin values and results were displayed as fold induction from untreated control.

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Figure legends

Figure 1. A Rip1- or Nemo- deficiency impairs NF- κ B response and sensitizes cells to genotoxic stress. (A) Wild type, Rip1- or Nemo-deficient MEF were left untreated or treated with doxorubicin (2 μ g/ml) and cell viability determined at 24 and 48 hours. Samples were done in triplicate; error bars represent the standard deviation. Statistical evaluation was performed using an unpaired student's t test and * indicates $p < 0.05$. (B) DNA damage-induced NF- κ B activation is impaired in the absence of Rip1 and Nemo. Wild type, Rip1- or Nemo-deficient MEFs were left untreated, treated with TNF- α (10 ng/ml) for 10min or treated with doxorubicin (10 μ g/ml) for the time periods indicated. NF- κ B activity was monitored by immunoblotting with an I κ B α antibody. (C) NF- κ B-dependent survival gene expression is impaired in Rip1- and Nemo-deficient MEF. Wild type, Rip1-deficient and Nemo-deficient MEFs were left untreated or treated with etoposide (40 μ M) for 6 hours and *Birc3* (cIap2), *Xiap* and *Bcl2l1* (*Bcl-xL*) mRNA levels were quantified using real time PCR. Gene expression was normalized to β -actin then normalized to untreated control to estimate fold induction. Samples were assayed in triplicate; error bars represent the standard deviation.

Figure 2. DNA damage stimulates the SUMO- and ubiquitin-modification of RIP1. (A) DNA damage stimulates the SUMO-1 modification of RIP1. Human U2OS cells were left untreated or treated with etoposide (40 μ M) for the time periods indicated. Protein extracts were boiled and then immunoprecipitated with an anti-RIP1 antibody and sumoylated proteins detected by immunoblotting with a SUMO-1 antibody. (B) A PIASy-deficiency reduces SUMO-RIP1 levels in etoposide-treated cells. Human U2OS cells were transfected with control or PIASy-specific siRNAs and then left untreated or treated with etoposide (40 μ M) for one hour. Protein extracts were boiled and then immunoprecipitated with an anti-RIP1 antibody followed by immunoblotting with an anti-SUMO-1 antibody. (C) DNA damage stimulates RIP1 polyubiquitination. Human U2OS cells were left untreated or treated with TNF- α (10 ng/ml) for 10 min or etoposide (40 μ M) for the time periods indicated. Protein extracts were boiled and then immunoprecipitated with an anti-RIP1 antibody followed by immunoblotting with anti-ubiquitin. (D) DNA damage stimulates the K63 specific ubiquitin-modification of RIP1. Human U2OS

cells were left untreated or treated with human TNF- α (10ng/ml) for 10 min or etoposide (40 μ M) for the time periods indicated. Protein extracts were boiled and then immunoprecipitated with K63-ubiquitin-specific antibodies and analyzed by immunoblotting with an anti RIP1 antibody.

Figure 3. DNA damage induces the formation of a large cytosolic complex containing ATM, NEMO, RIP1, TAK1 and IKK β . (A) A RIP1/NEMO/TAK1/IKK β complex is detected in the cytosol of DNA damaged cells. U2OS cytosolic extracts were fractionated using FPLC protein purification system (Pharmacia) on a Superose 6 column (Amersham Biosciences). Fractions 10-37 were separated by SDS-PAGE and immunoblotted with NEMO, Rip1, Tak1 and IKK β antibodies. The column was calibrated with gel filtration standards (Bio-Rad Laboratories) (B). Fractions 17-21 from untreated or etoposide treated cells were immunoprecipitated with a Tak1 antibody followed by immunoblotting with either a RIP1, NEMO or Tak1 antibody. (C) TAK1 binds modified RIP1 in etoposide treated cells. Human U2OS were left untreated, treated with TNF- α (10ng/ml) or etoposide (40 μ M) and cell lysates were immunoprecipitated with a TAK1 antibody followed by immunoblotting with a RIP1 antibody. (D) ATM levels in the nuclear and cytosolic extracts were examined by immunoblotting with an ATM antibody. Lysates were also probed with anti-PARP, anti-caspase3 and β -actin antibodies to monitor the purity of the nuclear/cytosolic lysates and the total protein levels. (E) DNA damage stimulates interactions between ATM and TAK1, RIP1 and TAK1 and RIP1 and NEMO. Human U2OS cells were left untreated or treated with etoposide (40 μ M) for the time periods indicated. Nuclear and cytosolic extracts were immunoprecipitated with a TAK1 or NEMO antibody followed by immunoblotting with either an ATM or RIP1 antibody.

Figure 4. DNA double strand breaks stimulate TAK1 kinase activity and TAK1 inhibition impairs the NF- κ B response and sensitizes cells to genotoxic stress. (A) Multiple DNA damaging agents stimulate TAK1 kinase activity. Human U2OS tumor cells were left untreated or treated with doxorubicin (10 μ g/ml), etoposide (40 μ M) or exposed to 10Gy γ -irradiation for the time periods indicated. TAK1 activity was measured by immunoblotting with a phospho-specific TAK1 antibody. Total TAK1 levels were measured by immunoblotting with a TAK1 antibody. (B) NF- κ B response to genotoxic stress is impaired in Tak1-deficient MEFs. SV40T

antigen-immortalized wild type or Tak1-deficient murine embryonic fibroblasts were left untreated, treated with 10ng/ml TNF- α for 10 min or treated with 10 μ g/ml doxorubicin (Dox) for the time periods indicated and NF- κ B activity was measured by monitoring I κ B α degradation. Cell lysates were probed with anti- β -actin antibody to insure equal amounts of total protein were loaded. **(C)** Tak1-deficient MEFs are hypersensitive to doxorubicin treatment. Wild type or Tak1-deficient MEFs were treated with 2 μ g/ml doxorubicin for 24 and 48 hours and viable cells were quantified by trypan blue exclusion. Samples were assayed in triplicate; error bars represent the standard deviation. The experiment shown is representative of at least three. Statistical evaluation was performed using an unpaired student's t test and ** indicates $p < 0.01$. **(D)** Treatment with a TAK1 kinase inhibitor impairs the NF- κ B response to DNA damage. Human U2OS cells were pretreated with the TAK1 kinase inhibitor 5Z7 (Bioaustralis) (1 μ M) or DMSO for 2 hours. The cells were left untreated or treated with doxorubicin (10 μ g/ml) for the time periods indicated or treated with TNF- α (10ng/ml) for 10 min. NF- κ B activity was measured by immunoblotting the cell lysates with an I κ B α antibody. Total protein levels were determined by immunoblotting with an anti- β -actin antibody. **(E)** TAK1 kinase inhibition sensitizes human tumor cells to doxorubicin treatment. Human MCF7 tumor cells were pretreated with the TAK1 kinase inhibitor 5Z7 (1 μ M) or DMSO control for 2 hours. Cells were then left untreated or treated with doxorubicin (10 μ g/ml) for 24 and 48 hours. Viable cells were quantified by trypan blue exclusion. Samples were assayed in triplicate; error bars represent the standard deviation. The experiment shown is representative of at least three. Statistical evaluation was performed using an unpaired student's t test and * indicates $p < 0.05$.

Figure 5. DNA damage fails to activate TAK1 or NF- κ B in the absence of ATM, NEMO or RIP1. Murine embryonic fibroblasts from *Atm*^{-/-}, *Nemo*^{-/-} or *Rip1*^{-/-} mice and control littermates were left untreated, treated with TNF- α or IL-1 β (10ng/ml) for 10 min or treated with etoposide (40 μ M) for the time periods indicated. TAK1 was immunoprecipitated from the cell lysates and TAK1 kinase activity measured using an *in vitro* kinase assay with a His-tagged, kinase-inactive MKK6 as a substrate. MKK6 phosphorylation was then measured using a phospho-specific MKK6 antibody as described in (Xia et al., 2009). Total MKK6 input levels were measured by immunoblotting with an anti-MKK6 antibody **(A and C)**. Alternatively,

TAK1 activity was measured by immunoblotting cell lysates with a phospho-specific TAK1 antibody (**B**, **D** and **E**). Total TAK1 levels were measured by immunoblotting with a TAK1 antibody. (**F**) RIP1 is also required for TAK1 and NF- κ B activation in etoposide-treated human tumor cells. To determine if acute RIP1 ablation altered the tumor NF- κ B response, human U2OS cells were transfected with a control or RIP1 specific siRNA oligonucleotides. Forty-eight hours later, cells were treated with human TNF- α for 10 min or with etoposide for the time periods indicated and phospho-TAK1 and -I κ B α reactivity measured. TAK1 and RIP1 levels were measured by immunoblotting.

Figure 6. TAK1 silencing impairs the tumor NF- κ B response to multiple DNA damaging agents. Human U2OS tumor cells were transfected with a control siRNA or a TAK1 siRNA, cells were left untreated or treated with doxorubicin (10 μ g/ml) (**A**), or etoposide (40 μ M) (**B**). Alternatively, human U2OS tumor cells were infected with a control GFP shRNA or TAK1-specific shRNA lentivirus and following selection, cells were left untreated or exposed to 10Gy γ -irradiation (**C**). Cells were lysed and NF- κ B activity measured by immunoblotting with phospho-I κ B α antibody or total I κ B α antibody. To monitor knockdown and to insure that equal amounts of total protein are compared, the cell lysates were probed with an anti-TAK1 or anti- β -actin antibody. Numbers under the blot indicate the relative abundance of phospho-I κ B α or total-I κ B α in each sample compare to β -actin as determined by densitometry.

Figure 7. Failure to induce NF- κ B-dependent survival gene expression and sensitivity to DNA damage in RIP1- or TAK1-depleted human tumor cells. (**A** and **B**) A TAK1 deficiency sensitizes human tumor cell lines to etoposide treatment or γ -irradiation. Human U2OS cells were infected with a control GFP shRNA or TAK1-specific shRNA lentivirus and following selection, cells were left untreated, treated with etoposide (40 μ M) for 24 and 48 hours, or exposed to 10Gy γ -irradiation. Viable cells were quantified by trypan blue exclusion. Samples were assayed in triplicate; error bars represent the standard deviation. The experiment shown is representative of at least three. Statistical evaluation was performed using an unpaired student's t test and * indicates $p < 0.05$. (**C** and **D**) DNA damage fails to induce expression of NF- κ B-dependent survival genes in TAK1- or RIP1-silenced human tumor cell lines. Human U2OS cells

were transfected with a control siRNA, a TAK1 siRNA or a RIP1 siRNA for 48 hours. Cells were then left untreated or treated with etoposide (40 μ M) for 0.5, 1 or 2 hours and RNA was harvested. *XIAP* and *BCL2L1(BCL-xL)* mRNA levels were quantified using real time PCR. Gene expression is reported as copy number per 1,000 copies of β -actin. Samples were assayed in triplicate; error bars represent the standard deviation. **(E)** A RIP1 and TAK1 deficiency does not result in increased doxorubicin sensitivity. Human HeLa cells were left untransfected or transfected with a control siRNA, a TAK1 siRNA, RIP1 siRNA or TAK1 plus RIP1 siRNA oligonucleotides for 48 hours. Cells were then left untreated or treated with doxorubicin (2 μ g/ml) for 12 and 24 hours. Viable cells were quantified by trypan blue exclusion. Samples were assayed in triplicate; error bars represent the standard deviation. Statistical evaluation was performed using an unpaired student's t test and * indicates $p < 0.05$, ns indicates not significant. **(F)** RIP1 and/or TAK1 knockdown efficiency was monitored by immunoblotting the cell lysates with RIP1 or TAK1 antibodies.

Figure 8. TAK1 mediates the p38 MAP kinase-MK-2 checkpoint in p53-deficient human tumor cells. **(A and C)** A TAK1 deficiency impairs p38 MAP kinase, MK2 and JNK activation in response to DNA damage. Human U2OS cells were infected with a control GFP or TAK1 shRNA lentivirus. The cells were left untreated, treated with TNF- α (10ng/ml) for 10 min or treated with etoposide (40 μ M) for the time periods indicated. Cell lysates were probed with a phospho-specific p38 MAP kinase, -MK2 or -JNK1/2 antibodies. Total p38 MAP kinase, MK2 and JNK1/2 levels were measured by immunoblotting the cell lysates with total p38 MAP kinase, MK2 or JNK1/2 antibodies. **(B)** Inhibiting TAK1 kinase activity impairs the p38 MAP kinase and JNK1/2 response to DNA damage. U2OS cells were pretreated with vehicle (DMSO) or the TAK1-selective kinase inhibitor 5Z7 (Bioaustralis) and cells were left untreated, treated with TNF- α for 10 min or treated with etoposide for various time periods. JNK and p38 MAP kinase activation was measured by immunoblotting the cell lysates with phospho-JNK1/2 or phospho-p38 MAP kinase antibodies. Total p38 MAP kinase and JNK1/2 levels were measured by immunoblotting the cell lysates with p38 MAP kinase or JNK1/2 antibodies. **(D)** Impaired cell cycle arrest upon DNA damage in TAK1-deficient, p53-deficient human tumor cells. H1299 lung adenocarcinoma cells were infected with a control GFP shRNA or TAK1 shRNA lentivirus and selected cells were left untreated or treated with etoposide (40 μ M) for 24 or 48

hours. Cell cycle analysis was performed by staining the cells with PI followed by flow cytometry. To confirm the flow cytometry data, phospho-CDC2 levels were compared by immunoblotting the cell lysates with phospho-specific CDC2 antibodies. Total protein levels were determined by immunoblotting with an anti- β -actin antibody.

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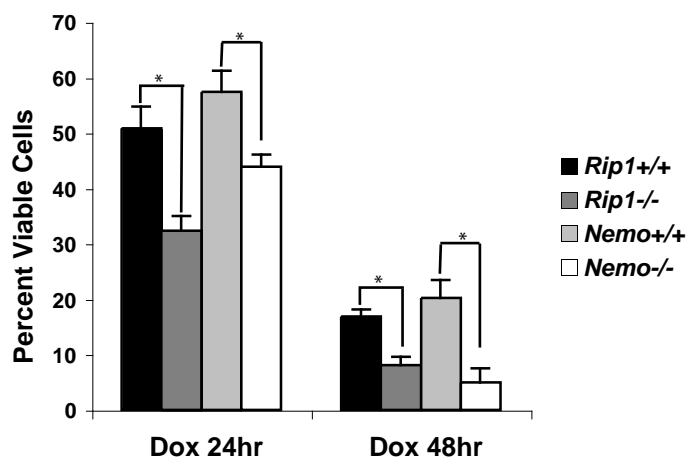
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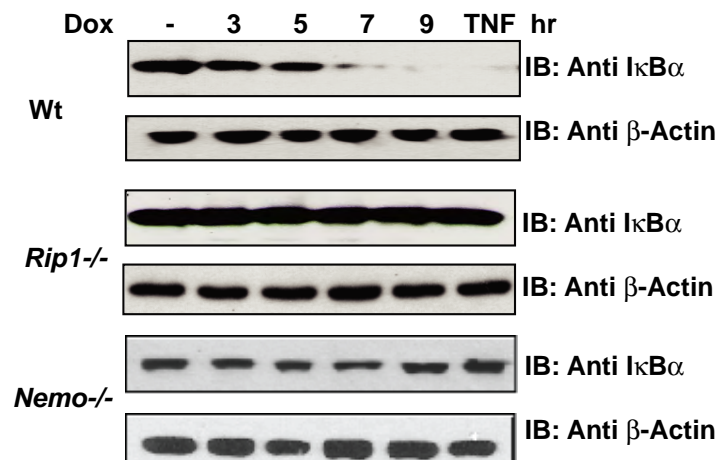
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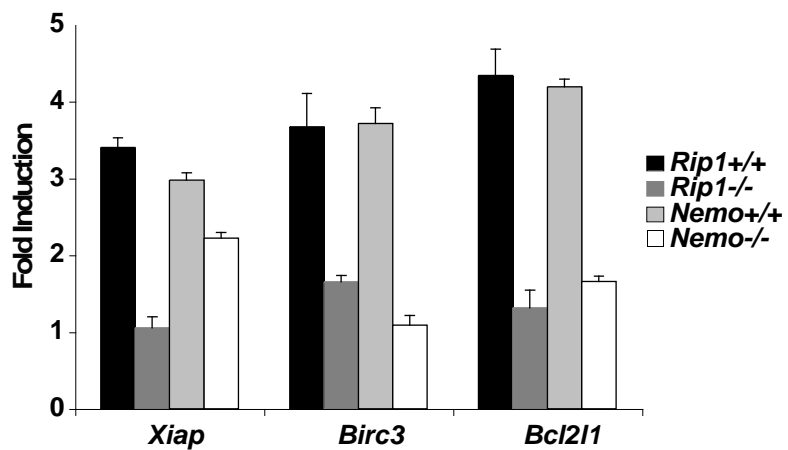
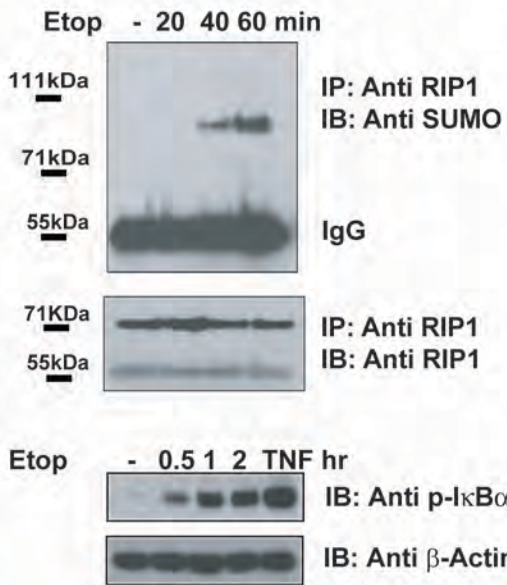
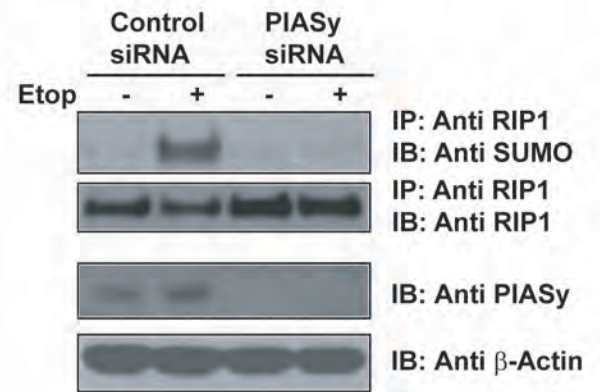


Fig.2

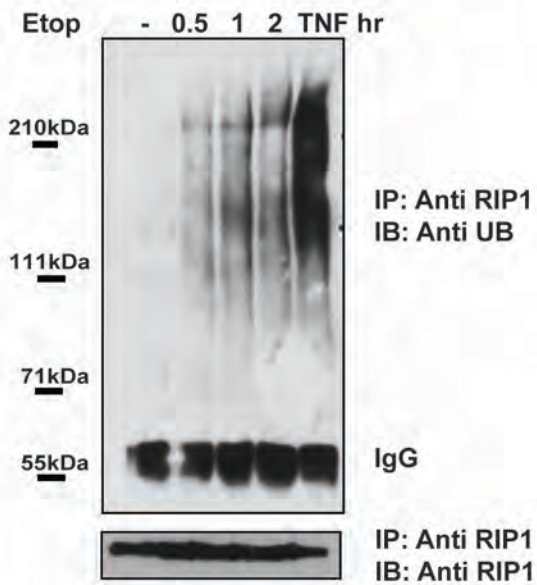
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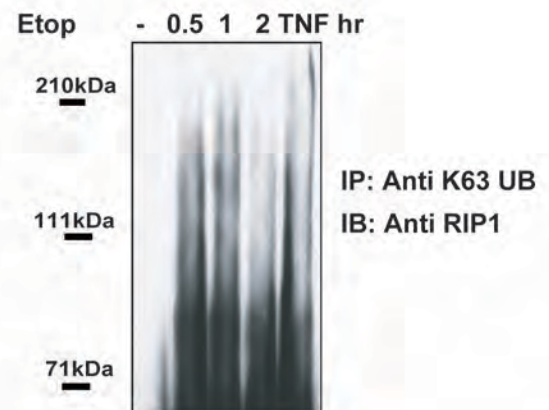
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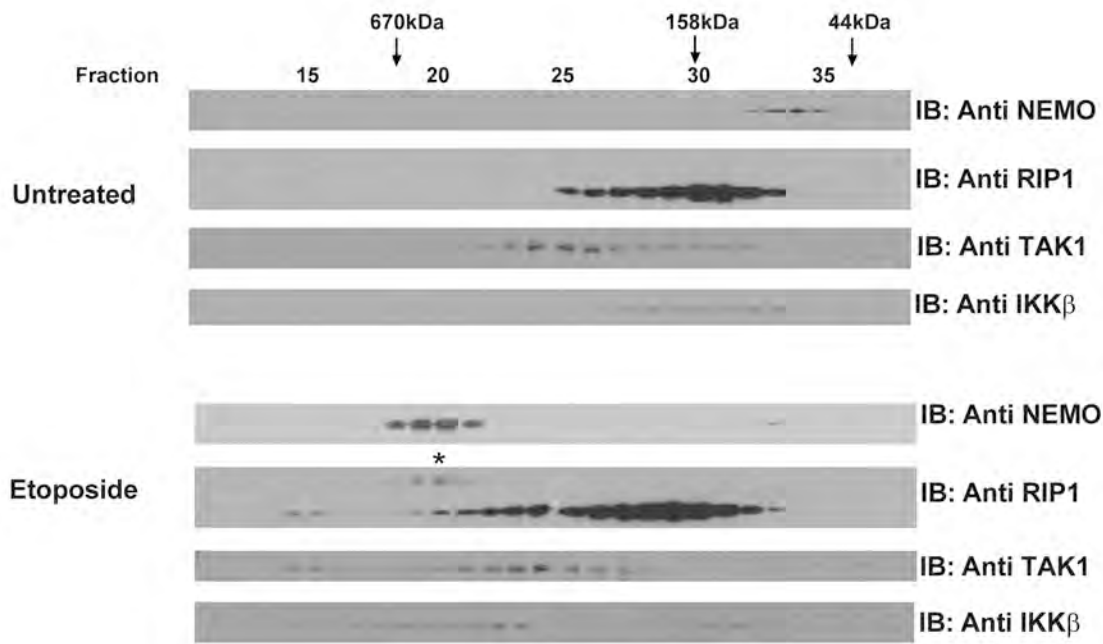
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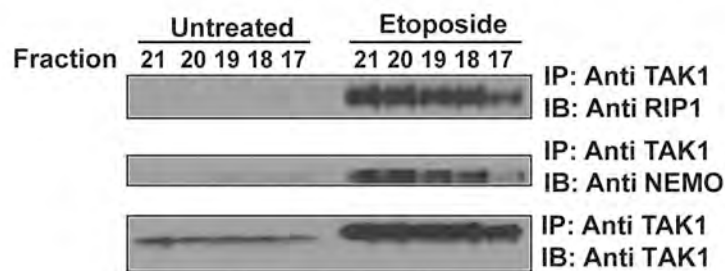
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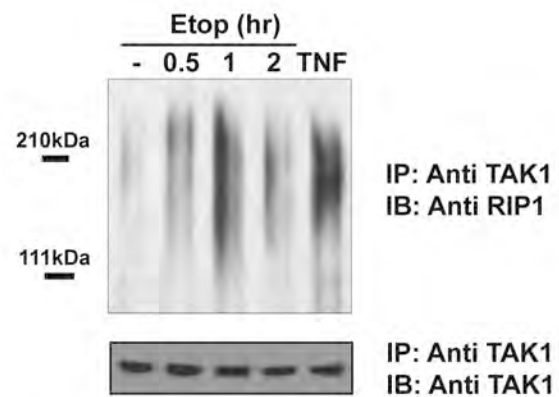
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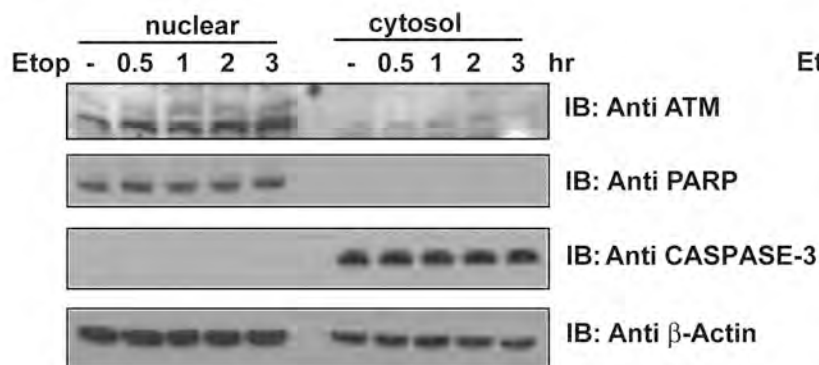
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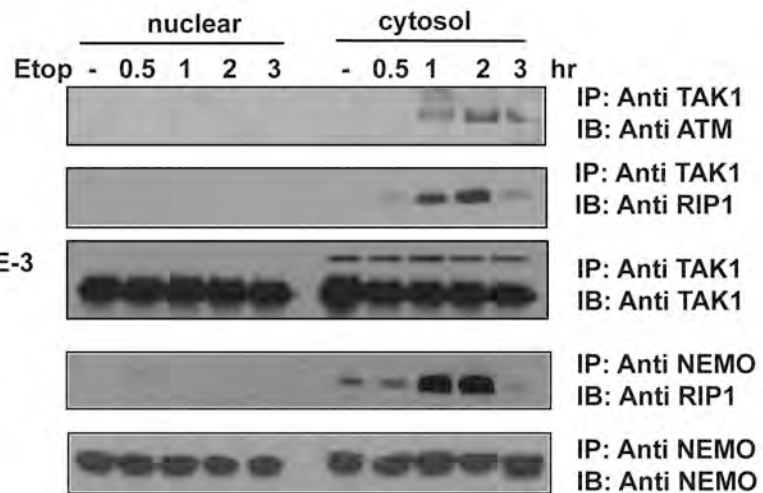
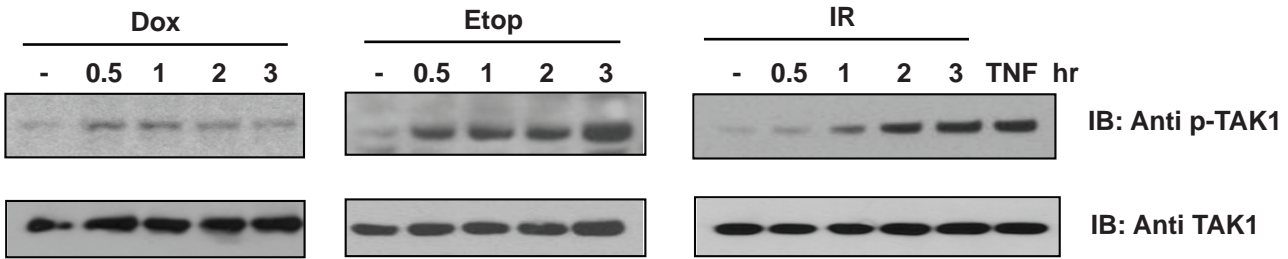
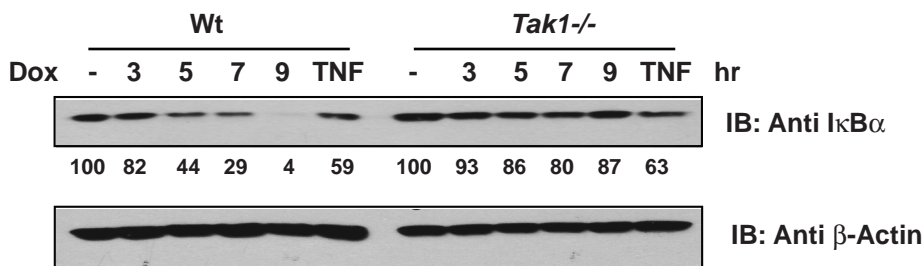


Fig. 4

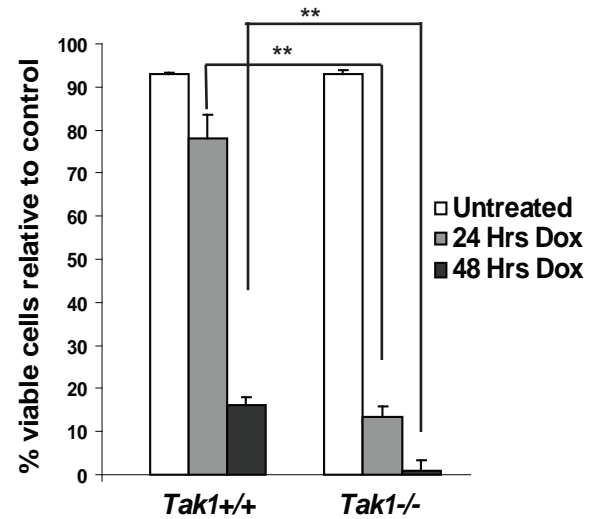
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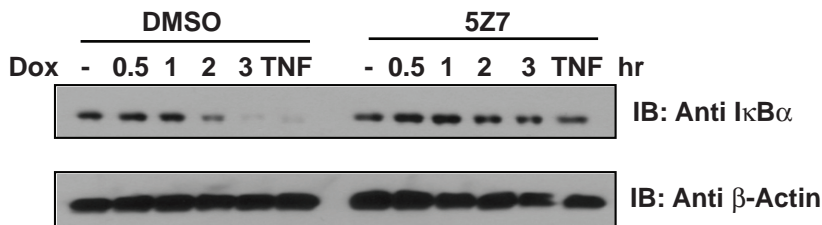
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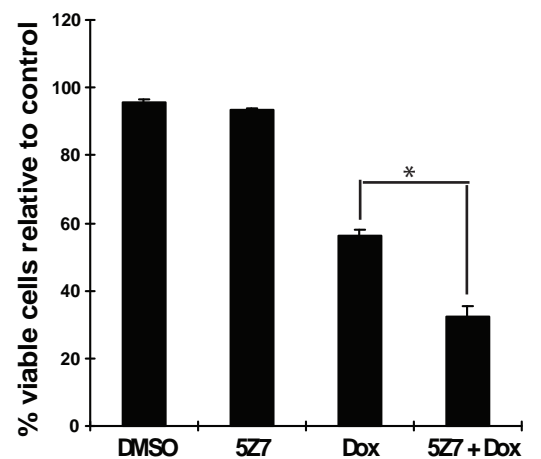
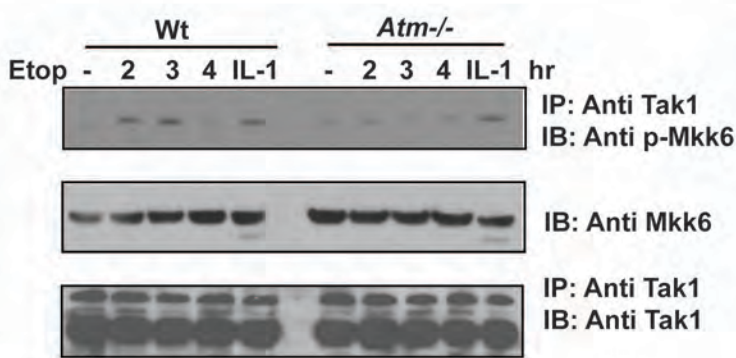
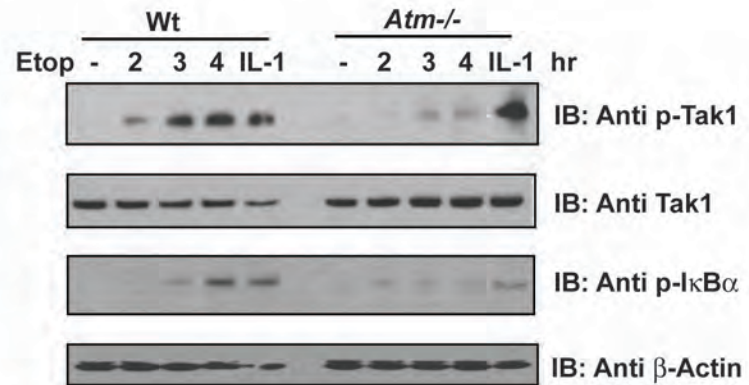


Fig. 5

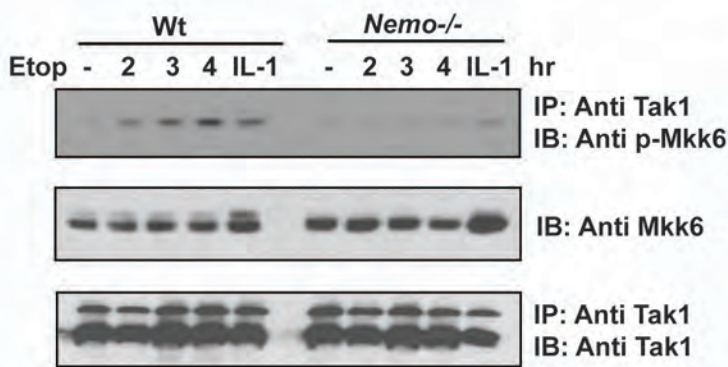
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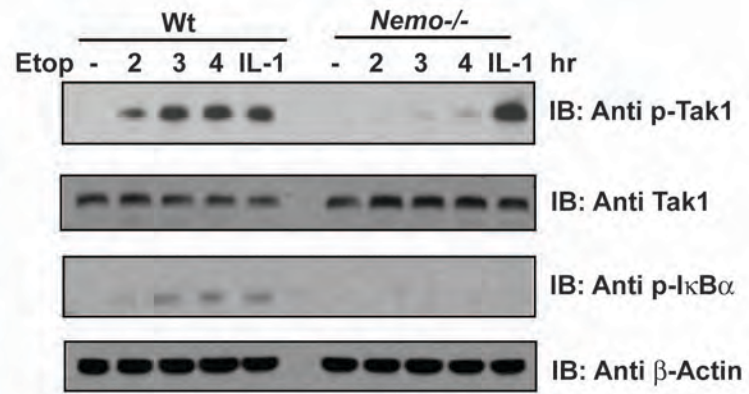
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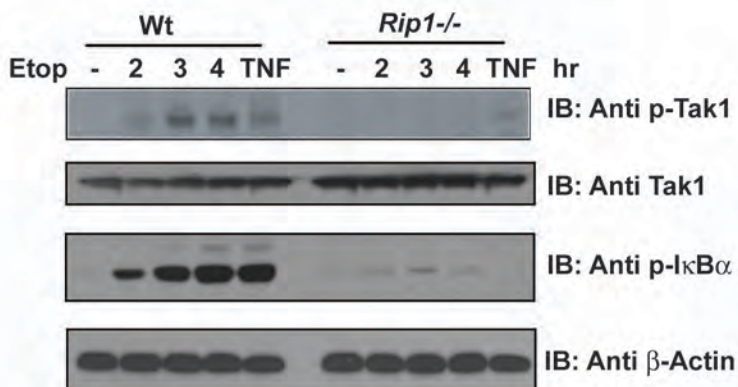
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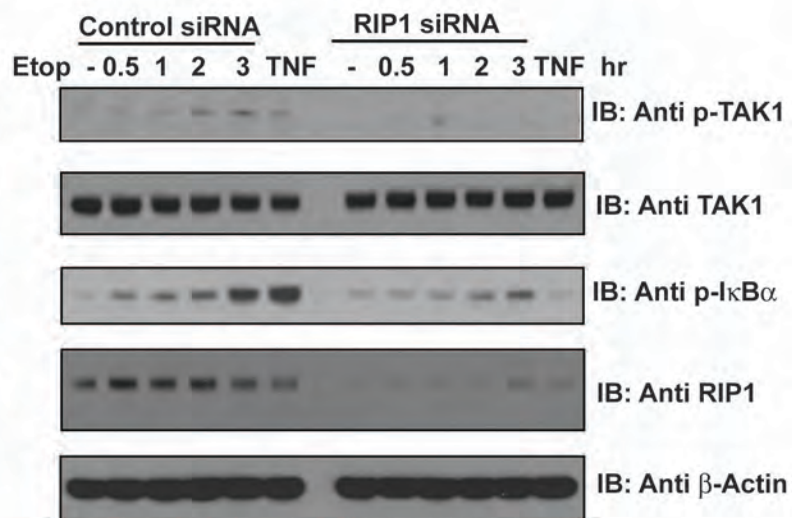
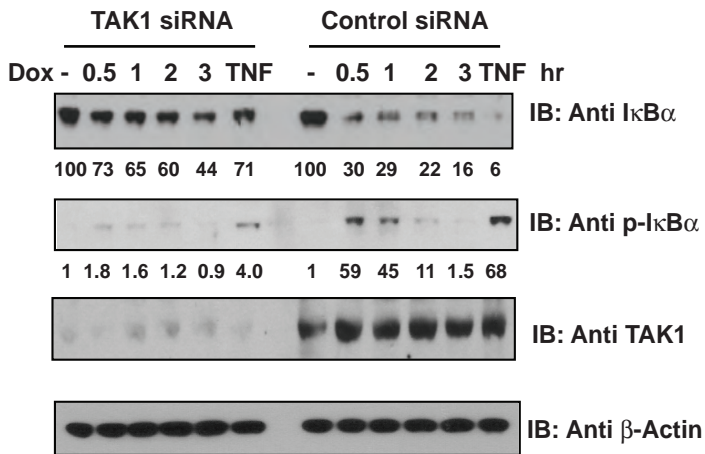
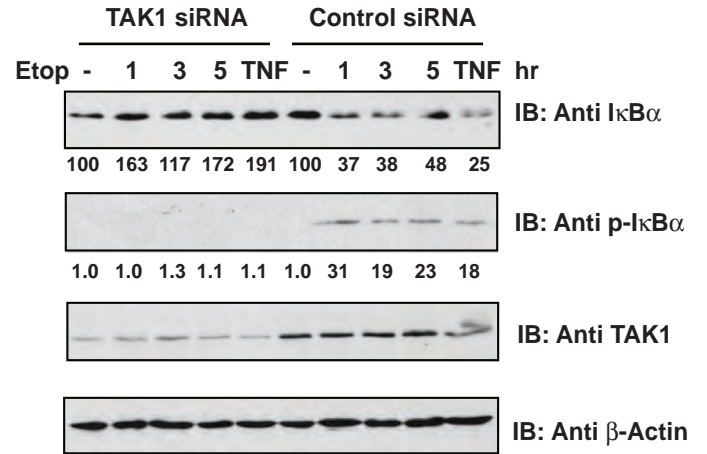


Fig. 6

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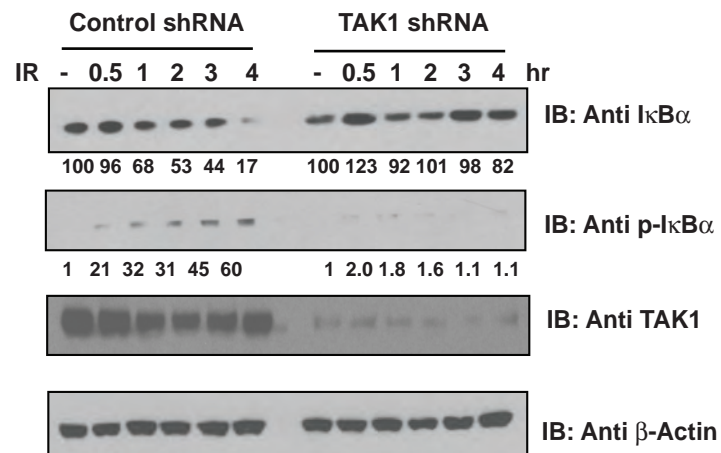
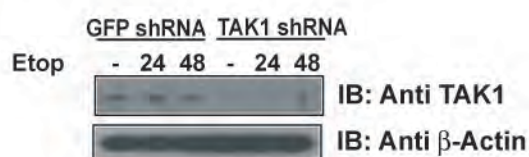
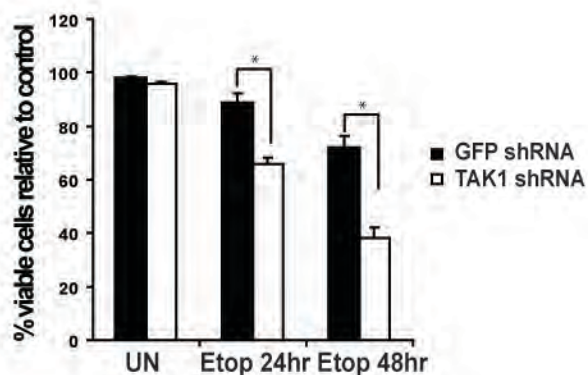
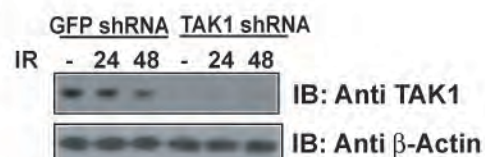
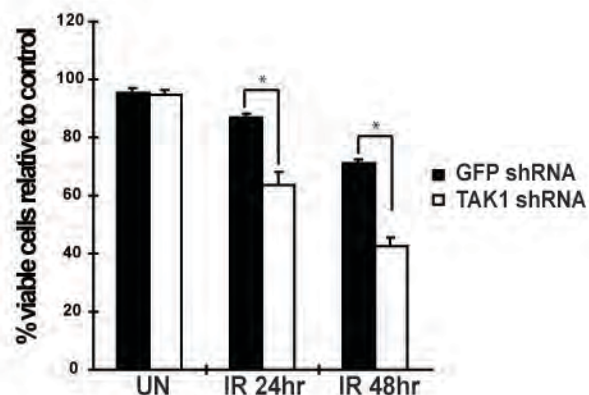


Fig. 7

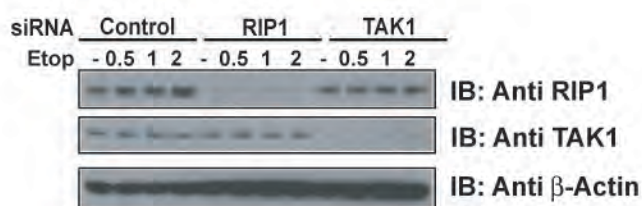
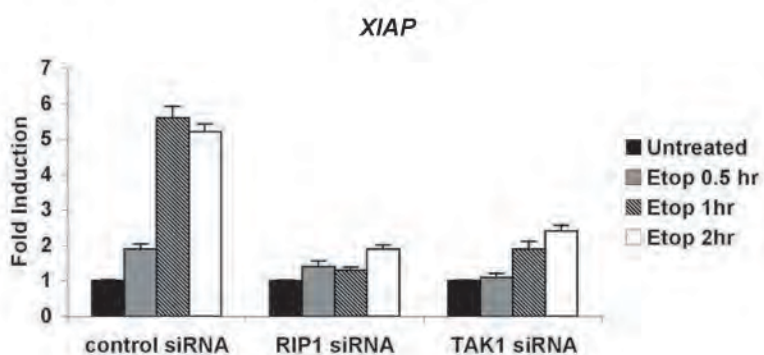
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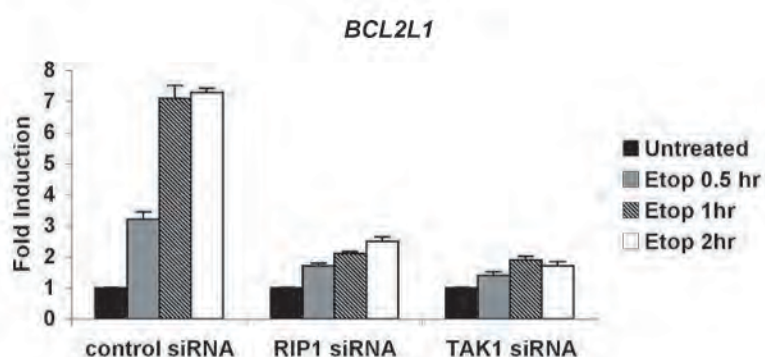
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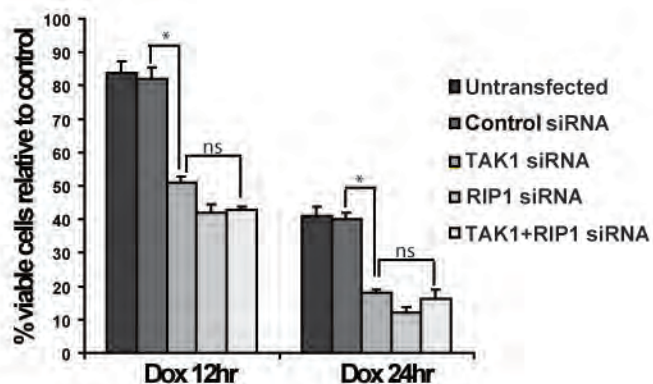
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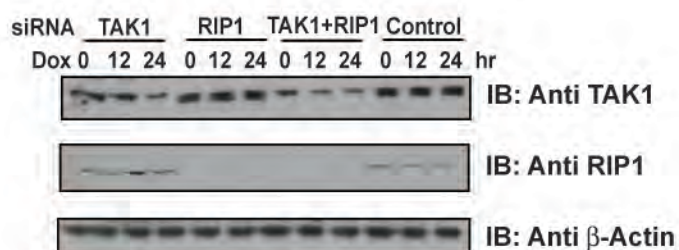


Fig. 8

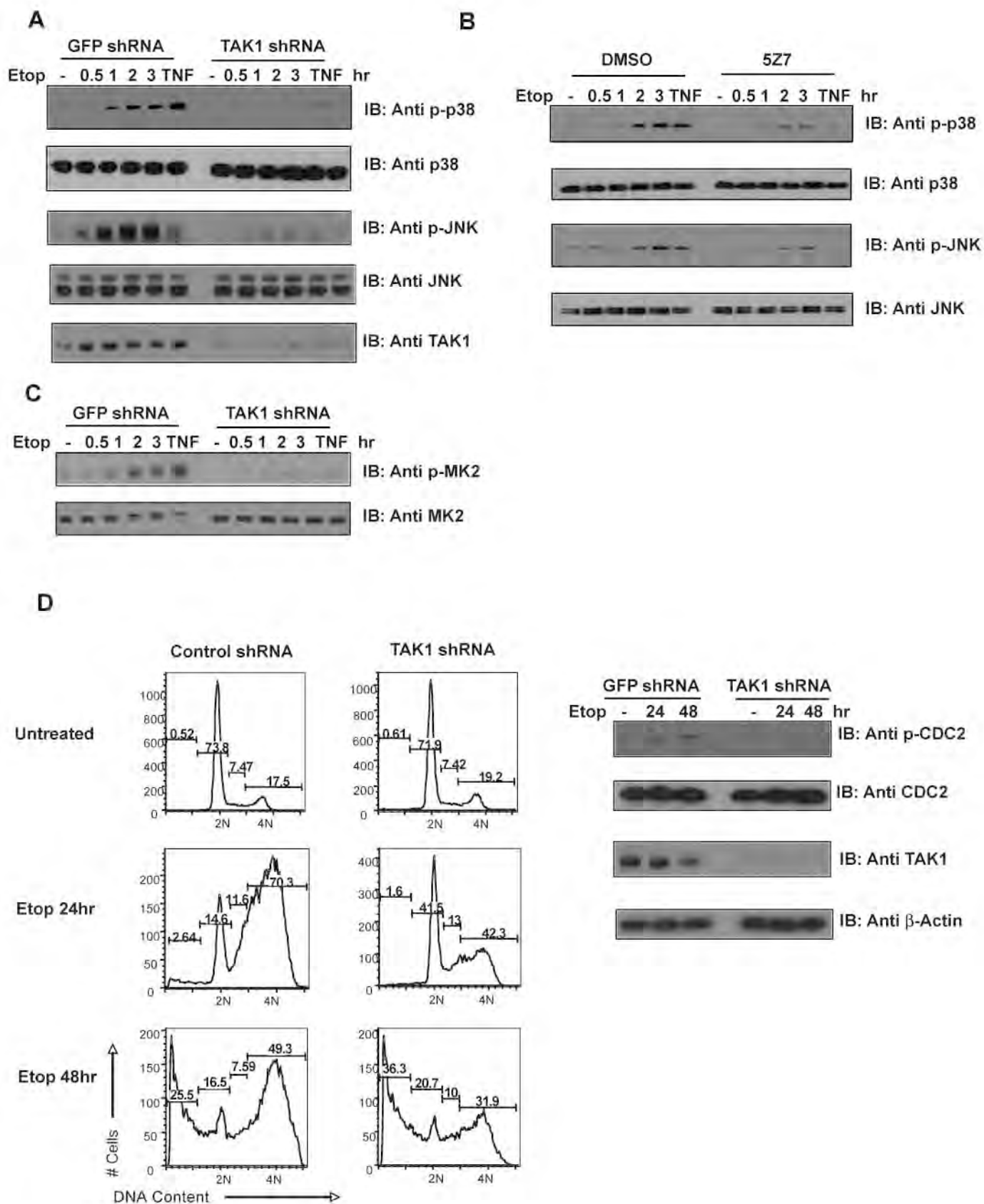


Figure Legends for Supplementary Data

Supplementary Figure 1. DNA damage stimulates the SUMO-1-modification of NEMO. (A) DNA damage stimulates the SUMO-1 modification of NEMO. Human U2OS cells were left untreated or treated with etoposide (40 μ M) for the time periods indicated. Protein extracts were boiled and then immunoprecipitated with an anti-NEMO antibody and sumoylated proteins detected by immunoblotting with a SUMO-1 antibody. (B) A PIASy-deficiency reduces sumoylated NEMO levels in etoposide-treated cells. Human U2OS cells were transfected with control or PIASy-specific siRNAs and the cells left untreated or treated with etoposide (40 μ M) for one hour. Protein extracts were boiled and then immunoprecipitated with an anti-NEMO antibody followed by immunoblotting with an anti-SUMO-1 antibody.

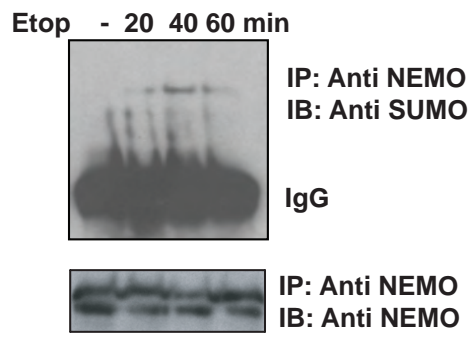
Supplementary Figure 2. The DNA damage-induced cytosolic complex contains modified RIP1. U2OS cytosolic extracts from untreated or etoposide-treated cells were fractionated using FPLC protein purification system (Pharmacia) on a Superose 6 column (Amersham Biosciences). Fractions 17-22 from the etoposide-treated cells were separated on a 3-8% gradient SDS-PAGE gel, transferred to nitrocellulose and immunoblotted with a RIP1 antibody.

Supplementary Figure 3. Expression of a K63-only form of ubiquitin is sufficient to stimulate a RIP1/TAK1 interaction in human tumor cells. Human U2OS cells were left untreated, transfected with a K63-only form of ubiquitin, or treated with etoposide for the time periods indicated. Cell lysates were immunoprecipitated with a TAK1 antibody followed by immunoblotting with a RIP1 antibody. Immunoprecipitates were also immunoblotted with a TAK1 antibody (lower panel) to insure that an equal amount of TAK1 protein was immunoprecipitated.

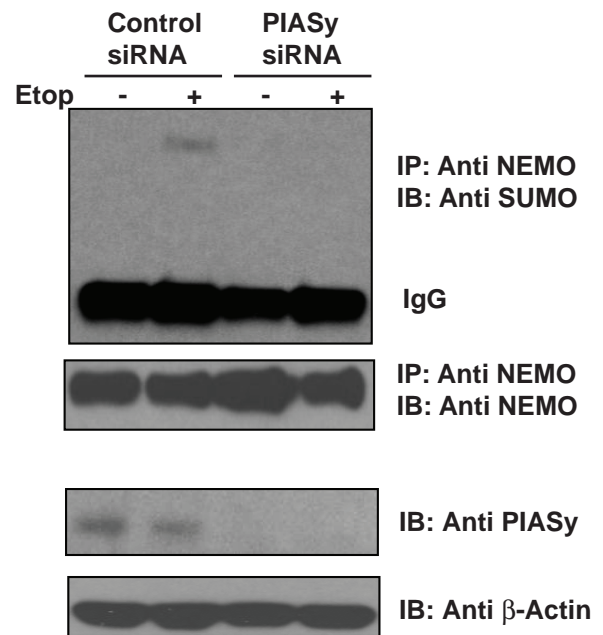
Supplementary Figure 4. TNF- α or doxorubicin treatment stimulates interactions between NEMO and RIP1 and TAK1 and RIP1. Human U2OS cells were left untreated, treated with 10ng/ml TNF- α for 5-15 min or treated with 10 μ g/ml doxorubicin (Dox) for the time periods indicated. Cell lysates were immunoprecipitated with a NEMO (A) or TAK1 (B) antibody followed by immunoblotting with a RIP1 antibody. Immunoprecipitates were also immunoblotted with a NEMO or TAK1 antibody (lower panel) to insure that equal amounts of protein were immunoprecipitated.

Supplementary Figure 5. UBC13 contributes to the NF- κ B response to DNA damage. (A) The human U373 astrocytoma cell line was infected with a lentivirus expressing a control GFP- or UBC13-specific shRNA (Open Biosystems), selected with 2.0 μ g/ml puromycin and UBC13 protein levels determined by immunoblotting. Cells were left untreated, treated with 10ng/ml TNF- α or treated with 10 μ g/ml doxorubicin (Dox) for the time periods indicated. Cells were lysed and NF- κ B activity measured by immunoblotting with total I κ B α antibody. To monitor knockdown and to insure that equal amounts of total protein are compared, the cell lysates were probed with an anti-UBC13 or anti- β -actin antibody. (B) Human U2OS tumor cells were transfected with control siRNA or UBC13 siRNA oligonucleotides. Cells were left untreated, treated with 10ng/ml TNF- α or treated with 10 μ g/ml doxorubicin (Dox) for the time periods indicated. Cells were lysed and NF- κ B activity measured by immunoblotting with total I κ B α antibody. To monitor knockdown and to insure that equal amounts of total protein are compared, the cell lysates were probed with an anti-UBC13 or anti- β -actin antibody.

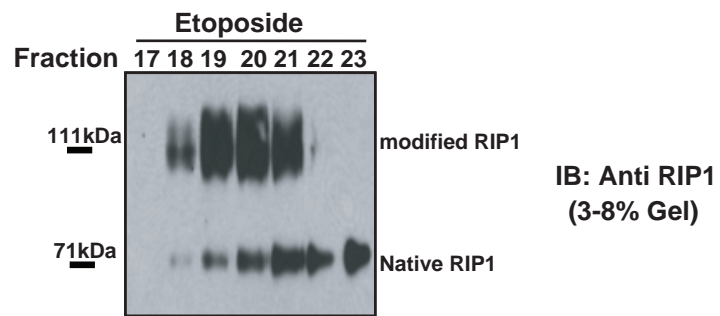
A



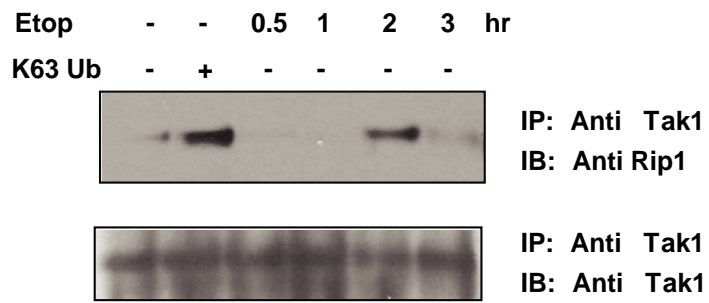
B

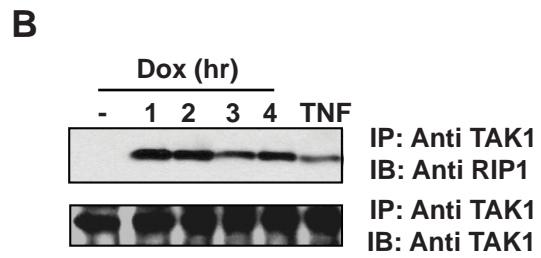
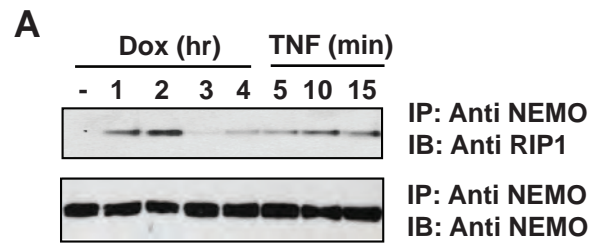


Supplementary Fig. 2

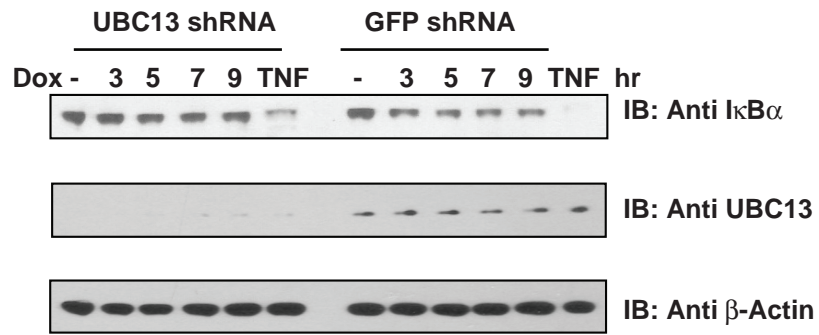


Supplementary Fig. 3

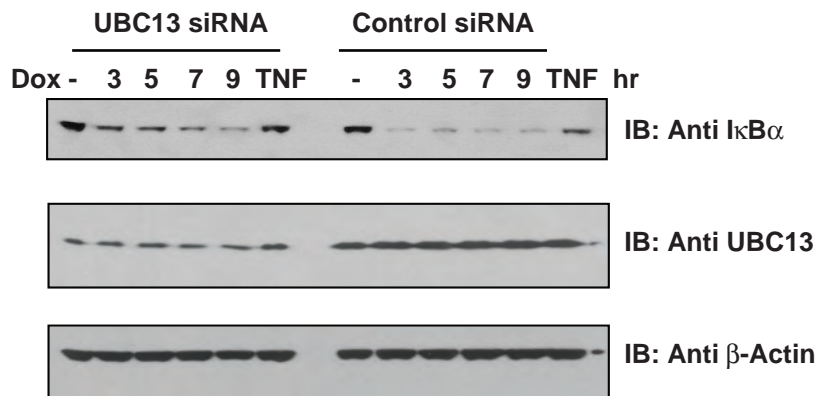




A



B



Supplementary Table 1. Real time PCR primers used in this study

Real time PCR primer	Sequences
Mouse β -actin-F	5'-CGAGGCCCCAGAGCAAGAGAG
Mouse β -actin-R	5'-CGGTTGGCCTTAGGGTTCAG
Mouse Birc3-F	5'-GCTGTGGCCTAATGCTAGACA
Mouse Birc3-R	5'-GGACAATCTTGATTTGCTCGGAA
Mouse Xiap-F	5'-CGAGCTGGGTTTCTTTATACCG
Mouse Xiap-R	5'-GATAGCACAGCCTGGATAGCAAC
Mouse Bcl2l1-F	5'-GACAAGGAGATGCAGGTATTGG
Mouse Bcl2l1-R	5'-TCCCGTAGAGATCCACAAAAGT
Human β -ACTIN-F	5'-CGCGAGAAGATGACCCAGAT
Human β -ACTIN-R	5'-GATAGGACAGCCTGGATAGCAAC
Human BCL2L1-F	5'-GGCCTTTTTCTCCTTCGGTG
Human BCL2L1-R	5'-CTCTCGGCTGCTGCATTGTT
Human XIAP-F	5'-GCAGGTTGGGTGTACGATGT
Human XIAP-R	5'-GCTGCCACAGTAGGACTCG